Purification Of A Serum Factor
That Triggers Cell Cycle Re-entry
In Differentiated Newt Myotubes

Dissertation

Zur Erlangung des akademischen Grades

Doctor rerum naturalium

(Dr. rer. nat.)

Vorgelegt

der Fakultät Mathematik und Naturwissenschaften
der Technischen Universität Dresden

von

Dipl.-Biochem. Werner Straube

Geboren am 9.09.1976 in Pössneck, Deutschland

Gutachter: Dr. Elly M. Tanaka

Prof. Bernard Hoflack

Prof. Jeremy P. Brockes

Eingereicht am: 22.2.2006
Summary

In contrast to mammals, some fish and amphibians have retained the ability to regenerate complex body structures or organs, such as the limb, the tail, the eye lens or even parts of the heart. One major difference in the response to injury is the appearance of a mesenchymal growth zone or blastema in these regenerative species instead of the scarring seen in mammals. This blastema is thought to largely derive from the dedifferentiation of various functional cell types, such as skeletal muscle, skin and cartilage. In the case of multinucleated skeletal muscle fibres, cell cycle re-entry into S-phase as well as fragmentation into mononucleated progenitors is observed both in vitro and in vivo.

In order to identify molecules that initiate dedifferentiation of cells at the wound site in amphibians we have established a cellular assay with a cultured newt myogenic cell line. Using this assay we have found a serum activity that stimulates cell cycle re-entry in differentiated multinucleated newt myotubes. The activity is present in serum of all mammalian species tested so far and, interestingly, thrombin proteolysis amplifies the activity from both serum and plasma. We think this serum factor provides a link between wounding and regeneration and its identification will be a key step in understanding the remarkable differences in wound healing between mammals and amphibians.

In the course of this PhD thesis we have characterized the serum factor as a thermo-labile, pH- and proteinase K-sensitive, high molecular weight protein that is resistant to denaturing conditions such as SDS, urea or organic solvents. Surprisingly, under denaturing conditions the activity behaves as a low molecular weight protein that displays charge heterogeneity on isoelectric focusing. Using these characteristics of the serum factor we have performed a systematic investigation of commonly used protein chromatography modes and separation techniques to develop a successful purification procedure. After four column chromatography steps -- cation exchange, hydrophobic interaction, heparin affinity and size exclusion chromatography under denaturing conditions -- we have achieved a 2,000-fold purification starting from a commercially available Crude Bovine Thrombin preparation. This represents about 40,000-fold purification over bovine serum. Silver stained gels of the most purified fractions revealed ten major protein bands. In order to finally identify the cell cycle re-entry factor, we are currently analyzing the purification by quantitative mass spectrometry by correlating the abundance of tryptic peptides with activity in sequential fractions across a chromatography run.
# Table of Contents

Index of Figures ........................................................................................................ VIII
Index of Contents ................................................................................................ XVI
Abbreviations ........................................................................................................... XII

## 1 Introduction ........................................................................................................ 1

1.1 Regeneration In Urodele Amphibians ............................................................... 1

1.2 Reversal Of The Differentiated State ................................................................. 4

1.2.1 Regeneration Of Skeletal Muscle ................................................................. 4

1.2.2 Regeneration Of The Retina And Lens ......................................................... 6

1.2.3 Cardiac Muscle Regeneration ..................................................................... 7

1.3 Muscle Dedifferentiation In Vitro ................................................................... 7

1.3.1 Newt A1 Cells ............................................................................................. 7

1.3.2 Mouse C2C12 Cells ................................................................................... 8

1.3.3 Cell Cycle Re-entry Of Cultured Myotubes ............................................... 8

1.3.3.1 Cell Cycle Re-entry In Newt A1 Myotubes .............................................. 8

1.3.3.2 S-Phase Re-entry In Mouse C2C12 Myotubes ....................................... 10

1.3.4 Fragmentation And Cellularization Of Myotubes .................................... 12

1.3.4.1 Blastema Extract Induces Fragmentation Of Cultured Newt And Mouse Myotubes .......................................................................................... 12

1.3.4.2 Myoseverin, A Microtubule Destabilizing Agent, Causes Fragmentation Of Cultured Myotubes ............................................................. 13

1.3.5 The Role Of Msx1 And Dedifferentiation Of Muscle Cells ...................... 14

1.4 Characterization Of S-Phase Re-entry Factor (SPRF) ..................................... 16

1.4.1 Initial Characterization Of S-Phase Re-entry Factor In Serum .................. 16

1.4.2 S-Phase Re-Entry Activity In Crude Bovine Thrombin ............................. 16

1.4.3 Thrombin Regulates S-Phase Re-entry In Cultured Newt Myotubes ..... 17

1.4.3.1 Thrombin Promotes Re-entry Into S-Phase ......................................... 17

1.4.3.2 Thrombin And Plasmin Generate Cell Cycle Re-entry Activity In Serum ........................................................................................................ 18

1.5 Selective Activation Of Thrombin Is A Critical Event For Vertebrates In Limb And Lens Regeneration .............................................................. 18

1.6 Plasma, Serum, Growth Factors And Crude Bovine Thrombin - The Starting Material For The Purification ................................................................. 19

1.7 The Myotube Assay For Quantification Of The Purification ......................... 20
1.8 Starting Point And Work Of This Thesis ........................................... 23

2 Results ................................................................................................. 25

2.1 Characterization Of S-Phase Re-Entry Factor .................................. 25
2.1.1 Crude Bovine Thrombin: Inhibition Of Proteolytic Activity In The
Starting Material .................................................................................... 25
2.1.2 Protease Sensitivity ........................................................................ 25
2.1.3 Temperature Sensitivity and pH-Stability ........................................ 25
2.1.4 Glycosylation Of SPRF ..................................................................... 26
2.1.5 Thrombin Treatment Of Starting Material ....................................... 27
2.1.6 Sensitivity Of SPRF To Reducing Agents ........................................ 27
2.1.7 Stability Of Cell Cycle Re-Entry Factor In Organic Solvents, SDS, Urea
And Water .............................................................................................. 28
2.1.8 Characterization Of Molecular Weight ............................................ 31

2.2 Chromatographic Fractionation Of The Serum Factor ....................... 34

2.2.1 Cation Exchange Chromatography .................................................... 34
2.2.1.1 Screening Of Cation Exchange Columns (pH 6.5) ......................... 34
2.2.1.2 Test Of Different pHs On Cation Exchange Columns .................. 35
2.2.1.3 Cation Exchange Chromatography On HiTrap CMFF As First Step Of
The Purification ....................................................................................... 37
2.2.2 Anion Exchange Chromatography .................................................... 38
2.2.2.1 Screening Of Different Anion Exchange Columns At pH 7.0 .......... 38
2.2.2.2 Optimization Of HiTrap Q After Cation Exchange Chromatography On
HiTrap CMFF ......................................................................................... 39
2.2.2.2.1 Step Gradient On Hitrap Q .......................................................... 39
2.2.2.2.2 Optimization Of Step Gradient On HiTrap Q ............................. 40
2.2.2.2.3 Linear Gradient On HiTrap Q ...................................................... 41
2.2.2.3 Mono Q Anion Exchange Chromatography After Heparin Affinity
Chromatography .................................................................................... 43
2.2.3 Hydrophobic Interaction Chromatography ........................................ 46
2.2.3.1 Test Of Different Hydrophobic Interaction Chromatography (HIC)
Columns After Cation Exchange Chromatography ................................ 46
2.2.3.2 Hydrophobic Interaction Chromatography On HiTrap Butyl As Second
Step Of The Purification ....................................................................... 48
2.2.4 Affinity Chromatography ................................................................. 50
2.2.4.1 Heparin Affinity Chromatography ................................................. 50
2.2.4.1.1  Heparin Affinity Chromatography After Cation Exchange Chromatography .................................................. 50
2.2.4.1.2  Heparin Affinity Chromatography After Cation Exchange Chromatography Using A Step Gradient ....................... 50
2.2.4.1.3  Heparin Affinity Chromatography After Cation Exchange Chromatography Using A Linear Gradient .................... 51
2.2.4.1.4  Heparin Affinity Chromatography After Hydrophobic Interaction Chromatography ................................................. 52
2.2.4.1.5  Heparin Affinity Chromatography After Hydrophobic Interaction Chromatography Using A Step Gradient ............... 52
2.2.4.1.6  Heparin Affinity Chromatography After Hydrophobic Interaction Chromatography Using A Linear Gradient ............ 53
2.2.4.2  Affinity Chromatography On Cibacron Blue F3G-A (HiTrap Blue) ...... 56
2.2.4.3  Lectin Affinity Chromatography ................................................................. 57
2.2.5  Chromatography on Hydroxyapatite .......................................................... 60
2.2.6  Chromatofocusing .................................................................................. 63
2.2.7  Reversed Phase Chromatography .............................................................. 64
2.2.8  Ultrafiltration .......................................................................................... 67
2.2.9  Gel Filtration Of Partially Purified Fractions ............................................. 68
2.2.9.1  Native Gel filtration (starting with Hep3-Conc) ..................................... 68
2.2.9.2  Gel Filtration In 0.1% SDS (starting with Hep3-Conc) ............................ 69
2.2.9.3  Gel Filtration At pH 11 ......................................................................... 70
2.2.9.3.1  Gel Filtration At pH 11 – Preparation Of The Starting Material ........ 70
2.2.9.3.2  Gel Filtration At pH 11 Starting With Hep-1M-Conc .......................... 71
2.2.9.3.3  Gel Filtration At pH 11 Starting With Hep3/4-Conc ........................... 74
2.2.10  Preparative PAGE And Isoelectric Focusing ........................................ 77
2.2.10.1  Preparative Non-reducing SDS-PAGE ................................................. 77
2.2.10.2  Preparative Native PAGE .................................................................... 78
2.2.10.3  Preparative Isoelectric Focusing On IPG Strips ................................. 81
2.2.10.4  2D-PAGE Preparative 2D-PAGE Including Staining With Simply Blue Safestain .......................................................... 83
2.2.11  Mass Spectrometry Of Fractions From The Purification ....................... 84
2.2.11.1  Mass Spectrometry Of Gel Slices After Preparative 2D-PAGE: Correlation Of Activity With Protein Identity ............... 84
2.2.11.2  Mass Spectrometry After SDS-PAGE Of Hep3-Butyl ......................... 85
2.2.12  Raising Blocking Monoclonal Antibodies Against SPRF ...................... 88
3 Discussion

3.1 Crude Bovine Thrombin – The Starting Material For The Purification

3.2 Characterization Of The Serum Factor In The Starting Material

3.3 Investigation Of Chromatography Techniques For The Purification

3.4 Heterogeneity Of SPRF

3.5 Summary Of The Final Purification Scheme

3.6 How Close Are We To Identify Candidates

3.7 Quantitative Mass Spectrometry

3.8 Future Purification Strategy

3.9 Identification And Validation Of SPRF Candidates

3.10 Perspective

4 Materials And Methods

4.1 Cell Culture And Myotube Assay

4.1.1 Cell Culture Solutions

4.1.2 Growing And Maintenance Of Newt A1 Cells And A1 Myotubes

4.1.2.1 Freezing Of A1 Cells

4.1.2.2 Thawing Of Frozen A1 Cells

4.1.2.3 Passage Of A1 Cells

4.1.2.4 Initiation Of Myogenesis

4.1.2.5 Myotube Purification

4.1.2.6 Sample Application For Myotube S-Phase Re-Entry Assay

4.1.2.7 BrdU-Addition

4.1.2.8 Fixation And Staining Of Myotubes

4.1.2.8.1 Buffers For Fixation And Staining Of Myotubes

4.1.2.8.2 Fixation Of Myotubes

4.1.2.8.3 Staining Of Myotubes

4.1.2.9 Counting Of Myotubes

4.1.2.10 Preparation Of Labeled Antibodies

4.2 General Methods For Purification

4.2.1 FPLC System For Chromatography

4.2.2 Calculation Of Protein Concentration

4.2.3 Cap Dialysis

4.2.4 Desalting On Nap-5 Columns
4.2.5 Concentrating And Desalting Of Diluted Protein Samples ............... 115
4.2.6 Thrombin Inhibition Assay .......................................................... 116
4.2.7 Preparation of Carrier-Mix ............................................................ 116
4.2.8 Analytical SDS-PAGE ................................................................. 117
4.2.9 Staining Of Protein Gels ................................................................. 117
4.2.9.1 SimplyBlue Safestain (Invitrogen) ............................................... 117
4.2.9.2 Coomassie Staining (Coomassie Brilliant Blue R-250) ................. 118
4.2.9.3 Silver Staining ................................................................................ 118
4.2.10 Protein Recovery From Dilute Solution - Precipitation .................... 119
4.2.10.1 Trichloroacetic Acid (TCA) Precipitation For SDS-PAGE .............. 119
4.2.10.2 Protein Precipitation After Wessel And Fluegge ......................... 120
4.3 Characterization And Purification Of S-Phase Re-Entry Factor .. 121
4.3.1 Common Buffers Used Across The Purification ................................. 121
4.3.2 Important Fractions Across The Purification ..................................... 123
4.3.3 Crude Bovine Thrombin - The Starting Material For The Purification 127
4.3.4 Inactivation Of Thrombin And Other Contaminating Proteases (PPACK Treatment) ................................................................. 127
4.3.5 Characterization Of S-Phase Re-Entry Factor ................................... 128
4.3.6 Chromatographic Fractionation Of The Serum Factor ....................... 132
4.3.6.1 Cation Exchange Chromatography ................................................. 132
4.3.6.1.1 Charging Of Cation Exchange Columns ...................................... 132
4.3.6.1.2 Screening Of Cation Exchange Columns at pH 6.5 .................. 132
4.3.6.1.3 Test Of Different pHs On Cation Exchange Columns ................ 132
4.3.6.1.4 Cation Exchange Chromatography On HiTrap CMFF As First Step Of Purification (Preparation of CMFF-FT) ........................................ 133
4.3.6.2 Anion Exchange Chromatography ............................................... 133
4.3.6.2.1 Charging Of Anion Exchange Columns ....................................... 133
4.3.6.2.2 Screening Of Anion Columns At pH 7.0 With Or Without 20% Ethanol ................................................................. 133
4.3.6.2.3 Optimization Of HiTrap Q .......................................................... 134
4.3.6.2.4 Anion Exchange Chromatography On MonoQ After Heparin Affinity Chromatography ................................................................. 134
4.3.6.3 Hydrophobic Interaction Chromatography (HIC) ........................... 134
4.3.6.3.1 Screening Of 1 ml HiTrap Butyl, HiTrap Phenyl And Valine Sepharose Columns ................................................................. 134
4.3.6.3.2 Comparison Of 5 ml HiTrap Butyl And HiTrap Octyl Columns ...... 135
4.3.6.3.3 Preparation Of Butyl-20% EtOH ............................................... 135
<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.3.6.4</td>
<td>Affinity Chromatography</td>
<td>136</td>
</tr>
<tr>
<td>4.3.6.4.1</td>
<td>Heparin Affinity Chromatography</td>
<td>136</td>
</tr>
<tr>
<td>4.3.6.4.1.1</td>
<td>Step Gradient On HiTrap Heparin</td>
<td>136</td>
</tr>
<tr>
<td>4.3.6.4.1.2</td>
<td>Linear Gradient On HiTrap Heparin - Preparation Of Hep3</td>
<td>136</td>
</tr>
<tr>
<td>4.3.6.4.1.3</td>
<td>Step Gradient On HiTrap Heparin After Hydrophobic Interaction Chromatography - Preparation Of Hep-1M</td>
<td>136</td>
</tr>
<tr>
<td>4.3.6.4.1.4</td>
<td>Linear Gradient On HiTrap Heparin After Hydrophobic Interaction Chromatography - Preparation of Hep3 -Butyl And Hep3/4-Pool</td>
<td>137</td>
</tr>
<tr>
<td>4.3.6.4.2</td>
<td>Affinity Chromatography On Cibacron Blue F3G-A (HiTrap Blue)</td>
<td>137</td>
</tr>
<tr>
<td>4.3.6.4.3</td>
<td>Lectin Affinity Chromatography</td>
<td>137</td>
</tr>
<tr>
<td>4.3.6.4.3.1</td>
<td>Buffer For Wheat Germ Agglutinin (WGA) Lectin Affinity Chromatography</td>
<td>137</td>
</tr>
<tr>
<td>4.3.6.4.3.2</td>
<td>Buffer For Concanavalin A (ConA) And Lentil Lectin Affinity Chromatography</td>
<td>138</td>
</tr>
<tr>
<td>4.3.6.4.3.3</td>
<td>Screening Of Lectin Affinity Columns</td>
<td>138</td>
</tr>
<tr>
<td>4.3.6.5</td>
<td>Chromatography On Hydroxyapatite</td>
<td>139</td>
</tr>
<tr>
<td>4.3.6.6</td>
<td>Chromatofocusing on Mono P</td>
<td>139</td>
</tr>
<tr>
<td>4.3.6.7</td>
<td>Reversed Phase Chromatography</td>
<td>140</td>
</tr>
<tr>
<td>4.3.6.8</td>
<td>Ultrafiltration and Concentration On A 100 kD Membrane</td>
<td>141</td>
</tr>
<tr>
<td>4.3.6.8.1</td>
<td>Preparation Of Hep3-Conc</td>
<td>141</td>
</tr>
<tr>
<td>4.3.6.8.2</td>
<td>Preparation Of Hep-1M-Conc And Hep3/4-Conc (For Gel Filtration At pH 11 And Native PAGE)</td>
<td>141</td>
</tr>
<tr>
<td>4.3.6.9</td>
<td>Gel filtration Of Partially Purified Fraction</td>
<td>142</td>
</tr>
<tr>
<td>4.3.6.9.1</td>
<td>Gel Filtration Marker And Running Conditions</td>
<td>142</td>
</tr>
<tr>
<td>4.3.6.9.2</td>
<td>Native Gel Filtration Of Hep3-Conc</td>
<td>142</td>
</tr>
<tr>
<td>4.3.6.9.3</td>
<td>Gel Filtration Of Hep3-Conc Under Denaturing Conditions In 0.1% SDS</td>
<td>143</td>
</tr>
<tr>
<td>4.3.6.9.3.1</td>
<td>Analytical Gel Filtration In 0.1% SDS</td>
<td>143</td>
</tr>
<tr>
<td>4.3.6.9.3.2</td>
<td>Preparation Of SD200-Pool</td>
<td>143</td>
</tr>
<tr>
<td>4.3.6.9.4</td>
<td>Gel Filtration At pH 11 (Starting With Hep-1M-Conc Or Hep3/4-Conc)</td>
<td>143</td>
</tr>
<tr>
<td>4.3.6.10</td>
<td>Preparative PAGE And Isoelectric Focusing</td>
<td>144</td>
</tr>
<tr>
<td>4.3.6.10.1</td>
<td>Preparative SDS-PAGE (Starting With Hep3-Conc)</td>
<td>144</td>
</tr>
<tr>
<td>4.3.6.10.2</td>
<td>Preparative Native PAGE (Starting With Hep-1M-Conc)</td>
<td>144</td>
</tr>
<tr>
<td>4.3.6.10.3</td>
<td>Isoelectric Focusing (Starting With Hep3-Conc)</td>
<td>145</td>
</tr>
<tr>
<td>4.3.6.10.4</td>
<td>2D-PAGE (Starting With Hep3-Conc)</td>
<td>146</td>
</tr>
<tr>
<td>4.3.7</td>
<td>Mass Spectrometry Analysis</td>
<td>146</td>
</tr>
</tbody>
</table>
5 Acknowledgments.................................................................147

6 References .................................................................149

7 Publications .......................................................................157

8 Declaration According To §5.5...............................................158
Index of Figures

Figure 1.1 - The red-spotted newt (Notophthalmus viridescens) 1

Figure 1.2 - Photograph of a regenerating limb that illustrates the ability of the salamander to regenerate limbs over a course of time after loss ...............2

Figure 1.3 - Phase contrast photomicrograph of longitudinal section of a regenerating larval axolotl (Ambystoma punctatum) limb five days after amputation. ....3

Figure 1.4 – Schematic diagrams of S-phase re-entry model and summary of cellular dedifferentiation effects observed *in vitro* on newt and mouse myotubes....15

Figure 1.5 - The myotube assay for the quantification of the purification of S-phase re-entry factor (SPRF) from Crude Bovine Thrombin ..........................22

Figure 2.1 - Characterization of the SPRF activity found in Crude Bovine Thrombin preparations .........................................................................................29

Figure 2.2 - Assessment of glycosylation of cell cycle re-entry activity.....................30

Figure 2.3 - Size exclusion chromatography of Crude Bovine Thrombin preparation under native and denaturing conditions .................................................33

Figure 2.4 - Cation exchange chromatography on HiTrap CMFF for removal of thrombin ...........................................................................................................37

Figure 2.5 - Anion exchange chromatography on HiTrap Q - step gradient..............42

Figure 2.6 - Anion exchange chromatography on HiTrap Q - linear gradient............42

Figure 2.7 - Anion exchange chromatography of Hep3 pool on Mono Q ..................45

Figure 2.8 - Hydrophobic interaction chromatography on HiTrap Butyl ...................49

Figure 2.9 - Heparin affinity chromatography after cation exchange chromatography using a linear gradient of sodium chloride.............................................55

Figure 2.10 - Heparin affinity chromatography after cation exchange chromatography and hydrophobic interaction chromatography using a linear gradient of sodium chloride .........................................................56

Figure 2.11 - Hydroxyapatite chromatography on CHT-II after cation exchange chromatography and hydrophobic interaction chromatography using a linear gradient of sodium phosphate .........................................................62

Figure 2.12 - Reversed phase chromatography of the low molecular weight form of SPRF after gel filtration in 0.1% SDS.........................................................66
Figure 2.13 - Ultrafiltration of Hep3 on a membrane with a MWCO of 100 kD ..........68

Figure 2.14 - Size exclusion chromatography of Hep3-Conc on Superdex-200 under denaturing conditions in PBS, 0.1% SDS ..............................................70

Figure 2.15 - Gel filtration at pH 11 of Hep-1M-Conc........................................73

Figure 2.16 - Gel filtration at pH 11 starting with a 200-fold purified fraction (Hep3/4-Conc) ............................................................................................................76

Figure 2.17 - Identification of SPRF activity after SDS-polyacrylamide gel electrophoresis ...........................................................................................................78

Figure 2.18 - Preparative native PAGE of a partially purified fraction (Hep-1M-Conc)....80

Figure 2.19 - Isoelectric focusing of SPRF (Hep3-Conc) in 8 M urea on IPG-strips (with a pH-range from 3 to 10) .........................................................................................82

Figure 2.20 - Preparative 2D-PAGE of Hep3-Conc with Simply Blue staining for correlation of protein spots with activity .........................................................84

Figure 3.1 - Non-reducing SDS-PAGE of fractions across the best purification scheme so far ..................................................................................................................97

Figure 3.2 - Quantitative mass spectrometry of fraction across gel filtration at pH 11 ..100
Table 1.1 - Summary of growth factor, serum and blastema extract sensitivity of mammalian (C2C12) and Newt (A1) cells.................................................12
Table 1.2 - Characterization of S-phase re-entry activity from serum and Crude Bovine Thrombin, fractionated on Q-sepharose, on newt A1 cells..........................17
Table 1.3 - Summary of the best purification of the SPRF activity so far ..................24
Table 2.1 - Comparison of purification and recovery of activity on different cation exchange media .....................................................................................35
Table 2.2 - Comparison of activity yield and purification factor for cation exchange chromatography at pH 4.5, 5.5 and 6.5.............................................................36
Table 2.3 - Test of anion exchange columns as initial purification step .................39
Table 2.4 - Anion exchange chromatography on HiTrap Q after cation exchange chromatography (HiTrap CMFF) - Step gradient of sodium chloride on HiTrap Q..................................................................................................40
Table 2.5 - Anion exchange chromatography on MonoQ .......................................44
Table 2.6 - Hydrophobic interaction chromatography: comparison of 1 ml HiTrap Butyl, HiTrap Phenyl and HiTrap columns .........................................................47
Table 2.7 - Hydrophobic interaction chromatography: Comparison of 5 ml HiTrap Butyl and HiTrap Octyl columns .................................................................47
Table 2.8 - Heparin affinity chromatography using a step gradient.........................51
Table 2.9 - Heparin affinity chromatography using a linear gradient of sodium chloride after cation exchange chromatography (CMFF) or after cation exchange and hydrophobic interaction chromatography (CMFF – Butyl). .......................54
Table 2.10 - Affinity purification on HiTrap Blue ..................................................57
Table 2.11 - Lectin affinity chromatography on Concanavalin A (ConA), Lens culinaris lectin (lentil lectin) and wheat germ agglutinin (WGA)..............................59
Table 2.12 - Chromatography on hydroxyapatite (CHT-II) ....................................61
Table 2.13 - Purification chart of a five-step purification protocol ending with reversed phase chromatography .................................................................65
Table 2.14 - Gel filtration at pH 11 after heparin affinity chromatography with step gradient elution (Hep-1M-Conc).................................................................72
Table 2.15 - Gel filtration at pH 11 after heparin affinity chromatography using a linear gradient of NaCl (Hep-3/4-Conc) ................................................................. 75

Table 2.16 - Identified proteins from active gel slices after preparative 2D-PAGE ........ 86

Table 2.17 - Identified proteins after SDS-PAGE of Hep3-Butyl in the region between 28 and 39 kD ......................................................................................... 87

Table 3.1 - Overview of all investigated chromatography modes .................................. 93

Table 3.2 - The current purification scheme of SPRF starting from Crude Bovine Thrombin ............................................................................................................ 96

Table 4.1 - Overview of all purifications and important fractions across the purifications ............................................................................................................. 125

Table 4.2 - Immobilized lectins used for the characterization of the glycosylation pattern of SPRF ................................................................................................. 130

Table 4.3 - Characteristics of cation exchange columns ............................................... 132
Abbreviations

AMEM  amphibi-an-MEM
APBS  amphibi-an-PBS
AS  ammonium sulfate
Bis-Tris Propane  1,3-bis[tris(hydroxymethyl)methylamino]propane
BrdU  5-bromo-2'-deoxyuridine
BSA  bovine serum albumin
CAPS  3-(cyclohexylamino)-1-propanesulfonic acid
CHAPS  3-[[3-Cholamidopropyl]dimethylammonio]-1-propanesulfonate
CHES  2-(Cyclohexylamino)ethanesulfonic acid
CV  column volume
ddH₂O  bi-distilled water
ddwater  bi-distilled water
DTT  dithiothreitol
EDTA  ethylenediaminetetraacetic acid
ETOH  ethanol
FBS  fetal bovine serum
FCS  fetal calf serum
FITC  fluorescein isothiocyanate
g  g force
HEPES  N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)
HIC  hydrophobic interaction chromatography
I  liter
LC-MS/MS  liquid chromatography mass spectrometry
M  molar
MEM  minimum essential medium
MES  2-(N-morpholino)ethanesulfonic acid
ml  milliliter
mM  millimolar
MOPS  3-(N-morpholino)propanesulfonic acid
MS  mass spectrometry
N.A.  not applicable
N.D.  not determined
NaAcetate  sodium acetate
PBS  phosphate-buffered saline
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>PFA</td>
<td>paraformaldehyde</td>
</tr>
<tr>
<td>pH</td>
<td>pH value</td>
</tr>
<tr>
<td>PNGaseF</td>
<td>Peptide: N-Glycosidase F</td>
</tr>
<tr>
<td>PPACK</td>
<td>D-Phe-Pro-Arg-chloromethylketone, HCl</td>
</tr>
<tr>
<td>ppm</td>
<td>parts per million</td>
</tr>
<tr>
<td>RT</td>
<td>room temperature</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SPRF</td>
<td>S-phase re-entry factor</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris-buffered saline</td>
</tr>
<tr>
<td>TCA</td>
<td>trichloroacetic acid (CCl₃COOH)</td>
</tr>
<tr>
<td>TE</td>
<td>trypsin-EDTA</td>
</tr>
<tr>
<td>TFA</td>
<td>trifluoroacetic acid (CF₃COOH)</td>
</tr>
<tr>
<td>Tris</td>
<td>Tris-(hydroxymethyl)-aminomethane</td>
</tr>
<tr>
<td>TRITC</td>
<td>tetramethylrhodamine isothiocyanate</td>
</tr>
<tr>
<td>µl</td>
<td>microliter</td>
</tr>
</tbody>
</table>
1 Introduction

1.1 Regeneration In Urodele Amphibians

The unique ability of adult urodele amphibians such as the newt and axolotl to replace lost parts of the body and regenerate after wounding has fascinated researchers for over 100 years. In response to tissue damage or injury an adult newt is capable of regenerating complex structures such as a limb, tail, spinal cord, heart ventricle, retina, lens and jaws that are composed of a variety of tissues including muscle, skin, bone, cartilage and nerves. This remarkable regenerative capacity is thought to at least partly depend on dedifferentiation of cells at the site of injury, a process that is lacking in mammals and other vertebrates, where regeneration is limited to a small spectrum of organs (Brockes and Kumar, 2002; Hay, 1966; Stocum, 1995; Tanaka, 2003).

Figure 1.1 - The red-spotted newt (*Notophthalmus viridescens*)

The regeneration of an amputated urodele limb proceeds through a typical series of morphological and histological stages (Brockes, 1997; Nye et al., 2003; Stocum, 2004). Following amputation of the limb, epithelial cells begin to crawl over the amputation site to form a wound epithelium. Then in response to undefined signals in the early regenerate, internal stump cells start to lose their specialized character in a process referred to as dedifferentiation. These dedifferentiated cells then proliferate to form a mesenchymal growth zone, known as the blastema, which harbors the cells that will later redifferentiate to form the regenerated limb (Figure 1.2, Figure 1.3). Moreover, blastema cells do not only differentiate back into the cell type which they derived from, but also transdifferentiate into other cell types (Echeverri and Tanaka, 2002a). The formation of
the blastema in response to wounding represents a key-difference compared to other vertebrates including mammals, which are not able to regenerate to this extent.

![Photograph of a regenerating limb that illustrates the ability of the salamander to regenerate limbs over a course of time after loss](image)

**Figure 1.2** - Photograph of a regenerating limb that illustrates the ability of the salamander to regenerate limbs over a course of time after loss

Forelimbs of adult newts (*Triturus viridescens*) were amputated at the level of lower arm (right) and upper arm (left) and photographs taken at 7, 21, 25, 28, 32, 42 and 70 days after amputation. During the third week the blastema appears as a bud at the tip of the regenerating limb (red circle). Note that only structures distal to the amputation plane are replaced, indicating the blastema contains a positional identity (Goss, 1969).

Historically, Charles S. Thornton first suggested a contribution of mesodermal tissue to the blastema. His studies on limb regeneration in larval axolotl (*Ambystoma punctatum*) and newt (*Triturus viridescens*) revealed a transformation of muscle, cartilage and other inner tissues of the limb into mesenchyma-like cells (Thornton, 1938; Thornton, 1942). A converse opinion at this time was that the dedifferentiating epidermis is a major contributor to the arising blastema (Rose, 1948). However, this was contradicted by observations of Chalkley who showed that cell division begins in the inner limb tissues much earlier and more proximally than understood before, and that epidermis may not be required (Chalkley, 1954). Further confirmation of these results was obtained with autoradiographic studies using tritiated thymidine. By tracing labeled cells, Hay and Fischman could provide the first direct evidence, that blastema cells in regenerating limbs of the newt indeed originate from the dedifferentiating internal tissues and not from the apical limb epidermis (Hay and Fischman, 1960; Hay and Fischman, 1961).
To date a contribution of reserve stem cells to the blastema has never been ruled out, but there is no direct evidence for such a mechanism (Stocum, 2004). Therefore dedifferentiation of cells at the wound site appears to be the crucial cellular response to injury that initiates blastema formation.

A key question is how injury does initiate the regeneration response and what are the molecular events occurring inside and outside the cells? Although experiments following labeled cells or grafts in animals after injury have accumulated much information about tissue behavior still very little is known about the process of regeneration at the molecular and cellular level and no factor, responsible for the initiation of dedifferentiation, has been identified so far.

![Figure 1.3 - Phase contrast photomicrograph of longitudinal section of a regenerating larval axolotl (Ambystoma punctatum) limb five days after amputation.](image)

(A) After 5 days the regenerating limb is covered by at least a five layer thick apical wound epidermis (EP) underneath which the blastema has already appeared (Bl). Most of the blastema seems to have arisen from dedifferentiating muscle fibers (Mus), but Schwann cells from cut nerves (Ne) and connective tissue also contribute. (B) Magnification of the dedifferentiating muscle fibers from (A): During the process of muscle fibers dedifferentiation, the normally elongated nucleus (N") enlarges (N') and becomes rounded in shape (N). These changes indicate the initiation of DNA synthesis. Furthermore, dedifferentiation leads to a breakdown of myofibrils and a fragmentation of the multinucleated muscle cell into mononucleated cells (N), which contribute to the blastema (Bl) (Hay, 1959)
1.2 Reversal Of The Differentiated State

The two definitive examples of cellular dedifferentiation during amphibian regeneration are the transdifferentiation of pigmented epithelial cells of iris or retina into lens and the dedifferentiation of muscle during limb and tail regeneration (Brockes and Kumar, 2002; Henry, 2003). In both cases, cells lose their differentiated phenotype to produce proliferating cells that contribute to the regenerated structure.

1.2.1 Regeneration Of Skeletal Muscle

Studies of regenerating limbs or tails have revealed enormous tissue re-organization near the wound site. These histological changes affect all tissues in the regenerating stump; however, the most visible and dramatic effect is found for multinucleated skeletal muscle cells. Therefore, muscle has become the most intensively studied cell type for understanding regeneration at the cellular and molecular level.

The first proposal that dedifferentiation of multinucleated muscle fibers at the wound site might contribute to the developing blastema came from Charles S. Thornton and were confirmed by Elizabeth Hay’s elegant electron microscopy observations already 40 years ago (Hay, 1959; Thornton, 1938). In longitudinal sections of a regenerating axolotl limb Hay interpreted changes of the nuclear shape in myofibers, from a normally elongated to an enlarged and rounded nucleus, as the beginning of DNA-synthesis. In addition myofibrils disappeared and the syncytial fibers broke up into individual cells during dedifferentiation (Hay, 1959). However, this conclusion was derived from static pictures and one could argue as well that the reverse process was occurring, that of cells fusing to form a muscle fiber.

More experimental evidence for the dedifferentiation theory of muscle cells was provided by implantation of labeled myotubes into a regenerating newt limb (Lo et al., 1993). Cultured newt limb myotubes were selectively microinjected with the lineage tracer rhodamine-dextran and introduced into regenerating limbs. One week after implantation accumulation of dextran labeled mononucleated cells was seen, strongly suggesting that the labeled myotubes had fragmented. Furthermore, these mononucleated cells were found to proliferate, as they were double-labeled with the cytoplasmic lineage tracer and $^3$H-Thymidine that had been incorporated into the nucleus. In contrast dedifferentiation of newt myotubes, left in cell culture, was never observed, suggesting a specific environment for reversal of the differentiated state in the regenerating limb. In later work,
these experiments were more elegantly repeated using retrovirus-labeled implanted myotubes (Kumar et al., 2000). Although the experiments by Lo and Kumar et al. strongly supported the reversal of differentiation during the course of regeneration, it remained unclear whether endogenous muscle fibers close to a wound can undergo dedifferentiation and contribute to the mass of proliferating blastema cells. However, this was demonstrated by labeling of single muscle fibers in axolotl tail (Echeverri et al., 2001). Specifically, a single fiber was labeled by pressure injection of rhodamine-dextran, followed by distal amputation of the tail close to the labeled fiber. Dedifferentiation of mature muscle fibers occurred between 3 and 5 days after an amputation by the synchronous fragmentation of the multinucleate muscle fiber into mononucleated cells followed by rapid proliferation of these cells. Remarkably, in addition to amputation or severe tissue damage a direct clipping of the muscle fiber was required to initiate this process. Based on these experiments it was calculated, that about 17% of the blastema cell mass derives from muscle dedifferentiation alone (Echeverri and Tanaka, 2002b). In parallel, similar observations were made using isolated myofibers from axolotl limb and dissociated in culture. When striated myofibers were labeled with a cell tracker dye and then implanted back into the environment of a limb blastema many examples of labeled mononucleated cells were observed in the regenerating limb two to four days later (Kumar et al., 2004).

Besides fragmentation of multinucleated cells, the second characteristic of dedifferentiating muscle cells is the re-entry from a postmitotic state into cell cycle accompanied by DNA-replication. Studies with tritiated thymidine both in vivo (Hay and Fischman, 1961) and with implanted myotubes (Kumar et al., 2000) indicated that DNA-synthesis occurs in the multinucleated myotube before fragmentation. Using injection of tritiated thymidine into regenerating limbs, followed by fixation of these tissues, Hay and Fishman found, in sections of the regenerating tissue, clear evidence for thymidine incorporation in polynucleated muscle fibers as early as the fourth day after amputation. However, between 10 to 20 days, incorporation was seen more commonly in rounded nuclei of mononucleated muscle fragments derived from the syncytial muscle fiber. Similar results were obtained by implanting retrovirus-labeled cultured myotubes into the environment of a regenerating limb using incorporation of BrdU as a marker for DNA-synthesis. After injection of BrdU into regenerates nine days after implantation followed 24 hours later by fixation and analysis, all the nuclei in several retrovirally labeled myotubes were found to be BrdU-positive, indicating S-phase re-entry of these myotubes (Kumar et al., 2000).
Neither of these studies addressed the issue of whether cell cycle re-entry and budding are independent from each other or if both linked in one pathway. The first support for autonomous mechanisms came from implantation of cultured newt myotubes where S-phase re-entry was irreversibly blocked. When myotubes, inhibited from progressing through the cell cycle by X-irradiation or transfection with the cell cycle inhibitor p16^INK4, were implanted into the regenerating newt blastema, fragmentation still occurred (Velloso et al., 2000). In contrast, p16^INK4-injected or irradiated myotubes either did not or at least at only very low frequency entered S-phase upon serum stimulation, as determined by labeling with tritiated thymidine.

Additional evidence for the independence of S-phase re-entry and fragmentation was obtained from isolated and dissociated myofibers of axolotl. When dissociated, many of these myofibers underwent breakage into viable multinucleated fragments or even mononucleated cells (cellularization). However, labeling with tritiated thymidine did not reveal any S-phase re-entry of myofibers up to 48 h after isolation, even in myofibers, showing clear signs of fragmentation and cellularization (Kumar et al., 2004). Therefore, cell cycle progression and fragmentation of myofibers are likely independent events controlled by at least two distinct signaling pathways during regeneration.

1.2.2 Regeneration Of The Retina And Lens

Amazingly, when the lens is removed from a larval or adult urodele (lentectomy), it can be regenerated by adjacent tissue. However, amphibians greatly differ in their ability to regenerate a lens and in the types of cells, which are used for the purpose. While salamanders of the family Ambystomidae are able to grow a new lens from a fragment of the old lens, some members of the family Salamandridae have the remarkable ability of growing new lens from the dorsal iris (Hay, 1966).

Regeneration of the lens from the dorsal iris (Wolffian regeneration) has been examined more extensively than any other type of lens regeneration. After removal of a newt lens, a defined population of dorsal iris cells loses their pigmentation, and then begins to divide to form a bulge in the epithelium. Over the next weeks, these cells continue to divide until they form a sphere of cells that eventually expresses the characteristic lens crystallins (Henry, 2003). These transitions have been reproduced in vitro by Eguchi and his colleagues who have demonstrated that the depigmenting drug, phenylthiourea, and also basic fibroblast growth factor (FGF) are required to induce retinal pigmented epithelial cells of the chick embryo to form lens in vitro (Hyuga et al., 1993). Furthermore, it was recently shown that active thrombin and a yet unknown thrombin generated
activity in serum are involved in this transition as these pigmented epithelial cells, like newt A1 myotubes, respond in culture to a thrombin-activated serum factor and re-enter S-phase (Simon and Brockes, 2002).

1.2.3 Cardiac Muscle Regeneration

A third well-studied cell type in newt regeneration is the cardiac myocyte. In contrast to mammals, newts are able to functionally replace 30 to 50% of the heart ventricle after excision. Removal of ventricular tissue strongly increased DNA synthesis and mitosis in cells adjacent to the wound (Oberpriller and Oberpriller, 1974; Oberpriller et al., 1995). Autoradiographic studies of tritiated thymidine labelled along with electron microscopy studies by Bader and Oberpriller failed to discover a reserve of undifferentiated satellite cells, but clearly identified differentiated cardiomyocytes as contributors to the regenerating heart ventricle (Bader and Oberpriller, 1979; Campion, 1984; Carlson, 1989).

Using primary cultures of adult newt cardiac muscle, a very recent study revealed heterogeneity of these cardiomyocytes with respect to proliferation. Interestingly, as with muscle and also pigmented epithelial cells, they are stimulated to re-enter cell cycle by fetal bovine serum in a dose-dependent manner. Although the majority can enter S-phase, only one third of the cells traverse mitosis to then undergo additional rounds of cell division. Furthermore, cell cycle re-entry is associated with phosphorylation of the retinoblastoma protein and injection of a plasmid encoding human CDK inhibitor p16\textsuperscript{INK4} into cultured cardiac myocytes decreased S-phase re-entry approximately 13-fold (Bettencourt-Dias et al., 2003). However it is unclear from this study, whether this serum stimulation of cardiomyocytes is triggered by a thrombin activated factor or other growth factors such as basic FGF, acidic FGF or PDGF (Oberpriller et al., 1995).

1.3 Muscle Dedifferentiation In Vitro

1.3.1 Newt A1 Cells

In order to study muscle dedifferentiation of urodeles in vitro on the cellular level a myogenic cell line derived from the red-spotted newt (Notophthalmus viridescens) limb is commonly employed. These A1 cells were originally isolated from cultures of normal limb mesenchymal tissue and can be propagated without any sign of senescence for more than one year in culture (Ferretti and Brockes, 1988). Moreover, upon serum reduction (0.5% fetal calf serum) A1 mononucleated cells exit the cell cycle, fuse to form a
polynucleated syncytium and start to express late markers of muscle differentiation, such as myosin-heavy chain, troponin T, myogenin and myoD. Functionally these differentiated myotubes become contractile in response to mechanical stimulation but on the other hand fail to display features in culture found in myofibers such as striation and peripheral alignment of nuclei. In contrast to plating at high density, when few cells are cultured on substrates such as fibronectin, vitronectin or laminin, newt A1 myotubes usually spread and form large myosacs containing one or more clusters of five to ten nuclei.

1.3.2 Mouse C2C12 Cells

For comparison of mammalian to newt muscle cells in the context of regeneration a mouse myogenic cell line, C2C12, is traditionally used. Mouse C2C12 muscle cells are a diploid, continuous cell line originally isolated by Yaffe from the thigh muscle of a two-month-old C3H mouse (Yaffe and Saxel, 1977). These cells were later subcloned and referred then as C2C12 (Blau et al., 1983). C2C12 cells are maintained as undifferentiated myoblasts in medium containing 20% fetal calf serum. To induce myoblast fusion and the formation of multinucleates, the culture medium is changed to 2% horse serum. This is associated with the expression of muscle markers such as myosin-heavy chain, troponin T, myogenin and myoD (McGann et al., 2001). Within two to three days after the onset of cell fusion, muscle fibers exhibit spontaneous contraction (Yaffe and Saxel, 1977). Very recently, it was reported that C2C12 are even able to form highly differentiated, contractile myotubes with peripheral nuclei and adult fast myosin expression. C2C12 co-culture on a fibroblast monolayer resulted in a well-defined mature sarcomeric structure and a response to electrical stimulation comparable to mature myotubes (Cooper et al., 2004). This suggests an important influence of the extracellular environment on differentiation and likely on dedifferentiation.

1.3.3 Cell Cycle Re-entry Of Cultured Myotubes

1.3.3.1 Cell Cycle Re-entry In Newt A1 Myotubes

An unanswered question in the context of regeneration is what molecules trigger cell cycle re-entry and mitosis in newt A1 myotubes, pigmented epithelial cells and cardiomyocytes. When the ability of cultured newt A1 myotubes to re-enter the cell cycle, to proliferate and to undergo mitosis in response to known mitogens was examined, they
remained withdrawn from cell cycle. In contrast to their A1 monoculeated progenitors, newt myotubes were refractory to several common growth factors, such as bFGF, PDGF, IGF or EGF. Upon incubation with elevated serum concentration, however, these terminally differentiated cells could re-enter the cell cycle and undergo DNA-synthesis (Tanaka et al., 1997).

One striking consequence of stimulation with serum is the phosphorylation of an intracellular cell cycle regulator, the retinoblastoma protein (pRb) that regulates progression through the G1 to S transition by complexing with the E2F family of transcription factors. The phosphorylation of pRb, by the action of the cyclin dependent kinase 4 (CDK 4) or CDK 6, releases members of the E2F family from this inhibitory Rb complex allowing them to control the expression of several genes required for entry into S-phase (Hatakeyama and Weinberg, 1995; Weinberg, 1995).

In order to determine whether phosphorylation is required for cell cycle re-entry, newt myotubes were injected with plasmids encoding the cell cycle inhibitor p16 or a pRb mutant. When human p16, which specifically inhibits the cyclin-dependent kinase 4 (CDK 4) or CDK 6 was expressed in newt myotubes, no DNA-synthesis was observed compared to control injections. More direct evidence for the central role of pRB-phosphorylation derived from injection with the pRb mutant Δ34 Rb, in which all eight CDK consensus phosphorylation sites had been mutated. Overexpression of Δ34 Rb competes with wild type pRB for binding to E2F members as well as CDK 4/6 and therefore prevents all actions, which require phosphorylation of endogenous pRB. Indeed injection of Δ34 Rb led to an increased inhibition of DNA-synthesis in myotubes compared to control injections, suggesting that phosphorylation of pRb is a critical step in cell cycle re-entry of these cells (Tanaka et al., 1997).

In addition to pRb phosphorylation, elevated serum stimulation of newt A1 myotubes causes entry into S-phase. As demonstrated in a number of investigations, described below, this DNA-synthesis is not an artifact but leads to accumulation of myotube nuclei with 4N DNA without any signs of apoptosis. Two days after serum addition, DNA-synthesis in myotubes starts asynchronously with a peak of nuclei incorporating BrdU around day four. A window of serum stimulation for as little as 8 h is sufficient to generate this response. When uptake of BrdU into myotube nuclei was judged against normal proliferating mononucleated cells, both incorporation rates were comparable and therefore activation of DNA repair machinery seems unlikely. Moreover, pulse chase experiments using tritiated thymidine and BrdU revealed, that S-phase lasts about 48 to 72 h in the myotube nuclei, which is similar to the length of S-phase in mononucleated
cells of newt limb (Tassava et al., 1987; Wallace and Maden, 1976). Finally, quantification of DNA-content in nuclei of stimulated and non-stimulated myotubes, suggest the complete traverse of S-phase with an exact doubling of DNA-content in each nucleus.

In order to develop an assay for the identification of the serum factor, the linearity of the myotube response was investigated. Increasing concentrations of fetal bovine serum displayed a dose-dependent response with a linear range from 5 to 20% positive myotubes using a BrdU pulse of 8 h. Higher concentration than 20% serum led to a loss of linearity and finally to a saturation of myotube response around 25 to 30% BrdU-positive myotubes (Tanaka et al., 1997).

Interestingly, the S-phase re-entry of myotubes is sensitive to contact inhibition. When purified myotubes were plated within high and low-density areas of mononucleated cells on the same dish, serum stimulation and BrdU labeling resulted in a high myotube response in the low-density environment compared with the high-density side (Tanaka and Brockes, 1998). This indicates that, in addition to stimulation of soluble factors in serum, a loss of cell-cell contact may required for the cell-cycle response of myotubes. It is also consistent with the in vitro finding, that myofibers require direct clipping for fragmentation (Echeverri and Tanaka, 2002b).

1.3.3.2 S-Phase Re-entry In Mouse C2C12 Myotubes

In comparison to newt A1 myotubes, their mammalian counterparts are not stimulated to undergo S-phase re-entry by elevated serum. When, for example, mouse C2C12 myotubes were generated and purified in a similar manner, no BrdU incorporation was found up to 72 h after serum stimulation (Tanaka et al., 1997). This significant difference in response to the serum factor might be one reason for the inability of mammals to achieve the regenerative potential of salamanders. In contrast, mononucleated mouse cells responded normally to serum. Table 1.1 summarizes the comparison of myogenic mouse C2C12 and newt A1 cells. It is unclear as yet, whether the lack of response derives from a missing or unresponsive receptor after muscle differentiation on the cell surface, an intracellular block of the S-phase pathway or an obstruction within the nucleus at the chromatin level.

Nevertheless, terminally differentiated C2C12 myotubes are not totally unresponsive to extracellular, proliferative signals. Incubation with serum led to an up-regulation of immediate-early genes such as c-fos, c-jun, c-myc and Id-1, indicating these cells are not confined to G0 but can partially traverse G1 (Tiainen et al., 1996). Moreover the
capability of C2C12 myotubes to undergo DNA-synthesis was demonstrated by transfection with viral proteins such as SV40 large T antigen. Expression of this oncogene, which binds to the unphosphorylated form of the retinoblastoma protein forced myotubes to re-enter the cell cycle. Moreover, the pRb became rephosphorylated upon stimulation with serum in myotubes expressing SV40 large T antigen but not in non-transfected C2C12 myotubes (Gu et al., 1993). This suggests a link between the ability for cell cycle re-entry and phosphorylation of pRb. However, like most retroviral oncogenes, the SV40 T antigen has no known cellular counterpart. The critical role of pRB was verified using cultured skeletal muscle cells derived from an Rb -/- mouse. In contrast to their wild type counterparts these Rb -/- myotubes re-entered the cell cycle and synthesized DNA (Schneider et al., 1994).

Very recently, Camarda and colleagues reported that terminal proliferation arrest is maintained in skeletal muscle cells by a pRb-independent mechanism. In contrast to the earlier work, they used conditional knockout myotubes. In this case excision of pRb after complete differentiation of myotubes caused reexpression of cell cycle regulators and down-regulation of muscle specific genes but did not trigger DNA-synthesis. Further investigation revealed the presence of a second pocket-protein independent block in myotubes, which could be relieved by cyclin D1/ CDK 4 co-expression (Camarda et al., 2004).

Finally, evidence for the potential of mouse myotube nuclei to undergo DNA-replication in response to serum was obtained from the formation of interspecies hybrid myotubes by fusing mouse C2C12 and newt A1 myogenic cells (Velloso et al., 2001). As expected under these conditions C2C12 homokaryons remained arrested. In contrast, C2C12 nuclei in hybrids re-entered the cell cycle upon serum stimulation, indicating that a pathway activated in newt cytoplasm can overcome the postmitotic arrest. Further experimental evidence, supporting this idea came from treatment of mouse myotubes with a blastema extract of a regenerating limb. This latter result will be discussed below in more detail.
Table 1.1 - Summary of growth factor, serum and blastema extract sensitivity of mammalian (C2C12) and Newt (A1) cells.

Myotubes and mononucleates were assayed for bromodeoxyuridine (BrdU) incorporation in response to growth factors such as platelet-derived growth factor (PDGF), serum from various species or blastema extract from a regenerating newt limb (McGann et al., 2001; Tanaka et al., 1999).

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Proliferate response</th>
<th>Proliferation + Fragmentation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell form</td>
<td>Serum*</td>
<td>PDGF**</td>
</tr>
<tr>
<td>Mouse C2C12 Mononucleates</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Myotubes</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Newt A1 Mononucleates</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Myotubes</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

* other tested sera were from fetal bovine, adult bovine, sheep, porcine, chicken and human
** other tested growth factors were bFGF, EGF, IFG-1 and keratinocyte growth factor
* not applicable

1.3.4 Fragmentation And Cellularization Of Myotubes

1.3.4.1 Blastema Extract Induces Fragmentation Of Cultured Newt And Mouse Myotubes

While newt myotubes clearly complete S-phase, neither mitotic figures nor budding were observed in culture, even up to 10 days after serum application, suggesting that the nuclei arrest in the G2-phase of the cell cycle. This also supports the theory of a second block in these myotubes, whereby another soluble factor provided by nerves and/or the wound epithelium or special requirements of the environment, such as signals from the extracellular matrix, are necessary for full dedifferentiation of muscle cells and the formation of proliferating mononucleated blastema cells (Mescher, 1996; Tassava and Mescher, 1975).

Recently, it has been shown that protein extracts prepared from regenerating newt limbs can also induce cultured newt as well as murine myotubes to re-enter the cell cycle. More importantly, after addition of blastema extract, a budding of myotubes into smaller myotubes or even proliferating mononucleates was observed (McGann et al., 2001). This treatment initially results in the reduction of expression of muscle differentiation proteins such as MyoD, myogenin and troponin T. Subsequently about 10% of the polynucleated murine myotubes undergo fission into proliferating cells. Almost no evidence for cellular
dedifferentiation was found when myotubes were treated with extracts from nonregenerating limbs, suggesting an activation of a factor or a set of factors after limb amputation, capable of inducing both DNA-synthesis and cellularization (Odelberg, 2002). It is not understood so far, whether the activities in serum and blastema extract act through similar mechanisms on myotubes. Compared to serum, blastema extract is a mixture of proteins and other biomolecules from several tissues and blood. Therefore it seems likely that the extract might contain two factors, one essential for fragmentation and another for S-phase re-entry.

1.3.4.2 Myoseverin, A Microtubule Destabilizing Agent, Causes Fragmentation Of Cultured Myotubes

The potential of mouse C2C12 to undergo fragmentation and cellularization was also demonstrated with of a novel microtubule-depolymerizing drug (Rosania et al., 2000). Myoseverin, a 2,6,9-trisubstituted purine, forced differentiated C2C12 myotubes to break into monoculeated cells, which in turn could proliferate again. Immunoblot analysis after myoseverin treatment with subsequent addition of growth medium, identified downregulation of the differentiation markers Myf5, MyoD and myosin heavy chain with a concomitant upregulation of cell cycle proteins, cyclin A and CDK2, indicating a reversal of the differentiated into a proliferate state. Moreover, this fragmentation occurred in the absence of S-phase re-entry (Perez et al., 2002). This implies again that cellularization is independent of S-phase traversal and points to a crucial function of myotube microtubule organization for generation of mononucleated progeny.

Myoseverin was also shown to act on newt A1 myotubes with similar cellularization effects (Imokawa et al., 2004a). Studying the expression of myogenic regulatory factors (MRFs), Imokawa and his colleagues found an upregulation of Myf5 associated with myotube formation in culture as well as with myofibers in the newt. Conversely, stimulation with serum or treatment with myoseverin led to the downregulation of Myf5 in cultured newt myotubes. Furthermore, after incubation with myoseverin, mononucleated cells positive for Myf5 and myosin heavy chain appeared adjacent to myotubes indicating that these cells may arise by fragmentation of the neighboring myotubes. These results suggest that local depolymerization of microtubules could be a significant event in the pathway leading to cellularization of multinucleated myofibers.

Recently, another line evidence for the importance of microtubules disassembly during fragmentation derived also from studies where cultured striated myofibers from axolotl limbs were treated with taxol. Typically, after isolation and dissociation most untreated
myofibers (80%) displayed morphological signs of fragmentation or even cellularization without S-phase re-entry in culture. When, however, these cultures were exposed to the microtubule-stabilizing agent taxol, this number decreased to 16% (Kumar et al., 2004).

1.3.5 The Role Of Msx1 And Dedifferentiation Of Muscle Cells

Over the last few years one molecule, Msx1, has emerged as the focus of several investigations into the mechanism of muscle dedifferentiation. This protein, a homeobox-containing transcriptional repressor, is expressed in rapidly proliferating mesenchymal cells during normal limb development. In studies of digit tip regeneration in fetal and newborn mice, Reginelli and colleagues demonstrated a restriction of the regenerative ability of mouse digit tips to an area where Msx1 is expressed (Reginelli et al., 1995). This led to the hypothesis, that expression of Msx1 (and Msx2) might be crucial for digit cells to take part in a regenerative response. The first direct evidence for an involvement of Msx1 in dedifferentiation came from a study of the temporal expression pattern in regenerating newt limbs. Msx1 was found to be strongly up-regulated during the initiation of regeneration, remained expressed throughout regeneration, but was not detectable in the fully regenerated limb, suggesting a correlation between the undifferentiated state and the expression of Msx1 (Simon et al., 1995). Another sign for a regulative role in the dedifferentiation of cells was obtained by the ectopic expression of the Msx1 gene in mouse C2C12 myotubes. Overexpression of Msx1 reduced the expression levels of myogenic factors such as MyoD, myogenin and MRF4. Moreover, expression of Msx1 induced myotube fragmentation with subsequent proliferation and even transdifferentiation into cells expressing chondrogenic, adipogenic or osteogenic markers (Odelberg et al., 2000). Recently, more functional evidence for the importance of Msx1 was provided using isolated muscle fibers from larval axolotls. Dissociated single fibers underwent fragmentation or even cellularization and this coincided with the selective appearance of Msx1 mRNA and protein. Furthermore, the uptake of Msx1 antisense morpholinos from culture medium caused a significant decrease in the expression of Msx1 protein and a marked inhibition of fragmentation (Kumar et al., 2004). Taken together, these results suggest a key role for Msx1 during regeneration. However, no study has yet addressed whether extracellular signals, such as the factors present in serum or the blastema extract, lead to elevated levels of Msx1 expression in newt A1 or mouse C2C12 myotubes. A summary of the factors acting on newt and mouse myotube that lead to cell cycle re-entry and also the generation of mononucleated cells is presented in Figure 1.4.
Figure 1.4 – Schematic diagrams of S-phase re-entry model and summary of cellular dedifferentiation effects observed in vitro on newt and mouse myotubes

(A) Schematic diagram of the activation of S-phase re-entry by newt A1 myotubes in the context of the wound healing responses that leads to regeneration:
Amputation of the limb triggers multiple responses such as inflammation, remodeling of the extracellular matrix and cell migration. A major aspect of wound healing is activation of the conversion of prothrombin to thrombin. In mammals, this induces the conversion of fibrinogen to fibrin polymers and formation of a clot. In newt, thrombin activation, in addition, leads to cell-cycle re-entry from the differentiated state. This involves the conversion of a latent activity (SPRF to SPRFa) within blood (or serum), which can selectively stimulate newt A1 myotubes, but not their mononucleate precursors to undergo S-phase. Although the SPRFa activity was found in all animal sera tested so far and hence is likely to be a general product of thrombin activation, mouse C2C12 myotubes were refractory to this activity (Tanaka et al., 1999).

(B) Schematic diagram of muscle dedifferentiation in vitro – comparison of newt A1 and mouse C2C12 myotubes:
In vitro an extracellular proteinaceous factor found both in serum and newt limb blastema extract is capable of pulling newt A1 myotubes out of G0 and allowing them to progress S-phase, where they become arrested in a 4N state. The G1-S transition is mediated via the phosphorylation of the retinoblastoma protein (pRB). In contrast to newt myotubes, mouse C2C12 myotubes can be stimulated to re-enter cell cycle by newt limb blastema extract but not by the serum factor. Besides cell cycle re-entry, a second characteristic for dedifferentiation of muscle cells is the fragmentation of a multinucleated cell into mononucleated derivatives referred to as cellularization. Newt limb blastema extract and the microtubule-destabilizing drug myoseverin are capable to induce fragmentation as well as cellularization in both newt A1 and mouse C2C12 myotubes. Furthermore it was shown that overexpression of Msx1, a homeobox-containing transcriptional repressor, in mouse C2C12 myotubes causes fragmentation and even cellularization. Interestingly, isolated and dissociated myofibers from axolotl limb can undergo cellularization concomitant with the expression of Msx1 but without any signs of S-phase re-entry. The red arrow indicates that cell cycle re-entry and fragmentation are thought to be independent events, as cell-cycle re-entry is not a requirement for fragmentation and the formation of viable mononucleated cells. The factors responsible for inducing myotubes to undergo DNA-synthesis, fragment, divide and eventually re-differentiate are still unknown (Echeverri and Tanaka, 2002b).
1.4 Characterization Of S-Phase Re-entry Factor (SPRF)

1.4.1 Initial Characterization Of S-Phase Re-entry Factor In Serum

The cell cycle re-entry of newt A1 myotubes was first discovered upon incubation with fetal bovine serum. However, sera of all species so far tested, including adult bovine, sheep, porcine, chicken and human, are able to generate a comparable response (Tanaka et al., 1999). This indicates a conserved property of serum and therefore a conserved mechanism for initiating the regenerative response throughout the animal kingdom.

Unfortunately, all attempts to test axolotl or newt serum have so far failed due to either the inability of obtaining sufficient amounts for testing or due to the high toxicity of axolotl serum. Nevertheless, as discussed below in more detail, there is strong evidence, that coagulation of blood is required in urodele regeneration and also that members of the coagulation cascade are involved.

In an initial characterization of the activity in bovine serum neither delipidation nor dialysis against a membrane with a molecular weight cut off between 6000 and 8000 Daltons abolished activity. Gel filtration on Superose-12 suggested that the native molecular weight of the factor in serum is 150,000-300,000 Daltons (Tanaka et al., 1997). Furthermore the activity appeared rather robust, as it showed resistant to denaturation by SDS. In this work the activity found in serum is always referred to as cell cycle re-entry factor or S-Phase Re-entry Factor (SPRF).

1.4.2 S-Phase Re-Entry Activity In Crude Bovine Thrombin

In order to find a suitable starting material for the characterization and purification of the cell cycle re-entry factor, several crude fractions derived from plasma or serum were tested. While, for example, platelet lysate, a potent source of growth factor activities, did not contain any detectable amount of activity, another plasma derivative enriched in thrombin contained significant amounts of SPRF activity. This commercially available Crude Bovine Thrombin preparation was prepared from plasma and in addition to high concentrations of active thrombin also contained at least 10-fold more cell cycle re-entry activity per unit protein compared to bovine serum (Tanaka et al., 1999). This led to the initial model that thrombin, a known mitogen, might be the sought-after S-phase re-entry activity (Chen and Buchanan, 1975).

Chromatographic fractionation disproved this theory and clearly distinguished the S-phase re-entry activity from thrombin (Tanaka and Brockes, 1998). When Crude Bovine
Thrombin was applied to a HiTrap Q anion exchange column and bound proteins eluted with a linear gradient of NaCl, the activity fractionated as two distinct peaks at 100 mM (Q-100) and 400 mM NaCl (Q-400), respectively. Additional chromatographic fractionation of the Q-100 fraction on a HiTrap SP cation exchange column displayed one major peak, containing mostly α, β and γ-forms of thrombin, eluting at 500 mM NaCl. Furthermore, treatment of fractions with PPACK, a potent irreversible inhibitor of thrombin protease activity (Bode et al., 1989), completely abolished cell cycle re-entry activity in the Q-100 but not in the Q-400 fraction when assayed in medium containing 0.5% serum (Table 1.2). This data demonstrated that the activities in these two fractions are distinct and that the activity in Q-100 is linked to active thrombin.

**Table 1.2 - Characterization of S-phase re-entry activity from serum and Crude Bovine Thrombin, fractionated on Q-sepharose, on newt A1 cells**

<table>
<thead>
<tr>
<th>Starting material (Crude Bovine Thrombin, CB-Thrombin) and fractions of the HiTrap Q column (flow through, Q-FT; 100 mM eluate, Q-100; and 400 mM eluate, Q-400) were added to mononucleated A1 cells and myotubes. Starting material and flow through both stimulated mononucleates, whereas with myotubes, stimulation was found for all fractions except Q-FT. However, when HiTrap Q fractions were treated with PPACK for inhibition of thrombin, S-phase re-entry activity of myotubes was abolished in the Q-100 fraction. Fetal bovine serum (FBS) was used as a control (Tanaka and Brockes, 1998). N.A. = not applicable.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A1 Mononucleates</strong></td>
</tr>
<tr>
<td>FBS</td>
</tr>
<tr>
<td>CB-Thrombin</td>
</tr>
<tr>
<td>Q-FT</td>
</tr>
<tr>
<td>Q-100</td>
</tr>
<tr>
<td>Q-400</td>
</tr>
</tbody>
</table>

**1.4.3 Thrombin Regulates S-Phase Re-entry In Cultured Newt Myotubes**

**1.4.3.1 Thrombin Promotes Re-entry Into S-Phase**

The characterization of Crude Bovine Thrombin on the HiTrap Q column raised some questions about the role of thrombin for cell cycle re-entry of newt A1 myotubes. Thus pure thrombin was assayed on myotubes in medium containing either 1.5% fetal bovine serum or 1% bovine serum albumin (BSA). Only myotubes in 1.5% fetal bovine serum re-entered the cell cycle, suggesting that thrombin-mediated re-entry requires sub-threshold concentrations of serum. This latter condition, however, suggests two
possibilities: either that thrombin acts directly through cleavage of a thrombin receptor (Chen and Buchanan, 1975; Goldsack et al., 1998) or indirectly by cleavage of molecules in serum that then, in turn, act on myotubes.

1.4.3.2 Thrombin And Plasmin Generate Cell Cycle Re-entry Activity In Serum

In order to test whether thrombin has a direct or indirect effect on myotubes, medium containing 1.5% serum was incubated with pure thrombin. Typically no myotube response was found for medium containing up to 1.5% serum alone. After 24 h of incubation with thrombin, the sample was then treated with PPACK to completely inhibit thrombin protease activity. As a control, thrombin was inhibited with PPACK and afterwards incubated with 1.5% serum. No stimulated myotubes were found in samples where thrombin was inhibited prior to incubation. In contrast, thrombin treatment of samples with subsequent inhibition of proteolytic activity generated a strong response, demonstrating that thrombin generated additional cell cycle re-entry activity in serum. A similar result was obtained when serum was incubated with the serum protease plasmin using, in this case α2-antiplasmin for inhibition. Other proteases that were tested in similar assays were found to be negative including trypsin, Factor Xa, Protein Ca, Factor IX and Factor XII (Tanaka et al., 1999).

1.5 Selective Activation Of Thrombin Is A Critical Event For Vertebrates In Limb And Lens Regeneration

Physiological evidence that regeneration in urodeles involves the action of thrombin derived from thrombin substrate overlay assays on frozen sections of regenerating newt limbs prepared eight days after amputation (Tanaka et al., 1999). By this time point cells of the mesenchymal tissue close to the amputation site had dedifferentiated and contributed to the forming blastema. When membranes impregnated with thrombin substrate were placed on top of sections, elevated thrombin proteolytic activity was localized exclusively in the mesenchyme at the distal tip of the regenerating limb underneath the wound epidermis. Moreover, this signal was completely inhibited by inclusion of PPACK, an irreversible inhibitor of thrombin.

Another line of evidence pointing to the general importance of thrombin in the initiation of dedifferentiation comes from in vitro studies with newt pigmented epithelial cells that, like
newt myotubes, respond in culture to a thrombin-activated serum factor (Simon and Brockes, 2002). In adult newts, lens regeneration only takes place at the pupillary margin of the dorsal iris where pigmented epithelial cells re-enter the cell cycle and transdifferentiate into the lens (Eguchi and Shingai, 1971). However, in cell culture pigmented epithelial cells both from dorsal and ventral iris posses the capacity to transdifferentiate suggesting a missing stimulus in the ventral part of the iris after lens removal (Abe and Eguchi, 1977). Recently, Imokawa and Brockes demonstrated that the transient and selective activation of thrombin at the dorsal margin in the newt eye after lentectomy is absolutely required for the activation of pigmented epithelial cells to undergo these regenerative events (Imokawa and Brockes, 2003). These results indicate that the selective activation of thrombin is a fundamental signal linking tissue injury to the initiation of regeneration in vertebrates.

The role of thrombin in this aspect of dedifferentiation of cells provides an interesting link between injury, the initiation of blood coagulation and the local activation of factors for regeneration of missing tissue in urodeles (Imokawa et al., 2004b; Maden, 2003). It is also in agreement with experimental evidence, that injury alone elicits dedifferentiation and cell cycle re-entry of cells (Tassava and Mescher, 1975; Thornton, 1953).

### 1.6 Plasma, Serum, Growth Factors And Crude Bovine Thrombin - The Starting Material For The Purification

The purification of proteins and in particular growth factors from plasma or serum is a challenging task. Plasma, the soluble component of the blood, is not only the largest and deepest version of the mammalian-derived proteome, it also has an extraordinary range in the concentration of proteins of more than 10 orders of magnitude from mg/ml to low pg/ml (Anderson and Anderson, 2002). Serum, the liquid fraction of clotted blood or plasma, is an even more complex mixture than plasma since extensive proteolytic processing and degradation occurs during coagulation and serum mitogens are released from their precursors in plasma and from blood platelets during serum preparation. In addition serum contains many other proteins, which are synthesized and secreted, shed or lost from cells and tissues throughout the body. From the 10 000 proteins estimated to be commonly present in serum (Adkins et al., 2002), only 10 proteins represent 90% of the total proteins. Moreover, the 22 most abundant proteins make up to 99% all proteins in plasma and serum. Therefore all remaining proteins, including growth factors, are
found in the remaining 1% total protein in serum (Plasma Proteome Institute, www.plasmaproteome.org). The major constituents of serum, with a combined protein concentration of approximately 60 mg/ml, are albumin (35 to 50 mg/ml), immunoglobulins (10 mg/ml), transferrin, haptoglobins and lipoproteins. Thus, two difficulties in the purification of growth factors from serum are the great complexity of serum and the low concentration of mitogens. In culture, cells usually require for an optimal growth promoting effect between 10 and 20% serum, which is 6 to 12 mg of protein per ml. Assuming a proteinaceous mitogen is in the range of 5 000 to 100 000 Da, and is maximally active at 0.1 to 10 nM then the growth factor would account for 167 ppm to 0.04 ppm of the total protein in serum (Gospodarowicz and Moran, 1976). Therefore, in order to get the pure growth factor from serum without any contaminants, one would require between 6000 and 24 million fold purification. These numbers also illustrate the advantage of starting the purification with an already partially purified plasma or serum fraction. In the case of SPRF, the best preparation found yet, Crude Bovine Thrombin, is derived from a prothrombin complex of Factors II, VII, IX and X that is activated with beef lung thromboplastin and CaCl$_2$ (Clark et al., 1989; Lundblad et al., 1976; Mann, 1976; White et al., 1979). The prothrombin complex is recovered from plasma by anion-exchange chromatography (Celliance, personal communication). In comparison to bovine serum, this Crude Bovine Thrombin preparation has 10 to 20-old higher specific activity for the cell cycle re-entry activity, or in other words the isolation of SPRF might require between 300 and 1.2 million-fold purification from this material if SPRF acts at the level of growth factors.

1.7 The Myotube Assay For Quantification Of The Purification

In order to screen for factors effecting the dedifferentiation of myotubes, an *in vitro* assay with multinucleated skeletal muscle cells from newt, derived from cultures of mononucleate A1 cells, was established allowing analysis of dedifferentiation events under very defined conditions (Lo et al., 1993). DNA-synthesis in the myotubes, assayed by incorporation of BrdU, was used as a marker to characterize factors for their ability to initiate this early step in dedifferentiation (Tanaka et al., 1997). The myotube assay, shown in a schematic outline in Figure 1.5, forms the basis of the activity assay used for the quantification of all purification procedures in this thesis work. In culture newt A1 myoblasts are propagated in medium containing 10% fetal bovine serum and adjusted to the osmolarity of axolotl plasma (Ferretti and Brockes, 1988). Newt A1 cells have a cell-cycle length 48 to 72 hours and are passaged with threefold
dilution once a week in 164 cm² cell culture flasks. After myoblasts have reached confluency they are detached and plated onto 10 cm dishes. Between 12 and 48 hours after plating, when the cells have attached and completely covered the dish, the concentration of fetal bovine serum in the medium is changed to 0.5% (low serum medium). Lowering the serum concentration induces within four to five days the fusion of mononucleated myoblasts into multinucleated myotubes, which subsequently express markers of muscle differentiation such as myosin heavy chain.

As described above cell cycle re-entry is dependent on the cell density of surrounding mononucleated cells. Hence, in order to get a robust response in the assay, the polynucleated myotubes must be separated from mononucleated non-fused myoblasts. Therefore, after 4 to 5 days in low serum medium, the mixture of myotubes and myoblasts is sieved through a 100-µm mesh to remove clumps of cells, followed by passage through a 35-µm mesh. While mononucleated cells pass through, these 35-µm sieves retain the larger myotubes. Purified myotubes are then distributed in 96-well plates and allowed to attach for 24 hours. Following this procedure, one well contains between 50 and 100 myotubes and contamination with myoblasts is usually less than 10%, excluding an influence of mononucleated cells on S-phase re-entry of myotubes.

The next day after myotube purification, when most of the myotubes have attached and already spread, a portion of the medium from each well is removed and replaced with samples from the purification of SPRF, which were desalted or dialyzed into low serum medium. All samples are assayed in triplicate. Furthermore, samples are added at different concentrations to ensure that the response is in the linear range.

At day four after sample addition, the peak of DNA synthesis, 5-Bromo-2'-deoxyuridine (BrdU, a thymidine analog) is added to cells for 12 hours. Finally, myotubes are fixed and stained with fluorescein-coupled antibody against myosin heavy chain and against rhodamine labeled antibody, recognizing BrdU, to determine the fraction of BrdU-positive myotubes. One unit of SPRF activity is defined as one percent BrdU-positive myotubes per well. Considering the volume of a sample, the protein concentration and the total volume of each chromatographic fraction, the purification factor and recovery based on the starting material can be determined.
Figure 1.5 - The myotube assay for the quantification of the purification of S-phase re-entry factor (SPRF) from Crude Bovine Thrombin

(A) Myotube purification: Newt A1 cells were cultured for four days in Low Serum AMEM (0.5% FCS) to induce myogenic differentiation. The cells were then trypsinized, filtered through 100 µm and 35 µm meshes. The myotubes retained on the 35 µm meshes were washed into Low Serum medium and plated into a fibronectin-coated 96-well plate. (B) Myotube assay: Fractions of the purification were added 24 hours after plating and samples were assayed in triplicate and the results averaged. Finally, BrdU was added at 10 µg/ml on day 4 for 12 h. The cells were fixed with 3% paraformaldehyde and stained for bromodeoxyuridine (BrdU, red) and for muscle-specific myosin (green). (C) Serum stimulates newt A1 myotubes to enter S-phase: Left panel shows three unstimulated myotubes while right panel shows one stimulated myotube (upper half) that has incorporated BrdU during the 12 h pulse upon serum stimulation. For the quantification of the purification the number of stimulated myotubes with BrdU incorporation and total number of myotubes in each well were counted and the percentage of BrdU-positive cells calculated.
1.8 Starting Point And Work Of This Thesis

In a series of experiments the serum activity was characterized and a strategy for optimal purification conditions from Crude Bovine Thrombin was developed (Tanaka and Drechsel, unpublished). As already mentioned above, based on gel filtration on Superose 12 the molecular weight of the factor was estimated to be 250,000-300,000 Daltons. Using the percentage of BrdU positive myotubes as a quantitative assay for activity, the S-phase re-entry factor was purified approximately 200-fold with 3% activity yield starting from a commercially available Crude Bovine Thrombin preparation using a three-step purification protocol (Table 1.3). The Crude Bovine Thrombin preparation was first loaded onto a cation exchange column (Poros20 HS) and eluted with an increasing NaCl gradient. The activity in the unbound fraction was pooled (F1P), directly loaded onto an anion exchange column (Poros20 HQ) and eluted with increasing NaCl concentration. Finally, pooled active fractions (HQ10–12) were purified over a heparin-sepharose column developed with a linear NaCl gradient (Hep-A to Hep-D). As Crude Bovine Thrombin contains at least 10-fold higher specific activity than serum, this purification procedure provided an approximately 2000-fold purification starting from serum. The most purified fraction on a silver stained gel contained multiple bands and after mass spectrometry sequencing none of the identified candidates could be confirmed as SPRF. From these preliminary results it became also clear that additional purification, perhaps another 100-fold, was required to identify the serum factor and eliminate other candidates. These preliminary purification experiments were the starting point of the PhD thesis work presented here. Based on this initial work we have carried out a more detailed characterization of the S-phase re-entry factor in the starting material Crude Bovine Thrombin. Furthermore a systematic investigation of various chromatographic modes and separation techniques, commonly used in the purification of proteins and peptides, was performed. Although this effort has not yet identified the S-phase re-entry factor, we present a five-step purification scheme that has significantly advanced our understanding of the biochemical properties of the active protein and has achieved a final preparation with substantially higher specific activity. Ongoing experiments are discussed at the end of this PhD thesis.
Table 1.3 - Summary of the best purification of the SPRF activity so far

Starting with Crude Bovine Thrombin the activity was purified by cation exchange chromatography, anion exchange chromatography and heparin affinity chromatography about 200-fold in the most purified fraction.

After cation exchange chromatography on Poros 20 HS the flow through was pooled (HS F1P), treated with PPACK and loaded onto a Poros 20 HQ anion exchange column. Bound proteins were eluted with a linear gradient of sodium chloride and most active fractions pooled (HQ10-12) and directly loaded onto a heparin column. Again adsorbed proteins were eluted with a linear gradient of NaCl and active fractions grouped into 4 pools. Of these pools HEP-D displayed the highest specific activity with about 200-fold purification from starting material. N.A. = not applicable

<table>
<thead>
<tr>
<th>Column</th>
<th>Fraction</th>
<th>Purification (fold)</th>
<th>Activity yield (%)</th>
<th>Total Protein (mg)</th>
<th>Total activity in fraction (Units)*</th>
<th>Specific activity (units/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum</td>
<td>N.A.</td>
<td>N.A.</td>
<td>6</td>
<td>100</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>Starting material</td>
<td>Crude Bovine Thrombin</td>
<td>1</td>
<td>100</td>
<td>127</td>
<td>92666</td>
<td>730</td>
</tr>
<tr>
<td>Cation exchange (Poros 20 HS)</td>
<td>HS F1P</td>
<td>2</td>
<td>68</td>
<td>52.8</td>
<td>63333</td>
<td>1199</td>
</tr>
<tr>
<td>Anion exchange (Poros 20 HQ)</td>
<td>HQ F10-12</td>
<td>4</td>
<td>17</td>
<td>5.7</td>
<td>16128</td>
<td>2829</td>
</tr>
<tr>
<td>Heparin affinity (HiTrap Heparin)</td>
<td>HEP--A</td>
<td>5</td>
<td>2</td>
<td>0.594</td>
<td>2019</td>
<td>3392</td>
</tr>
<tr>
<td></td>
<td>HEP--B</td>
<td>21</td>
<td>2</td>
<td>0.139</td>
<td>2138</td>
<td>15521</td>
</tr>
<tr>
<td></td>
<td>HEP--C</td>
<td>118</td>
<td>3</td>
<td>0.035</td>
<td>2992</td>
<td>86244</td>
</tr>
<tr>
<td></td>
<td>HEP--D</td>
<td>182</td>
<td>3</td>
<td>0.023</td>
<td>3095</td>
<td>132645</td>
</tr>
</tbody>
</table>

** One unit is the defined as the amount of SPRF-activity that stimulates 1% of myotubes to undergo S-Phase (BrdU-positive myotubes in 150 µl medium volume)
2 Results

2.1 Characterization Of S-Phase Re-Entry Factor

2.1.1 Crude Bovine Thrombin: Inhibition Of Proteolytic Activity In The Starting Material

The basic biochemical properties of S-Phase Re-entry factor (SPRF) were examined in a series of experiments using the starting material for the purification, a Crude Bovine Thrombin preparation. This Crude Bovine Thrombin (or Bovine Thrombin) preparation is a commercially available, partially purified and lyophilized thrombin preparation that contains at least 10-fold enriched SPRF activity compared to fetal calf serum (Tanaka et al., 1999). In order to distinguish between our activity and thrombin, which is an activator of the factor, the thrombin preparation is treated with PPACK to irreversibly inhibit thrombin proteolytic activity before assaying on cells (Bode et al., 1989). Inhibition of thrombin is also necessary for survival of myotubes, which otherwise detach and undergo apoptosis.

2.1.2 Protease Sensitivity

First of all we tested whether the activity was attributable to a protein, or a non-proteinaceous entity associated with a carrier. For example Mauch et al, reported that cholesterol was the active entity involved in synaptic maturation associated with ApoE in supernatants from glial cells (Mauch et al., 2001). Therefore the sensitivity of the SPRF to proteolytic digestion was tested. Crude thrombin preparations were incubated with active proteinase K, or proteinase K that had been inactivated with PMSF or by boiling. Figure 2.1A shows that incubation with the active proteinase K reduced the cell cycle re-entry activity 10-fold. This result strongly suggests that the active moiety in serum is a protein.

2.1.3 Temperature Sensitivity and pH-Stability

As an initial test of the protein's properties its stability to incubation at different pH-values (Beynon and Easterby, 1996; Rehm, 2000) and temperatures was examined (Figure 2.1A, B). After 12 hours incubation at room temperature the protein shows a broad range of pH stability, with essentially full recovery of activity in the range between pH 5 and 11.
A reduced but significant proportion of activity was also recovered at pH 2 and 3. At pH 3 and 4 evidence of precipitation in the preparation was observed, indicating many proteins in the preparation may possess an isoelectric point around this pH. The activity is fully stable when incubated at temperatures up to 40°C for 4 h with progressively reduced recovery between 50-80°C (Figure 2.1). This heat lability also argues the activity is associated with a protein.

### 2.1.4 Glycosylation Of SPRF

Many extracellular proteins and in particular serum proteins become glycosylated during biosynthesis, and this modification is often important for function (Robertson and Kennedy, 1996). To ask if the activity is a glycoprotein it was determined which lectins could deplete the activity from Hep3, a partially purified fraction containing high levels of SPRF (Gerard, 1990; West and Goldring, 1996). Concavalin A (ConA), Jacalin, Sambucus Nigra Lectin (SNA), and Wheat Germ Agglutinin (WGA) fully depleted the activity, while Peanut Agglutinin partially depleted the activity (Figure 2.2A). No depletion was observed with Soybean Agglutinin (SBA) and Ulex Europaeus Agglutinin I (UEA I). This profile of lectin binding indicates that either the serum activity or a tightly associated protein likely harbors glucose or mannose, N-acetyl glucosamine, galactose, sialic acid and perhaps galactose sugar entities.

To test the functional role of glycosylation, the Crude Bovine Thrombin mix was incubated with Peptide: N-Glycosidase F (PNGFase), which cleaves between the innermost N-acetyl glucosamine and asparagine residues of high mannose, hybrid and complex oligosaccharides from N-linked glycoproteins (Maley et al., 1989; O’Neill, 1996). After such treatment approximately 50% of the activity was recovered, suggesting that N-glycosylation may exert a functional role in the molecule (Figure 2.2B). Efficient deglycosylation of the preparation was evidenced by the clear shift of bands (arrows, Figure 2.2C). It is yet unclear if this partial inactivation stems from incomplete deglycosylation of N-linked carbohydrates, the presence of O-linked glycans that are resistant to PNGase F or simply a partial requirement of carbohydrate modification for full SPRF activity.
2.1.5 Thrombin Treatment Of Starting Material

Initial characterization experiments of the cell cycle re-entry factor revealed that the SPRF activity could be generated in fetal calf serum upon incubation with thrombin (Tanaka et al., 1999). This suggests a proteolytic action of thrombin in a direct or indirect way on the activity. Although the Crude Bovine Thrombin starting material contains high concentrations of active thrombin, it was not clear whether all possible activity is already generated in the starting preparation. Therefore Crude Bovine Thrombin was mixed with partially purified thrombin (HiTrap CMFF 1.5 M NaCl eluate, “CMFF-1.5M”) and then incubated for 4, 8, 24 and 48 hours at room temperature before all thrombin was irreversibly inhibited with PPACK, prior to assay on cells. A comparison of different incubation times clearly showed no increase of specific activity. In fact, a significant reduction down to 50% after 48 h was observed, indicating a prolonged incubation with thrombin leads to non-specific proteolysis of SPRF. This result also proposes a rapid execution of the first purification step that serves to largely remove thrombin, in order to stabilize the preparation after reconstitution of the lyophilized starting material. After the first step residual thrombin activity should then be inhibited completely with PPACK in order to prevent any loss of the SPRF activity in the preparation.

2.1.6 Sensitivity Of SPRF To Reducing Agents

Disulfide bonds, essential for structure and activity, are often found in secreted proteins present in serum (Betz, 1993; Bulaj, 2005). In order to detect the presence of crucial disulfide bonds in SPRF, reducing agents were incubated with samples prior to assay on cells. Incubation of Crude Bovine Thrombin with 10 mM or 100 mM DTT for 12 hours decreased the amount of activity by half but did not remove it completely. From these results it is not clear, whether or not disulfide bonds are essential for the activity. It is also possible that after desalting the sample into medium disulfide bonds were reformed. A clearer answer to this question of the role of disulfide bonds would be obtained by the addition of iodoacetamide after DTT-treatment to irreversibly block reformation of disulfide bonds.
2.1.7 Stability Of Cell Cycle Re-Entry Factor In Organic Solvents, SDS, Urea And Water

Fractionation of proteins by precipitation with increasing concentration of ethanol or salt is often used as a first step in purifications starting from plasma (Burnouf, 1995; Clark et al., 1989). In addition precipitation is successfully used for concentrating proteins from very dilute solutions (Pohl, 1990). To test whether activity can survive precipitation with organic solvents, Crude Bovine Thrombin was incubated with increasing concentrations of ice-cold ethanol or acetone (Englard and Seifter, 1990; Scopes, 1994). Specifically, Crude Bovine Thrombin was mixed on ice with acetone or ethanol to a final concentration of 20 to 60% organic solvent. After 15 min incubation on ice, precipitated proteins were sedimented by centrifugation and the pellets resuspended in Anion Buffer, 100 mM NaCl (pH 7.0) prior to assay on cells. We found that in the presence of 30%, 40% and 60% ethanol or acetone, SPRF activity precipitated. Following resuspension of the pellet 50% of SPRF activity or more were recovered.

No attempt has been made so far to test selective precipitation of Crude Bovine Thrombin as a purification step but this technique may find use in later stages of the purification for either concentrating proteins or removing contaminants such as salts or SDS. In addition the robustness of SPRF activity to organic solvents may allow the use of chromatographic modes that require organic modifier such as reversed phase chromatography or hydrophilic interaction chromatography.

To find out whether purification procedures could be carried out under denaturing conditions, we tested how much activity could be recovered after incubation in sodium dodecyl sulfate (SDS) or urea. A partially purified fraction after heparin affinity chromatography (Hep3, see below) was incubated either with 1% SDS (for gel filtration in SDS or preparative SDS-PAGE) or in 8 M urea, 2% CHAPS (for isoelectric focusing) for 24 h and afterwards dialyzed against PBS and Serum Free AMEM. No significant loss of activity was found for either SDS or urea treatment, indicating once more that the activity is a very robust protein.

Finally, the stability of SPRF in water was investigated. Partially purified fractions (CMFF-FT and Butyl-20% EtOH, see below for details) were dialyzed against water at 4°C. Precipitation occurred after complete removal of salts, but after addition of 10X PBS all precipitates were immediately re-dissolved and full SPRF activity was recovered.
Figure 2.1 - Characterization of the SPRF activity found in Crude Bovine Thrombin preparations

(A) The activity is attributable to a protein. Incubation of Crude Bovine Thrombin with proteinase K destroyed the cell cycle re-entry activity. As a control, proteinase K was inactivated by PMSF treatment or boiling prior to incubation with thrombin. (B) The serum activity retains activity over a broad pH range. Crude Bovine Thrombin was incubated for 12 hours at various pHs. Significant activity was recovered between pH 5 and pH 11. (C) The serum factor is heat labile. The serum factor retained robust activity up to 40 °C. Activity was progressively lost at higher temperatures with no recovery at 100 °C.
Figure 2.2 - Assessment of glycosylation of cell cycle re-entry activity

(A) Lectin binding assays with partially purified material demonstrate that the activity is a glycoprotein or bound to glycoproteins. Active fractions (Hep3 pool) were incubated with various lectins with affinity for different sugars. ConA, Jacalin, SNA and WGA efficiently bound to the activity and depleted it from the active fractions. 

(B) Incubation of the serum factor with PNGaseF diminishes the activity. 

(C) Non-reducing SDS-PAGE of the PNGaseF-treated fraction. Note the discrete shift in bands compared to the untreated sample (arrows).
2.1.8 Characterization Of Molecular Weight

Earlier studies found that SPRF in serum fractionates on gel filtration at a molecular weight of 250,000 Daltons when run under native conditions (PBS) and at neutral pH (Tanaka et al., 1997). It is unclear whether this represents the native molecular weight of SPRF or reflects either its self-association under these conditions or its binding to a carrier protein present in serum. To investigate this issue we determined the apparent molecular weight of SPRF in the Crude Bovine Thrombin preparation, under native and denaturing conditions, by size exclusion chromatography on Superdex 200 (Cutler, 1996; Stellwagen, 1990). In order to separate non-covalently linked protein subunits from each other, gel filtration was carried out under two denaturing conditions, either PBS containing 0.1% SDS or at pH 11 (20 mM CAPS, 200 mM NaCl). Crude Bovine Thrombin was dissolved in Cation Buffer (pH 6.5) and, after thrombin inhibition, either desalted into PBS (pH 7.4) or 20 mM CAPS, 200 mM NaCl (pH 11). In order to completely denature proteins for SDS gel filtration, SDS was added to Crude Bovine Thrombin in PBS to a final concentration of 1%.

When run at high concentrations under native conditions, in PBS (pH 7.4), the activity in the Crude Bovine Thrombin preparation predominantly eluted between the 232 kD marker and the exclusion volume marker Blue Dextran 2000 (Figure 2.3A) with some trailing of activity towards lower molecular weights, from 230 to 43 kD. At lower concentrations of Crude Bovine Thrombin, activity was also found in a lower molecular weight range from 150 to 200 kD (data not shown). This concentration dependent migration behavior suggests that SPRF under native conditions undergoes a low affinity association either with itself or with a contaminating protein in Crude Bovine Thrombin.

When gel filtration was performed at pH 11, the activity eluted at a significantly lower molecular weight as a broad peak spanning 90 kD to 20 kD (Figure 2.3B). Compared to the resolution of runs with homogenous marker proteins, this elution pattern suggests that either multiple proteins, differing in their molecular weight, bear SPRF activity or that high pH may not fully denature the preparation. Nonetheless, gel filtration at pH 11 provides a simple and easy way to separate SPRF from the bulk of high molecular weight proteins.

In the presence of SDS, most proteins dissociate from each other, are completely denatured and migrate as unfolded polypeptide chains on size exclusion chromatography. Under these conditions SPRF displayed a sharp peak of activity at a molecular weight around 20 kD (Figure 2.3C). This implies that the active and functional
form of SPRF is a relatively homogeneous, low molecular weight protein. However, with this result one can still speculate whether the 20 kD form of the protein is sufficient to induce cell cycle re-entry or whether multimerization occurs upon transfer to medium for assay on cells and is required for activity.

In summary these results from molecular size exclusion chromatography indicate that the factor may multimerize or associate with a high molecular weight complex under native conditions and that high pH and addition of SDS may block these interactions. It is unclear so far, whether the complex formation is physiologically relevant or if it is an artifact of this preparation of Crude Bovine Thrombin.
Results

Figure 2.3 - Size exclusion chromatography of Crude Bovine Thrombin preparation under native and denaturing conditions

(A) Under native conditions the activity associates with a macromolecular complex. When applied to a Superdex-200 column in native, neutral pH conditions the activity elutes between the excluded volume and 200 kD. (B) High pH dissociates the macromolecular complex. At pH 11 the activity displays a molecular weight between 90 kD and 20 kD. (C) SPRF is a low molecular weight protein. Under denaturing conditions in SDS, SPRF activity migrates as a protein with a molecular weight of 20 kD. Arrows indicate molecular weight standards: 1, excluded volume; 2, 669 kD; 3, 440 kD; 3, 232 kD; 4, 158 kD; 5, 158 kD; 6, 67 kD; 7, 43 kD; 8, 25 kD; 9, 13.7 kD. Black line denotes the absorbance profile at 280 nm (left axis). The orange bars represent the activity found in each fraction (right axis).
2.2 Chromatographic Fractionation Of The Serum Factor

2.2.1 Cation Exchange Chromatography

2.2.1.1 Screening Of Cation Exchange Columns (pH 6.5)

The primary objectives of this first phase of purification were the removal of thrombin and unstable, precipitating contaminants from the Crude Bovine Thrombin preparation as well as achieving a high recovery of the activity. Various cation exchange column chemistries were tested as a first step. In small-scale experiments Crude Bovine Thrombin was loaded and bound proteins were eluted with step gradients of sodium chloride (Bollag, 1994; Choudhary and Horvath, 1996).

Lyophilized Crude Bovine Thrombin preparation, the starting material for the purification, has a pH of about 6.5, when dissolved in water. In contrast to thrombin many other serum proteins have an isoelectric point of less than 7 and would therefore not bind to a cation exchange medium at pH 6.5 (Berg et al., 1979; Brosstad, 1977). To test an efficient separation of thrombin and other proteins from the SPRF activity, in an initial screening cation exchange chromatography was performed at pH 6.5. Five columns with different cation exchange media were tested. These included strong cation exchangers such as HiTrap SPFF or Resource S and a weak cation exchanger, HiTrap CMFF.

Specifically Crude Bovine Thrombin was dissolved in Cation Buffer (pH 6.5) to a concentration of 5.5 mg/ml, loaded onto columns and bound proteins were eluted stepwise with 5 CV containing 0, 100, 200, 500 and 1500 mM sodium chloride. All tested columns had a slightly different elution profile with differences in recovery and purification in each single fraction. Furthermore, for all columns two peaks of activity were observed, one in the flow through fraction (FT) and one at 500 mM and 1500 mM sodium chloride (Table 2.1). Importantly, most of thrombin, as detected by protease activity, was eluted at higher concentrations of NaCl. Although every effort was made to fully inhibit thrombin with the specific and potent inhibitor, PPACK, some activity found in these high salt fractions could derive from residual thrombin, that was not completely inhibited and generated a background response when assayed in medium containing 0.5% FBS (Low Serum AMEM).

HiTrap CMFF (108%) and HiTrap SPFF (86%) columns were found to give the highest recovery in the flow through fractions with a purification factor of 1.6 and 1.7 respectively. In both cases thrombin was eluted with 500 mM and 1500 mM sodium chloride and thereby removed from the bulk of the SPRF activity. Based on these results both resins were considered as appropriate first steps in the overall purification scheme in order to separate SPRF activity from thrombin.
Table 2.1 - Comparison of purification and recovery of activity on different cation exchange media

Crude Bovine Thrombin was loaded onto each column and eluted with a step gradient of sodium chloride in Cation Buffer (pH 6.5). For each fraction yield (%) and purification factor (fold) for SPRF-activity were determined (e.g. 108 / 1.6 stands for 108% recovery with 1.6 fold purification). Fractions containing the bulk of activity in one run are shown in **bold**. FT: flow through; N.D.: not determined

<table>
<thead>
<tr>
<th>Column</th>
<th>Yield (%) / Purification (fold)</th>
<th>[NaCl] in elution step (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% fold</td>
<td>% fold</td>
</tr>
<tr>
<td>HiTrap CM FF</td>
<td>108</td>
<td>1.6</td>
</tr>
<tr>
<td>HiTrap SP FF</td>
<td>86</td>
<td>1.7</td>
</tr>
<tr>
<td>Resource S</td>
<td>76</td>
<td>1.2</td>
</tr>
<tr>
<td>Fractogel EMD (SO3)</td>
<td>36</td>
<td>0.8</td>
</tr>
<tr>
<td>Poros 20 HS</td>
<td>15</td>
<td>0.3</td>
</tr>
</tbody>
</table>

2.2.1.2 Test Of Different pHs On Cation Exchange Columns

Although we had already discovered conditions to separate SPRF activity from thrombin in the initial screen of cation exchangers, we were interested in finding out if at lower pH, where the net positive charge would be higher, the factor would bind the resin rather than flow through after loading (Choudhary and Horvath, 1996). Under these conditions a better purification might be achieved as activity might be separated from the bulk of contaminating proteins in the flow through at pH 6.5.

For comparison, a strong (HiTrap SPFF) and a weak cation exchanger (HiTrap CMFF) were chosen and chromatography performed at pH 4.5 and 5.5 with a step gradient of NaCl. However, when Bovine Crude Thrombin was dissolved in Cation Buffer at pH 5.5 and particularly pH 4.5 only a fraction of total protein was dissolved. In order to check if this precipitation could be used as a purification step, Bovine Crude Thrombin was centrifuged and then the supernatant loaded onto cation exchange columns. Subsequently, bound proteins were eluted with step gradients of 0, 100, 200 and 1000 mM sodium chloride.

When chromatography was performed at pH 5.5 on HiTrap CMFF, SPRF activity was still obtained in flow through while on HiTrap SPFF most of the activity was now recovered in 1000 mM sodium chloride fraction with little change in the purification factor,
compared to chromatography at pH 6.5 (Table 2.2). The total amount protein and SPRF activity at pH 5.5 was similar to experiments with Cation Buffer at pH 6.5.

In contrast, when bovine thrombin was dissolved in Cation Buffer at pH 4.5 only 30% of proteins were soluble and only 50% of SPRF activity was recovered. After chromatography at pH 4.5 almost no activity was recovered on HiTrap CMFF and only 20% in the 1000 mM fraction of HiTrap SPFF (Table 2.2). This loss might be due to either a precipitation on the columns when proteins are close at their isoelectric point or an irreversible loss of activity at this pH. In summary, neither cation exchange chromatography at pH 5.5 nor at 4.5 provided a comparative advantage and therefore we continued with pH 6.5 for the cation exchange step in the purification scheme.

Table 2.2 - Comparison of activity yield and purification factor for cation exchange chromatography at pH 4.5, 5.5 and 6.5

Crude Bovine Thrombin was dissolved in Cation Buffer at pH 4.5 or 5.5, soluble proteins were loaded onto each column and eluted with a step gradient of sodium chloride. For each fraction yield (%) and purification factor (fold) for SPRF-activity were determined (for example 50 / 0.7 stands for a 50% recovery with 0.7 fold purification). Fractions containing most of activity under each condition are shown in bold.

FT: flow through; N.D.: not determined

<table>
<thead>
<tr>
<th>Column</th>
<th>[NaCl] (mM)</th>
<th>pH (Cation Buffer)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>pH 4.5</td>
</tr>
<tr>
<td></td>
<td>Yield (%)</td>
<td>%</td>
</tr>
<tr>
<td></td>
<td>Purification (fold)</td>
<td>fold</td>
</tr>
<tr>
<td>HiTrap CM FF</td>
<td>FT (0)</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>N.D.</td>
</tr>
<tr>
<td></td>
<td>1000</td>
<td>6</td>
</tr>
<tr>
<td>HiTrap SP FF</td>
<td>FT (0)</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>N.D.</td>
</tr>
<tr>
<td></td>
<td>1000</td>
<td>19</td>
</tr>
</tbody>
</table>
2.2.1.3 Cation Exchange Chromatography On HiTrap CMFF As First Step Of The Purification

In all the descriptions of multi-step purifications that follow, the first step, cation exchange chromatography, was performed on a 5 ml HiTrap CMFF or 20 ml HiPrep CMFF column in Cation Buffer (pH 6.5). Crude Bovine Thrombin was loaded onto a HiTrap CMFF column, washed with 10 CV Cation Buffer and bound proteins eluted with 5 CV 1500 mM sodium chloride (Figure 2.4). Pooled flow through fractions (“CMFF-FT”) contained 100% of the SPRF activity with about 1.3-fold purification, whereas 30% of proteins including thrombin were bound to the column and eluted with 1500 mM NaCl (“CMFF-1.5M”). Although cation exchange chromatography does not provide a significant purification, it is an important step for following reasons. Thrombin, known to be an activator of SPRF and toxic to myotubes at high concentrations in the assay, is separated from the SPRF-activity without any loss of yield. Remaining thrombin activity in the flow fractions can now be easily inhibited by PPACK and therefore a background response in the myotubes assay can be excluded. Furthermore, compared to Crude Bovine Thrombin starting material, the pooled flow through is stabilized in solution and no precipitation occurs.

![Figure 2.4 - Cation exchange chromatography on HiTrap CMFF for removal of thrombin](image)

**Figure 2.4 - Cation exchange chromatography on HiTrap CMFF for removal of thrombin**

**(A) Chromatogram:** Crude Bovine Thrombin (CB-Thrombin), dissolved in Cation Buffer (pH 6.5), was loaded onto a HiTrap CMFF column, the flow through (CMFF-FT, 2.5 volumes of loading volume) pooled and remaining thrombin activity inhibited with PPACK. Bound proteins, including thrombin, were eluted from the column with 1.5 M NaCl (CMFF-1.5M).

**(B) Silver stained gel of non-reducing SDS-PAGE:** In each lane the same amount of protein (0.5 µg) was loaded.
2.2.2 Anion Exchange Chromatography

2.2.2.1 Screening Of Different Anion Exchange Columns At pH 7.0

Although cation exchange chromatography at pH 6.5 on HiTrap CMFF gave high recovery and separated SPRF-activity from unstable proteins as well as thrombin, almost no purification was achieved. Therefore we investigated anion exchange chromatography as the first step in the purification. Based on their behavior on cation exchange, where the factor failed to bind and thrombin bound strongly, it was anticipated that on anion exchange the opposite would occur: thrombin would flow through and SPRF would bind. In order to find the optimal resin we screened through several weak and strong anion exchangers (Bollag, 1994; Choudhary and Horvath, 1996). In an initial screen, strong anion exchangers such as HiTrap Q, Poros20 HQ or Resource Q and weak anion exchangers like HiTrap DEAE or Fractogel DEAE were tested and the assumption confirmed. Thrombin was found in the flow through and SPRF-activity was recovered when columns were eluted with more than 200 mM sodium chloride (data not shown).

For that reason a more careful experiment was performed using three columns with different anion exchange media. These included the strong anion exchangers, HiTrap Q, Resource Q, and the weak anion exchanger, HiTrap DEAE. Additionally, it was tested whether inclusion of a modifier, such as ethanol, to the mobile phase, blocks hydrophobic interactions with the backbone and has an effect on recovery and purification. Therefore a second series of identical runs was performed in Anion buffer (pH 7.0), containing 20% ethanol.

Crude Bovine Thrombin was dissolved in Anion Buffer (pH 7.0), loaded onto columns and eluted stepwise with 5 CV of 0, 100, 200, 500 and 1600 mM sodium chloride. For all columns, SPRF-activity was bound and eluted with high recovery in fractions containing 200, 400 or 800 mM sodium chloride. Addition of 20% ethanol to the buffer caused SPRF-activity to elute at lower concentrations of sodium chloride but did not significantly change the yield or purification for any column type (Table 2.3). Although excellent activity yield was achieved for all three columns (between 60 and 100% per run) no notable purification was accomplished, because SPRF-activity always eluted along with the bulk of proteins.
Table 2.3 - Test of anion exchange columns as initial purification step

Crude Bovine Thrombin was loaded onto each column and eluted with a step gradient of sodium chloride in Anion Buffer (pH 7.0) with or without 20% ethanol. The flow through (FT) fraction is represented by 0 mM NaCl. For each fraction yield (%) and purification factor (fold) for SPRF-activity were determined (for example, 27 / 0.6 stands for 27% recovery with 0.6 fold purification). Fractions containing most of activity in one run are shown in bold. FT: flow through; EtOH: ethanol.

<table>
<thead>
<tr>
<th>Column</th>
<th>Buffer additive</th>
<th>Yield (%) / Purification (fold)</th>
<th>[NaCl] (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>% fold</td>
<td>0</td>
</tr>
<tr>
<td>HiTrap DEAE</td>
<td>-</td>
<td>4 0.3</td>
<td>27</td>
</tr>
<tr>
<td></td>
<td>+ 20% EtOH</td>
<td>2 0.2</td>
<td>67</td>
</tr>
<tr>
<td>HiTrap Q</td>
<td>-</td>
<td>5 0.6</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>+ 20% EtOH</td>
<td>1 0.2</td>
<td>39</td>
</tr>
<tr>
<td>Resource Q</td>
<td>-</td>
<td>2 0.2</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>+ 20% EtOH</td>
<td>0 0.0</td>
<td>64</td>
</tr>
</tbody>
</table>

2.2.2.2 Optimization Of HiTrap Q After Cation Exchange Chromatography On HiTrap CMFF

In order to include an anion exchange chromatography step into the purification scheme a more careful examination of elution conditions that compared linear vs. step salt gradients was undertaken. After cation exchange chromatography on HiTrap CMFF, the flow through fraction (CMFF-FT) was directly loaded onto a HiTrap Q column and bound proteins eluted with either a linear or step gradient of sodium chloride in Anion Buffer (pH 7.0).

2.2.2.1 Step Gradient On Hitrap Q

In order to develop a fast and simple purification procedure we first tested a stepwise elution of bound proteins from the HiTrap Q anion exchange column. When activity was eluted with a step gradient of 200, 400, 600, 800 and 1600 mM NaCl most of the activity was found in fractions containing 400 mM and 600 mM NaCl with 1.1 or 2.6-fold purification respectively (Table 2.4, Figure 2.5). These results are consistent with those
from anion exchange column screening, where Crude Bovine Thrombin was directly loaded.

Table 2.4 - Anion exchange chromatography on HiTrap Q after cation exchange chromatography (HiTrap CMFF) - Step gradient of sodium chloride on HiTrap Q

The Flow through of HiTrap CMFF (CMFF-FT: 100% yield / 1.3-fold purification) is applied to the HiTrap Q column and eluted with a step gradient of sodium chloride in Anion Buffer (pH 7.0). Most of SPRF activity eluted both at 400 mM 600 mM NaCl. The flow through (FT) fraction is represented by 0 mM NaCl. For each fraction yield (%) and purification factor (fold) for SPRF-activity starting from Crude Bovine Thrombin were determined. Fractions containing most of activity in one run are shown in bold. FT: flow through

<table>
<thead>
<tr>
<th>[NaCl] (mM)</th>
<th>0 (FT)</th>
<th>200</th>
<th>400</th>
<th>600</th>
<th>800</th>
<th>1600</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yield (%) / Purification (fold)</td>
<td>% fold</td>
<td>% fold</td>
<td>% fold</td>
<td>% fold</td>
<td>% fold</td>
<td>% fold</td>
</tr>
<tr>
<td>Column</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HiTrap Q</td>
<td>2 1.9</td>
<td>4 0.3</td>
<td>50 1.1</td>
<td>42 2.6</td>
<td>0 0.0</td>
<td>0 0.0</td>
</tr>
</tbody>
</table>

2.2.2.2 Optimization Of Step Gradient On HiTrap Q

For further optimization of a step gradient on HiTrap Q an additional series of experiments was carried out with smaller stepwise increases in sodium chloride concentration between 200 and 600 mM. HiTrap CMFF flow through was loaded onto the HiTrap Q column and was then washed with 5 CV of Anion Buffer (pH 7.0). In order to find the lowest concentration of sodium chloride where our SPRF activity remained bound while contaminating proteins were removed; adsorbed proteins were eluted with either 200, 300 or 350 mM and afterwards with 600 mM NaCl. For the determination of the lowest sodium chloride concentration where all SPRF activity elutes, adsorbed proteins were eluted with 200 mM and afterwards with either 450, 500 or 600 mM NaCl. These experiments showed that SPRF-activity starts to elute around 300 mM and is completely eluted from the column at 600 mM NaCl. Intermediate concentrations of NaCl gave partial elution of the activity. No further significant purification could be achieved with one of these step gradients.
2.2.2.2.3 Linear Gradient On HiTrap Q

Linear gradients often give superior resolution of closely eluting proteins on ion exchange chromatography. Therefore we also tested a linear gradient of NaCl on a HiTrap Q anion exchange column after cation exchange chromatography on CMFF.

Using a linear gradient of sodium chloride from 0 to 1600 mM NaCl, SPRF-activity eluted from the column over a broad concentration with a peak of activity between 300 and 700 mM NaCl (Figure 2.6). If only those fractions showing increased specific activities (two to threefold higher than Crude Bovine Thrombin) were pooled, then the overall yield would be low. On the other hand, if all the active fractions were pooled, then the yield would be good but little or no net purification would be achieved. In conclusion, as SPRF activity elutes in a broad peak and is not well resolved from the bulk of contaminating protein, anion exchange on HiTrap Q is not an effective step at this stage in the overall purification.
Figure 2.5 - Anion exchange chromatography on HiTrap Q - step gradient
When the Flow through of cation-CMFF (CMFF-FT) is applied to the HiTrap Q column and eluted with a step gradient of sodium chloride, activity elutes both at 400 mM and 600 mM NaCl. No activity was found at higher concentrations of NaCl.

Figure 2.6 - Anion exchange chromatography on HiTrap Q - linear gradient
When the Flow through of cation-CMFF (CMFF-FT) is applied to HiTrap Q column and eluted with a linear gradient of sodium chloride from 0 to 1600 mM, activity elutes with the bulk of contaminating proteins between 300 mM and 700 mM NaCl.
2.2.2.3 Mono Q Anion Exchange Chromatography After Heparin Affinity Chromatography

Compared to HiTrap Q columns that are packed with 90µm beads, superior resolution is often achieved on columns packed with Mono Q resin as these are smaller (10 µm) and show a more uniform size distribution (Regnier, 1984). The drawback to using Mono Q is its expense and hence the difficulty in scaling up the purification for large protein loads. In order to test this resin we then loaded a more purified preparation after cation exchange (CMFF-FT) and heparin affinity chromatography (Hep3, see below), so that activity could be easily assayed after fractionation without exceeding the total capacity of the Mono Q resin. Hep3 protein was loaded onto a 1 ml–Mono Q column at 50 mM NaCl and a salt gradient up to from 50 to 1500 mM NaCl over 30 column volumes was used for elution of bound proteins. Fractions were desalted into Low Serum AMEM and assayed on cells.

The Mono Q column revealed a spread of activity over a number of fractions with a peak between 350 and 800 mM NaCl (corresponding to MonoQ fractions 4 – 9 in Table 2.5 and Figure 2.7A). No activity was recovered in the flow through and wash fractions. We found that the overall purification after cation exchange, heparin affinity and anion exchange chromatography was superior compared to the fractionation on Hitrap Q. Across the peak of activity we calculated purification factors from 50 to 136-fold, starting from Crude Bovine Thrombin. In addition, total recovery of SPRF for the Mono Q step was 100%. SDS-PAGE analysis of fractions across the gradient displayed well-resolved bands, however, the total number of different proteins was still too high to correlate SPRF-activity with certain bands from this gel (Figure 2.7B). In conclusion, anion exchange chromatography is not a good step at this stage of the purification, but might be a promising step at later stages of the purification, where we want to correlate bands with activity.
Table 2.5 - Anion exchange chromatography on MonoQ
Overview over the purification of S-phase stimulating activity with cation exchange (HiTrap CMFF), heparin-sepharose (HiTrap Heaprin) and anion exchange (MonoQ) chromatography; For each fraction yield (%) and purification factor (fold) is shown. Fractions containing most of activity in the Mono Q run are shown in bold.

<table>
<thead>
<tr>
<th>Column</th>
<th>Fraction name</th>
<th>Overall</th>
<th></th>
<th></th>
<th>Step</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Yield</td>
<td></td>
<td></td>
<td>Yield</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>(%)</td>
<td></td>
<td></td>
<td>(%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Purification</td>
<td>(fold)</td>
<td></td>
<td>Purification</td>
<td>(fold)</td>
<td></td>
</tr>
<tr>
<td>Starting material</td>
<td>CB-Thrombin</td>
<td>100</td>
<td>1.0</td>
<td></td>
<td>100</td>
<td></td>
<td>1.0</td>
</tr>
<tr>
<td>HiTrap CMFF</td>
<td>CMFF-FT</td>
<td>99</td>
<td>1.3</td>
<td></td>
<td>99</td>
<td></td>
<td>1.3</td>
</tr>
<tr>
<td>HiTrap Heparin</td>
<td>Hep3</td>
<td>21</td>
<td>21</td>
<td></td>
<td>21</td>
<td></td>
<td>16.6</td>
</tr>
<tr>
<td>Mono Q</td>
<td></td>
<td>0</td>
<td>0</td>
<td></td>
<td>0</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1</td>
<td>0</td>
<td></td>
<td>0</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>0</td>
<td>2</td>
<td>1</td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
<td>1</td>
<td>18</td>
<td>6</td>
<td>0.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>4</td>
<td>4</td>
<td>61</td>
<td>19</td>
<td>2.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>5</td>
<td>3</td>
<td>50</td>
<td>15</td>
<td>2.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>6</td>
<td>3</td>
<td>66</td>
<td>16</td>
<td>3.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>7</td>
<td>1</td>
<td>51</td>
<td>6</td>
<td>2.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>8</td>
<td>3</td>
<td>136</td>
<td>13</td>
<td>6.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>9</td>
<td>2</td>
<td>64</td>
<td>9</td>
<td>3.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>10</td>
<td>2</td>
<td>28</td>
<td>9</td>
<td>1.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>11</td>
<td>2</td>
<td>29</td>
<td>12</td>
<td>1.9</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>22</td>
<td></td>
<td></td>
<td>106</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Results

Figure 2.7 - Anion exchange chromatography of Hep3 pool on Mono Q

(A) Chromatogram: Following cation exchange chromatography on CMFF and heparin affinity chromatography the most purified pool, Hep3, was desalted into Anion Buffer (pH 7.0), 50 mM NaCl by concentration and dilution on a 10 kD ultrafiltration membrane. Desalted Hep3 pool was loaded onto a 1 ml MonoQ column (in Anion Buffer, pH 7.0) and eluted with an increasing linear gradient of sodium chloride from 50 to 1500 mM NaCl. Activity was recovered in fractions between 250 mM (Mono Q3) and 1000 mM NaCl (MonoQ 11).

(B) Silver stained gel across the MonoQ run: In each lane 0.5 µg total protein was loaded and separated under reducing conditions.
2.2.3 Hydrophobic Interaction Chromatography

2.2.3.1 Test Of Different Hydrophobic Interaction Chromatography (HIC) Columns After Cation Exchange Chromatography

Previous experiments had shown that the SPRF-activity was relatively hydrophobic as it stuck strongly to hydrophobic interaction chromatography (HIC) resins (Tanaka and Drechsel, unpublished). Thus hydrophobic interaction chromatography was further investigated as a mode for separation of SPRF activity from less hydrophobic proteins (el Rassi et al., 1990; Queiroz et al., 2001; Wu and Karger, 1996). In a first set of experiments three different resins were compared: butyl-sepharose, phenyl-sepharose and valine-sepharose. After removal of thrombin with HiTrap CMFF the flow through (CMFF-FT) was desalted into 1 M ammonium sulfate, 50 mM Phosphate Buffer (pH 7.0) for loading onto the HIC columns. One ml-HIC columns were loaded with equal volumes of sample, then washed with loading buffer and bound proteins eluted first with 0 M ammonium sulfate, 50 mM Phosphate Buffer (pH 7.0) and finally with 20% ethanol, 50 mM Phosphate Buffer (pH 7.0). From this first screen HiTrap Butyl FF gave the best purification in the 20% ethanol fraction (13fold) with a recovery of about 24% of starting material (Table 2.6). HiTrap Phenyl FF (low sub) had the same recovery but a lower purification factor than Butyl. In contrast, valine-sepharose gave a very good purification in 20% Ethanol (24-fold) but with a lower yield.

These promising results with hydrophobic columns suggested that further screening with larger columns and also more hydrophobic matrices might be profitable. Therefore in a second screen for hydrophobic interaction chromatography, 5 ml HiTrap Butyl and HiTrap Octyl columns were compared with a more detailed gradient for elution of proteins. The starting material was the flow through after cation exchange chromatography (CMFF-FT) and this fraction was directly adjusted to 1 M ammonium sulfate, 50 mM Phosphate Buffer (pH 7.0) and then loaded on a HiTrap Butyl or HiTrap Octyl column. Elution was achieved by a prolonged step gradient (10 column volumes each) of decreasing concentrations of ammonium sulfate, followed by addition of 20% or 40% ethanol to the phosphate buffer in order to elute very hydrophobic proteins. With this optimized protocol HiTrap Butyl gave now a yield of 79% with 21-fold purification in the protein fraction eluted with 20% ethanol. As expected HiTrap Octyl had a stronger affinity for proteins including SPRF as higher levels of ethanol (40%) were required for full elution (Table 2.7). This higher affinity was also reflected in a lower overall yield for Octyl compared to Butyl HIC.
Table 2.6 - Hydrophobic interaction chromatography: comparison of 1 ml HiTrap Butyl, HiTrap Phenyl and HiTrap columns
The flow through fraction from the CMFF column (CMFF-FT: 100% yield / 1.3-fold purification) was desalted into 50 mM Phosphate Buffer (pH 7.0), containing 1 M ammonium sulfate (1 M AS) and loaded onto HIC columns. Elution followed stepwise (each 5 CV) with no ammonium sulfate (0M AS) and 20% Ethanol (20% EtOH), respectively in 50 mM Phosphate Buffer (pH 7.0). For each fraction yield (%) and purification (fold) is shown, starting from Crude Bovine Thrombin.

<table>
<thead>
<tr>
<th>Column</th>
<th>50 mM Phosphate Buffer (pH 7.0)</th>
<th>+ 1 M AS</th>
<th>(0 M AS)</th>
<th>20% EtOH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Yield (%) / Purification (fold)</td>
<td>%</td>
<td>fold</td>
<td>%</td>
</tr>
<tr>
<td>HiTrap Butyl</td>
<td>0</td>
<td>0.0</td>
<td>32</td>
<td>0.7</td>
</tr>
<tr>
<td>HiTrap Phenyl</td>
<td>4</td>
<td>0.2</td>
<td>29</td>
<td>0.6</td>
</tr>
<tr>
<td>HiTrap Valine</td>
<td>4</td>
<td>0.1</td>
<td>7</td>
<td>1.8</td>
</tr>
</tbody>
</table>

Table 2.7 - Hydrophobic interaction chromatography: Comparison of 5 ml HiTrap Butyl and HiTrap Octyl columns
The flow through fraction from the CMFF column (CMFF-FT) was adjusted to 50 mM Phosphate Buffer (pH 7.0), containing 1 M ammonium sulfate and loaded onto columns. This was followed by a stepwise elution (each 10 CV) with 50 mM Phosphate Buffer with no ammonium sulfate (0 M AS), followed by 20% Ethanol (20% EtOH) then 40% Ethanol (40% EtOH), also in 50 mM Phosphate Buffer. For all fractions, yield (%) and purification (fold) starting from Crude Bovine Thrombin, are shown.

<table>
<thead>
<tr>
<th>Column</th>
<th>50 mM Phosphate Buffer (pH 7.0)</th>
<th>(0 M AS)</th>
<th>20% EtOH</th>
<th>40% EtOH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Yield (%) / Purification (fold)</td>
<td>%</td>
<td>fold</td>
<td>%</td>
</tr>
<tr>
<td>HiTrap Butyl</td>
<td>5</td>
<td>3.2</td>
<td>79</td>
<td>21.1</td>
</tr>
<tr>
<td>HiTrap Octyl</td>
<td>2</td>
<td>1.4</td>
<td>41</td>
<td>8.3</td>
</tr>
</tbody>
</table>
2.2.3.2 Hydrophobic Interaction Chromatography On HiTrap Butyl As Second Step Of The Purification

Based on the results above, hydrophobic interaction chromatography on HiTrap Butyl was included as the second step after cation exchange chromatography on HiTrap CMFF into the purification scheme. Analysis of the fractions by SDS-PAGE showed the enrichment of distinct bands in each fraction and suggested that the overall resolution of the HiTrap Butyl column was good (Figure 2.8). The significant reduction of total protein in the 20% EtOH fraction along with good recovery of activity (79%, Table 2.7), that together give a 21-fold purification, provides a great advantage for any subsequent steps as the total salt concentration is low and also 20-fold more protein can be loaded. In all following multi-step purification schemes using hydrophobic interaction on butyl, the active pool of fractions, containing 20% ethanol, was designated as Butyl-20%EtOH.
Figure 2.8 - Hydrophobic interaction chromatography on HiTrap Butyl

(A) Chromatogram of HiTrap Butyl run: Following cation exchange chromatography on CMFF, CMFF-FT was loaded in 50 mM Phosphate Buffer, 1 M AS onto a 5 ml HiTrap Butyl column. Elution of bound proteins was performed with a step gradient of 1 M AS, 0.1 M AS, 0 M AS, 20% EtOH and 40% EtOH, respectively in 50 mM Phosphate Buffer (pH 7.0). The orange bars represent the percentage of BrdU-positive myoutubes found in these fractions. Most of activity was eluted in the fraction containing 20% EtOH (Butyl-20%EtOH).

(B) Silver stained gel of fractions from HiTrap Butyl run: Equal amounts of protein (0.5 µg) of purified fractions were loaded and run under non-reducing conditions.
2.2.4  Affinity Chromatography

2.2.4.1  Heparin Affinity Chromatography

Many serum proteins and growth factors display an affinity for the proteoglycan heparin (Farooqui and Horrocks, 1984; George-Nascimento and Fedor, 1990; Lobb et al., 1986). Heparin can operate as an affinity ligand, for example in the case of coagulation factors, but has also a function as a high capacity cation exchanger due to its anionic sulphate groups (Farooqui, 1980). Previous chromatographic investigations on heparin columns after cation exchange chromatography and anion exchange chromatography have shown that SPRF activity binds specifically to heparin-sepharose with the peak of activity eluting at 450 mM on a linear gradient of NaCl (Tanaka and Drechsel, unpublished). Pooling the peak fractions gave a preparation with a specific activity that was 30 to 50-fold higher than the starting material with 35% recovery of the activity for this heparin affinity step.

2.2.4.1.1  Heparin Affinity Chromatography After Cation Exchange Chromatography

In an effort to improve the yield and to omit the ineffective anion exchange chromatography step, we tested loading the active fraction from cation exchange directly on a heparin-sepharose column and eluted proteins with either a step or a linear gradient of sodium chloride.

2.2.4.1.2  Heparin Affinity Chromatography After Cation Exchange Chromatography Using A Step Gradient

In order to streamline the purification procedure we first tested heparin affinity chromatography using step gradients of NaCl. The flow through fraction from cation exchange chromatography (CMFF-FT) was adjusted to 200 mM NaCl to prevent non-specific protein interactions and loaded onto a 5 ml HiTrap Heparin. The total amount of protein in the loaded CMFF-FT used in one run was calculated that it did not exceed the binding capacity of the HiTrap Heparin column (about 15 mg). Performing a stepwise gradient of NaCl in Anion Buffer (pH 7.0) for elution of bound proteins we found that activity started to elute at 200 mM and was completely desorbed by 750 mM NaCl (Table 2.8). In comparison to previous results (Tanaka and Drechsel, unpublished) no fraction
with a comparatively high yield and purification was identified. For this reason a linear gradient was tested for elution of the SPRF activity from the heparin-sepharose column.

Table 2.8 - Heparin affinity chromatography using a step gradient
The flow through from HiTrap CMFF CMFF-FT was adjusted to 200 mM NaCl and subsequently loaded onto HiTrap Heparin column, which was pre-equilibrated with Anion Buffer, 200 mM NaCl. Bound proteins were eluted with a stepwise gradient of NaCl (5 CV each). For each fraction yield (%) and purification factor (fold) compared to Crude Bovine Thrombin is shown. Fractions containing most of activity in the run are shown in bold.

<table>
<thead>
<tr>
<th>NaCl (mM)</th>
<th>Yield (%)</th>
<th>Purification (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>200 (FT)</td>
<td>1</td>
<td>0.0</td>
</tr>
<tr>
<td>200</td>
<td>5</td>
<td>0.3</td>
</tr>
<tr>
<td>250</td>
<td>18</td>
<td>1.5</td>
</tr>
<tr>
<td>300</td>
<td>11</td>
<td>1.1</td>
</tr>
<tr>
<td>350</td>
<td>14</td>
<td>4.4</td>
</tr>
<tr>
<td>400</td>
<td>14</td>
<td>12.1</td>
</tr>
<tr>
<td>450</td>
<td>2</td>
<td>6.2</td>
</tr>
<tr>
<td>500</td>
<td>3</td>
<td>17.1</td>
</tr>
<tr>
<td>750</td>
<td>9</td>
<td>8.7</td>
</tr>
<tr>
<td>1500</td>
<td>0</td>
<td>0.0</td>
</tr>
<tr>
<td>Total</td>
<td>77</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

2.2.4.1.3 Heparin Affinity Chromatography After Cation Exchange Chromatography Using A Linear Gradient

Due to the unsatisfying results using a step gradient we then investigated heparin affinity using a linear gradient. The flow through from a HiTrap CMFF cation exchange column in Cation Buffer (pH 6.5) was adjusted to 200 mM NaCl and loaded directly onto a HiTrap Heparin column. We determined the yield and purification factor for all fractions across a linear gradient of 0 to 1.0 M NaCl. Screening through all fractions of the heparin column developed with a linear gradient of sodium chloride revealed elution of the activity between 200 mM and 700 mM NaCl. We then pooled single fractions according to significant protein peaks in the chromatogram and assayed them on cells (Figure 2.9).
Between 430 - 590 mM NaCl a pool with 20-fold purification and 20% recovery (designated as Hep3) was identified (Table 2.9). In many of the following multi-step purification schemes described in this work, that include a heparin affinity column, this Hep3 pool was used for subsequent fractionation steps.

2.2.4.1.4 Heparin Affinity Chromatography After Hydrophobic Interaction Chromatography

After the development of hydrophobic interaction chromatography on HiTrap Butyl as a second purification step (see above), heparin affinity with a step or linear gradient of sodium chloride was considered again. The inclusion of hydrophobic interaction chromatography between cation exchange and heparin affinity chromatography reduced the total amount of proteins in the preparation and permitted the load of 20 times more SPRF activity in one run compared to a load directly after HiTrap CMFF. Cation exchange and hydrophobic interaction chromatography were performed as described above. The active fraction Butyl-20%EtOH (about 20-fold purification) was then loaded directly onto the HiTrap Heparin column and bound proteins eluted with a step or a linear gradient of sodium chloride from 0 to 1000 mM.

2.2.4.1.5 Heparin Affinity Chromatography After Hydrophobic Interaction Chromatography Using A Step Gradient

For a simplification of the protocol we first investigated a simple step gradient elution. The Butyl-20%EtOH fraction was loaded directly onto the Heparin column, non-specifically bound proteins washed from the column with 200 mM NaCl and then all adsorbed proteins eluted with 1 M NaCl. We obtained 30-fold purification with 44% activity recovery, starting from Crude Bovine Thrombin (Table 2.14). This Hep-1M fraction was then used as starting material for lectin affinity chromatography or after concentration on a 100 kD membrane (Hep-1M-Conc) for native PAGE as well as gel filtration at pH 11.
2.2.4.1.6 Heparin Affinity Chromatography After Hydrophobic Interaction Chromatography Using A Linear Gradient

In a second experiment we examined a linear gradient after hydrophobic interaction chromatography. Therefore the Butyl-20%EtOH fraction was adjusted to 200 mM NaCl, loaded onto a Heparin column and the column developed with a linear gradient of sodium chloride from 0 to 1000 mM. In order to compare the efficiency of this heparin step we pooled the same fractions as for heparin affinity chromatography after CMFF (Table 2.9, Figure 2.10). We obtained now for the pool between 430 and 590 mM NaCl (designated as Hep3-Butyl) 240-fold purification with 25% recovery, starting from Crude Bovine Thrombin. In addition we found an even more purified pool between 590 and 680 mM NaCl (Hep4-Butyl). In the following experiments we combined Hep3-Butyl and Hep4-Butyl and then referred this pool as Hep3/4-Pool. However, care has to be taken now to the concentration of proteins in many active fractions, that is now less than 10 µg/ml when using a linear gradient for elution. Therefore, in order to minimize activity loss due to unspecific adsorption, all Eppendorf-tubes were pre-incubated with Carriermix. In conclusion, the insertion of a hydrophobic interaction step between cation exchange and heparin affinity chromatography greatly improved the total purification of the activity and provided approximately 10-fold more purification with similar yield compared to heparin affinity directly after cation exchange chromatography.
Table 2.9 - Heparin affinity chromatography using a linear gradient of sodium chloride after cation exchange chromatography (CMFF) or after cation exchange and hydrophobic interaction chromatography (CMFF – Butyl).

After cation exchange chromatography (CMFF-FT) or hydrophobic interaction chromatography (Butyl-20%EtOH) the active pool was loaded onto a HiTrap Heparin column, pre-equilibrated in Anion Buffer (pH 7.0). Fractions across the linear gradient of NaCl were pooled according to characteristic peaks and region of the chromatogram and referred as FT-1 and FT-2 (flow through and wash fraction) Hep1 (80-200 mM NaCl), Hep2 (200-430 mM NaCl), Hep3 (430-590 mM NaCl) and Hep4 (590-680 mM NaCl). For each fraction yield (%) and purification factor (fold) compared to Crude Bovine Thrombin is shown. Fractions that gave good purification in the run are shown in bold. N.A. not applicable

* Heparin fractions after hydrophobic interaction chromatography on Butyl were designated with the suffix “—Butyl” (e.g. Hep3-Butyl)

<table>
<thead>
<tr>
<th>Column</th>
<th>Fraction name</th>
<th>CMFF</th>
<th>CMFF - Butyl</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Yield (%)</td>
<td>Purification (fold)</td>
</tr>
<tr>
<td>Starting material</td>
<td>CB-Thrombin</td>
<td>100</td>
<td>1.0</td>
</tr>
<tr>
<td>HiTrap CM FF</td>
<td>CMFF-FT</td>
<td>99</td>
<td>1.3</td>
</tr>
<tr>
<td>HiTrap Butyl</td>
<td>Butyl 20%EtOH</td>
<td>N.A.</td>
<td>N.A.</td>
</tr>
<tr>
<td>HiTrap Heparin</td>
<td>FT-1 (Butyl)*</td>
<td>3</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td>FT-2 (Butyl)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Hep1 (Butyl)</td>
<td>4</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>Hep2 (Butyl)</td>
<td>64</td>
<td>2.3</td>
</tr>
<tr>
<td></td>
<td>Hep3 (Butyl)</td>
<td>24</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td>Hep4 (Butyl)</td>
<td>9</td>
<td>10</td>
</tr>
</tbody>
</table>
Figure 2.9 - Heparin affinity chromatography after cation exchange chromatography using a linear gradient of sodium chloride

(A) Chromatogram: The CMFF flow through fraction was applied to a heparin sepharose column and eluted with an increasing linear salt gradient. Activity was recovered in several fractions with the highest purification achieved by pooling fractions between 430 and 590 mM NaCl (Hep3). Orange bars represent the percentage of BrdU incorporation for single fractions.

(B) Silver stained gel after reducing SDS-PAGE: In each lane equal amounts of protein were loaded.
2.2.4.2 Affinity Chromatography On Cibacron Blue F3G-A (HiTrap Blue)

In addition to heparin, another affinity chromatography mode used for serum proteins is based on protein binding to dyes such as Cibacron Blue F3G-A. This mode is particularly useful for the isolation and purification of albumin, interferon, α2-macro-globulin and also coagulation factors (Lowe and Pearson, 1984; Subramanian, 1984). Therefore we investigated the binding of SPRF to immobilized Cibacron Blue F3G-A (HiTrap Blue). The starting material for this experiment was the flow through (CMFF-FT) after chromatography of Crude Bovine Thrombin on a weak cation exchanger (HiTrap CMFF). The PPACK treated CMFF flow through was loaded onto a HiTrap Blue column, pre-equilibrated in Anion Buffer (pH 7.0), and bound proteins eluted with a stepwise gradient of NaCl. Surprisingly, activity was split into bound and unbound fractions. While 25% of activity did not bind and was recovered in the flow through as well as wash fractions, a similar amount was eluted with 500, 1000 and 1500 mM NaCl (Table 2.10). This indicates that either the activity is composed of two components that differ in their affinity.
for HiTrap Blue or it may be bound to other proteins that in turn have different affinities for Blue-sepharose. No substantial purification was achieved in any one of the fractions with activity and therefore affinity chromatography on HiTrap Blue was not further considered.

Table 2.10 - Affinity purification on HiTrap Blue

When flow through of HiTrap CMFF (CMFF-FT: 100% yield / 1.3-fold purification) is applied to a HiTrap Blue column and eluted with a step gradient of sodium chloride in Anion Buffer (pH 7.0), activity elutes both in the flow through fractions (0 mM) as well as at higher concentrations of NaCl (500 to 1500 mM). No substantial purification is achieved in one of the fractions. For each fraction yield (%) and purification (fold) compared to the starting material Crude Bovine Thrombin is shown.

<table>
<thead>
<tr>
<th>[NaCl] (mM)</th>
<th>Yield (%)</th>
<th>Purification (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (FT)</td>
<td>10</td>
<td>0.5</td>
</tr>
<tr>
<td>0 (Wash)</td>
<td>15</td>
<td>1.8</td>
</tr>
<tr>
<td>200</td>
<td>1</td>
<td>0.4</td>
</tr>
<tr>
<td>500</td>
<td>5</td>
<td>0.5</td>
</tr>
<tr>
<td>1000</td>
<td>13</td>
<td>1.3</td>
</tr>
<tr>
<td>1500</td>
<td>7</td>
<td>3.0</td>
</tr>
<tr>
<td>2000</td>
<td>1</td>
<td>2.5</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>52</strong></td>
<td><strong>N.D.</strong></td>
</tr>
</tbody>
</table>

2.2.4.3 Lectin Affinity Chromatography

Lectin affinity chromatography is often successfully used in the purification of serum proteins (Cartellieri et al., 2002; West and Goldring, 1996). In initial characterization studies with a partially purified fraction (Hep3, see above) it was demonstrated that SPRF activity could be depleted by incubation with immobilized lectins such as Concanavalin A, Jacalin, Sambucus Nigra agglutinin and Wheat Germ Agglutinin. Therefore it was tested whether lectin affinity chromatography could be included as a powerful step in the purification scheme. For a first test Concanavalin A (ConA), Lens culinaris lectin (Lentil Lectin) and Wheat Germ Agglutinin (WGA) were chosen. ConA and Lentil Lectin both bind to mannose and glucose, whereas WGA has an affinity to N-acetylglucosamine and N-acetylneuraminic acid.
In order to reduce the amount of total protein and to obtain material in the appropriate buffer for lectin affinity chromatography, SPRF activity was purified over cation exchange chromatography (CMFF-FT), hydrophobic interaction chromatography (Butyl-20%EtOH) and heparin affinity chromatography using a step gradient of sodium chloride (Hep-1M). Heparin affinity chromatography was performed in 20 mM Tris (pH 7.0), bound proteins were eluted with 1 M NaCl and subsequently diluted with 20 mM Tris to 500 mM NaCl. Afterwards a fraction was loaded onto lectin columns, non-bound proteins washed away and specifically bound proteins eluted with methyl-α-D-glucopyranoside (ConA), methyl-α-D-mannopyranoside (lentil lectin) or N-acetyl-D-glucosamine (WGA).

In contrast to earlier tests with less-purified material fractionated on immobilized lectins, this time SPRF activity was predominantly found in the flow through of the ConA and Lentil lectin columns (Table 2.11) along with the majority of applied proteins. Neither the flow through fraction nor the eluate gave a promising purification. In addition, the total recovery of activity for the runs were low: 17% and 24% for ConA and lentil lectin, respectively. On the other hand, when applied to a WGA column, SPRF activity was bound and eluted with N-acetylglucosamine (31% activity yield). However, the bulk of the protein loaded also bound to WGA and therefore no purification was achieved.

One cause for the contradiction between these results and earlier studies with immobilized lectins could be the different purification procedure used to obtain starting material for lectin affinity chromatography. In the current experiment, a step gradient of 1 M NaCl was used to elute SPRF activity from the heparin column (Hep-1M). When the heparin 1 M pool was concentrated on ultrafiltration membranes with a 100 kD MWCO, a substantial amount of activity was found in the flow through. This suggests that SPRF activity dissociates at high concentrations of NaCl to some extent from a high molecular weight complex and therefore changes its chromatographic behavior. In this scenario, the depletion of SPRF activity seen in initial lectin binding studies could be based on an indirect effect, where another protein of the complex, binds to Concanavalin A or Lentil Lectin.

In conclusion, lectin affinity chromatography on wheat germ agglutinin confirmed the previous lectin binding study results. In contrast to ConA or lentil lectin, WGA bound SPRF activity, suggesting a direct binding of SPRF to WGA. It cannot be ruled out in this case as well, that the binding might be indirect. Although with the presented purification scheme no further purification was achieved, affinity chromatography on WGA should be reconsidered at later stages of the purification scheme after the low molecular weight form has been separated from the complex. Furthermore, in order to increase purification
and yield, a more detailed step gradient for elution as well as addition of 20% ethanol to elute more tightly bound proteins should be tried.

Table 2.11 - Lectin affinity chromatography on Concanavalin A (ConA), Lens culinaris lectin (lentil lectin) and wheat germ agglutinin (WGA)

After cation exchange (CMFF-FT), hydrophobic interaction chromatography (Butyl-20%EtOH) and heparin affinity using a step gradient of NaCl (Hep-1M) the active pool was diluted to 20 mM Tris (pH 7.0), 500 mM NaCl and loaded onto lectin columns. All lectin affinity chromatography was performed in 20 mM Tris (pH 7.0), 500 mM NaCl. After washing the columns (FT), bound proteins were eluted (Eluate) with 0.5 M methyl-α-D-glucopyranoside (ConA), 0.3 M methyl-α-D-mannopyranoside (lentil lectin) or 0.5 M N-acetyl-D-glucosamine (WGA). For each fraction yield (%) and purification factor (fold) is shown. Fractions containing most of activity in the lectin runs are shown in bold.

<table>
<thead>
<tr>
<th>Column</th>
<th>Fraction name</th>
<th>Overall</th>
<th></th>
<th>Step</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Yield</td>
<td>Purification</td>
<td>Yield (%)</td>
</tr>
<tr>
<td>Starting material</td>
<td>CB-Thrombin</td>
<td>100</td>
<td>1.0</td>
<td>77</td>
</tr>
<tr>
<td>HiPrep CM FF</td>
<td>CMFF-FT</td>
<td>77</td>
<td>1.0</td>
<td>77</td>
</tr>
<tr>
<td>HiPrep Butyl</td>
<td>Butyl-20%EtOH</td>
<td>47</td>
<td>15.3</td>
<td>61</td>
</tr>
<tr>
<td>HiTrap Heparin</td>
<td>Hep-1M</td>
<td>25</td>
<td>17.1</td>
<td>53</td>
</tr>
<tr>
<td>HiTrap ConA FT</td>
<td>FT</td>
<td>3.1</td>
<td>6.0</td>
<td>12</td>
</tr>
<tr>
<td>HiTrap ConA Eluate</td>
<td></td>
<td>1.2</td>
<td>57.2</td>
<td>5</td>
</tr>
<tr>
<td>HiTrap ConA total</td>
<td></td>
<td>4.3</td>
<td></td>
<td>17</td>
</tr>
<tr>
<td>HiTrap Lentil Lectin FT</td>
<td></td>
<td>4.2</td>
<td>6.7</td>
<td>17</td>
</tr>
<tr>
<td>HiTrap Lentil Lectin Eluate</td>
<td></td>
<td>1.8</td>
<td>13.5</td>
<td>7</td>
</tr>
<tr>
<td>HiTrap Lentil Lectin total</td>
<td></td>
<td>6.0</td>
<td></td>
<td>24</td>
</tr>
<tr>
<td>HiTrap WGA FT</td>
<td></td>
<td>0.6</td>
<td>14.3</td>
<td>3</td>
</tr>
<tr>
<td>HiTrap WGA Eluate</td>
<td></td>
<td>7.7</td>
<td>11.4</td>
<td>31</td>
</tr>
<tr>
<td>HiTrap WGA total</td>
<td></td>
<td>8.4</td>
<td></td>
<td>34</td>
</tr>
</tbody>
</table>
2.2.5 Chromatography on Hydroxyapatite

Separation of proteins using hydroxyapatite (HAP) is a very useful complement to other separation techniques such as ion exchange and hydrophobic interaction chromatography, particularly at later stages of a purification scheme. Hydroxyapatite chromatography has unique separation properties and often separates proteins shown to be homogenous by other chromatographic and electrophoretic techniques (Bernardi et al., 1972; Gorbunoff, 1990). Therefore hydroxyapatite chromatography was performed after cation exchange chromatography and hydrophobic interaction chromatography on a CHT-II cartridge using a linear gradient of sodium phosphate (pH 7.0) for elution of bound proteins.

In order to streamline the purification procedure, hydrophobic interaction chromatography was performed in 10 mM phosphate for a direct load of the active pool onto the subsequent HAP-column. The decrease of phosphate buffer however caused an altered desorption behavior of bound proteins, which now started to elute earlier in the step gradient on HiTrap Butyl and led to a less purified Butyl-20%EtOH (7.5-fold with 41% recovery) fraction compared to elution in 50 mM phosphate buffer (21-fold with 79% recovery; Table 2.7).

After elution from a HiTrap Butyl column in 10 mM phosphate buffer (pH 7.0), 20% ethanol, the Butyl-20%EtOH pool was directly applied to a 5 ml CHT-II column, equilibrated in 10 mM phosphate (pH 7.0). The column was washed with 10 mM phosphate buffer and bound proteins eluted with a linear gradient from 10 to 500 mM phosphate. We found the activity spread across the whole gradient of sodium phosphate with a peak of activity between 200 and 400 mM (Figure 2.11). In fractions between 360 and 500 mM phosphate (Table 2.12, fractions 31 to 40) we obtained a specific activity approximately 30-fold higher than in the starting material. Furthermore, SDS-PAGE analysis of the run displayed a spreading of major bands over a large number of fractions. This result is similar to heparin affinity chromatography and anion exchange chromatography on HiTrap Q using a linear gradient of NaCl, where the SPRF activity was also found to elute as a broad peak.

In summary, hydroxyapatite after hydrophobic interaction did not give a superior purification, in spite of this the activity elute as a very broad peak with the bulk of proteins. According to the bad separation properties of CHT-II this mode of chromatography was not further investigated.
Table 2.12 - Chromatography on hydroxyapatite (CHT-II)

After cation exchange (CMFF-FT), hydrophobic interaction chromatography (Butyl-20%EtOH) in 10 mM Phosphate Buffer (pH 7.0), the Butyl-20%EtOH pool was loaded onto a CHT-II column. The HAP-column was washed with 10 mM Phosphate Buffer (pH 7.0) and bound proteins eluted with a linear gradient from 10 to 500 mM phosphate buffer (pH 7.0). Activity was eluted along the whole gradient, however only fractions between 360 mM and 500 mM phosphate (fractions 31 to 40) gave considerable purification and are shown here. For each fraction yield (%) and purification factor (fold) is shown.

<table>
<thead>
<tr>
<th>Column</th>
<th>Fraction name</th>
<th>Overall Purification (fold)</th>
<th>Overall Yield (%)</th>
<th>Step Purification (fold)</th>
<th>Step Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Starting material</td>
<td>CB-Thrombin</td>
<td>1.0</td>
<td>100</td>
<td>1.0</td>
<td>100</td>
</tr>
<tr>
<td>HiTrap CMFF</td>
<td>CMFF-FT</td>
<td>1.3</td>
<td>94</td>
<td>1.3</td>
<td>94</td>
</tr>
<tr>
<td>HiTrap Butyl</td>
<td>Butyl-20%EtOH (in 10 mM Phosphate)</td>
<td>7.5</td>
<td>41</td>
<td>5.6</td>
<td>44</td>
</tr>
<tr>
<td>HAP - CHT-II</td>
<td>Total run</td>
<td>34</td>
<td></td>
<td>34</td>
<td>83</td>
</tr>
<tr>
<td></td>
<td>31</td>
<td>27</td>
<td>2.6</td>
<td>3.6</td>
<td>6.3</td>
</tr>
<tr>
<td></td>
<td>32</td>
<td>35</td>
<td>2.6</td>
<td>4.6</td>
<td>6.4</td>
</tr>
<tr>
<td></td>
<td>33</td>
<td>25</td>
<td>1.5</td>
<td>3.4</td>
<td>3.7</td>
</tr>
<tr>
<td></td>
<td>34</td>
<td>20</td>
<td>0.9</td>
<td>2.7</td>
<td>2.2</td>
</tr>
<tr>
<td></td>
<td>35</td>
<td>31</td>
<td>1.2</td>
<td>4.2</td>
<td>2.8</td>
</tr>
<tr>
<td></td>
<td>36</td>
<td>37</td>
<td>1.1</td>
<td>5.0</td>
<td>2.8</td>
</tr>
<tr>
<td></td>
<td>37</td>
<td>34</td>
<td>0.9</td>
<td>4.6</td>
<td>2.1</td>
</tr>
<tr>
<td></td>
<td>38</td>
<td>41</td>
<td>0.9</td>
<td>5.4</td>
<td>2.1</td>
</tr>
<tr>
<td></td>
<td>39</td>
<td>57</td>
<td>1.0</td>
<td>7.7</td>
<td>2.5</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>27</td>
<td>0.4</td>
<td>3.6</td>
<td>1.0</td>
</tr>
</tbody>
</table>
Figure 2.11 - Hydroxyapatite chromatography on CHT-II after cation exchange chromatography and hydrophobic interaction chromatography using a linear gradient of sodium phosphate

The CMFF flow through fraction was applied to a HiTrap Butyl column and the Butyl-20% ETOH in 10 mM phosphate (pH 7.0) further fractionated on hydroxypapitate CHT-II column by eluting bound proteins with an increasing linear sodium phosphate gradient. Activity was recovered in several fractions with a peak between 200 and 400 mM. Fraction between 360 and 500 mM (31 to 40) gave approximately 30-fold purification. Orange bars represent the percentage of BrdU incorporation for single fractions.
2.2.6 Chromatofocusing

Chromatofocusing is a powerful chromatography method for separating proteins based on differences in their isoelectric point (pI) and particularly useful for later steps of protein purifications. Proteins with the same pI are focused during the run on the anion exchange resin and resolved from other proteins by applying a linear pH-gradient to the column. According to their isoelectric point, proteins are then eluted from the column (Fagerstam et al., 1983; Giri, 1990).

In order to test chromatofocusing as a potential purification step we applied a partially purified preparation containing SPRF activity to a Mono P chromatofocusing column. This starting material was Crude Bovine Thrombin purified by cation exchange (HiTrap CMFF) and then hydrophobic interaction chromatography on HiTrap Butyl. Elution from butyl column was performed in 10 mM phosphate buffer, 20% ethanol (Table 2.12) and subsequently the phosphate concentration adjusted to 25 mM for loading onto the Mono P chromatofocusing column, equilibrated with 25 mM phosphate buffer (pH 7.0). After loading the Mono P column, proteins were eluted using a linear gradient from pH 7 to 4.

Only 1% of total protein in the starting material did not bind to the Mono P column and was present in the flow through, which is similar to the behavior of this sample on other anion exchange resins. Across the pH gradient only 11% of loaded total protein was eluted, with the remaining protein desorbed from the column using 2 M sodium chloride. No activity was recovered in assays of all fractions along the gradient. Presumably, most of the proteins, including SPRF, precipitated on the column at their isoelectric points under these low salt conditions and therefore did not elute.

These results are consistent with initial pH characterization experiments, where at pH 3 and 4 considerable precipitation was observed. In addition, the activity may have an isoelectric point of 4 or even less and therefore has not eluted with the applied gradient.

From the result it is unclear if the loss of activity seen with chromatofocusing is an intrinsic property of SPRF or due to its strong interaction with a contaminating protein. Therefore chromatofocusing was not further considered as a step in the purification scheme but should perhaps be re-investigated in more purified fractions, especially after separation of the low molecular weight form of SPRF.
2.2.7 Reversed Phase Chromatography

Reversed phase chromatography is regularly used as a high-resolution step in the purification of peptides as well as low molecular weight proteins up to 30 kD and has often succeeded in purifying growth factors (Aguilar and Hearn, 1996; George-Nascimento and Fedor, 1990; Hearn, 1984). Initial tests indicated that SPRF activity is able to survive strong denaturing conditions that are used for reversed phase chromatography (60% acetonitrile, 0.1% TFA). Therefore it was further investigated if it is possible to include this method as a step in the purification of SPRF.

In order to minimize precipitation of high molecular weight proteins, which are often unstable under these conditions and could disturb recovery of SPRF, gel filtration in 0.1% SDS was carried out before reversed phase chromatography to separate high and low molecular weight proteins. Because SDS is incompatible with reversed phase, a SDS trap guard column was installed before a C4 reversed phase column.

After four purification steps, including cation exchange chromatography, heparin affinity chromatography, concentrating of the Heparin pool on a 100 kD membrane and gel filtration in 0.1% SDS, the six most active fractions were pooled and referred to as SD200-Pool (130-fold purified, 11% yield, Table 2.13). Following adjustment to 0.1% TFA the SD200-Pool was loaded onto a C4 column in 4% acetonitrile, 0.1% TFA. The column was developed with a linear gradient of acetonitrile from 4 to 70% final. In this initial reversed phase run, all of the SDS was bound to the SDS trap guard column and the SDS after the run eluted with 90% acetonitrile. Subsequently, fractions, mixed with BSA, were lyophilized to remove acetonitrile, then dissolved in PBS and dialyzed against PBS and medium before assay on myotubes. We recovered 41% of the activity in this step across the entire reversed phase run (7% total activity yield), with a peak of activity eluting at 45% acetonitrile. However, there was some spreading of activity and this might have been due to residual SDS in the sample (Figure 2.12A).

In order to rule out peak spreading due to residual SDS, the 10 most active fractions (86-fold purified, 4.5% yield, Table 2.13) were pooled and re-run on the C4 column. The pool from the first run on a C4 column was diluted two-fold (to 25% acetonitrile final) and run a second time with a shallower gradient between 40 and 60% acetonitrile to increase the resolution in this region. Fractions were prepared for the assay on cells as described above. In the re-run a peak of activity was found again around 45% acetonitrile and two fractions contained most of the activity (60-fold purified, 1.1% yield and 100-fold purified, 0.7% yield, Table 2.13). Running a silver-stained SDS-PAGE gel under non-reducing conditions across the second C4 run revealed a number of protein bands in the most
active fractions (Figure 2.12B,C). The purification starting with Crude Bovine Thrombin is summarized in Table 2.13.

In conclusion, the final step, reversed phase chromatography on a C4 column, did not provide a more purified fraction compared to the starting material after SDS gel filtration and based on the low overall yield of activity after this step, we did not further pursue this chromatographic mode. The activity eluted from the column as a broad peak and we cannot exclude an influence of residual SDS. Furthermore, glycoproteins often resolve poorly on reversed phase due to excess hydrophilic character and from the WGA lectin results it seems that most of the protein including the activity is glycosylated. However reversed phase chromatography should be re-investigated after separation of the low molecular weight SPRF by gel filtration at pH 11. In addition, a better running buffer might be phosphate pH 6 with n-propanol.

Table 2.13 - Purification chart of a five-step purification protocol ending with reversed phase chromatography

<table>
<thead>
<tr>
<th>Column</th>
<th>Fraction</th>
<th>Overall Purification (fold)</th>
<th>Overall Yield (%)</th>
<th>Step Purification (fold)</th>
<th>Step Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Starting material</td>
<td>CB-Thrombin</td>
<td>1</td>
<td>100</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Crude Bovine Thrombin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cation exchange (HiTrap CMFF)</td>
<td>CMFF-FT</td>
<td>1.3</td>
<td>100</td>
<td>1.3</td>
<td>100</td>
</tr>
<tr>
<td>Heparin affinity (HiTrap Heparin)</td>
<td>Hep3</td>
<td>20</td>
<td>25</td>
<td>15.4</td>
<td>25</td>
</tr>
<tr>
<td>Ultrafiltration (NMWL 100 kD)</td>
<td>Hep3-Conc</td>
<td>38</td>
<td>22</td>
<td>1.9</td>
<td>88</td>
</tr>
<tr>
<td>Gel filtration in 0.1% SDS</td>
<td>SD200-Pool</td>
<td>130</td>
<td>11</td>
<td>3.4</td>
<td>50</td>
</tr>
<tr>
<td>(Superdex-200)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1st run Reversed phase (GraceVydac C4)</td>
<td>Pool of 10 most active fractions</td>
<td>86</td>
<td>4.5</td>
<td>0.7</td>
<td>41</td>
</tr>
<tr>
<td>2nd run Reversed phase (GraceVydac C4)</td>
<td>2 most active fractions</td>
<td>60</td>
<td>1.1</td>
<td>0.5</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td></td>
<td>100</td>
<td>0.7</td>
<td>0.8</td>
<td>16</td>
</tr>
</tbody>
</table>
Figure 2.12 - Reversed phase chromatography of the low molecular weight form of SPRF after gel filtration in 0.1% SDS

(A) **First reversed phase run:** After gel filtration in SDS the SD200-Pool was applied to a Vydac C4 column and eluted with a linear gradient from 4 to 70 % acetonitrile. A peak of Activity eluted around 45% acetonitrile.  

(B) **Second reversed phase run:** The ten most active fractions from the first reversed phase run were pooled and re-run using a shallower gradient between 40 and 60%. Two fractions containing most of the activity (1 and 2) were identified.  

(C) **Silver stained gel across the second reversed phase run:** Equal amounts of protein (0.5 µg) were loaded in each lane. Fractions 1 and 2 in the myotube assay were a pool of 2 fractions from the reversed phase run, respectively.
2.2.8 Ultrafiltration

Under native conditions SPRF activity behaves as a protein with a molecular weight of 200 kD or greater. This was demonstrated by size exclusion chromatography of the starting material for the purification, Crude Bovine Thrombin, under native conditions in PBS. Similar results were also obtained with gel filtration under native conditions using a more purified fraction after cation exchange chromatography, heparin affinity chromatography and concentration of the active pool (Hep3) on a membrane with a MWCO of 10 kD. Interestingly, no loss of activity by aggregation or precipitation was observed during the concentration procedure. We therefore investigated more carefully ultrafiltration as a purification step and for the purpose of removing proteins with a molecular weight of less than 100 kD (Schratter, 2004; Scopes, 1994).

Initial ultrafiltration and concentration experiments were performed using a Hep3 pool as starting material that was obtained by cation exchange chromatography, heparin affinity chromatography and concentration on a 10 kD membrane. In later experiments this pre-concentrated Hep3-pool was diluted 10-fold and afterwards concentrated back to the starting volume on a 100 kD membrane. Dilution and concentration was then repeated four times. After five washing steps 50% of total protein was retained on the membrane with little loss of activity in the flow through (Figure 2.13). We obtained 88% recovery of activity in this step with approximately 2-fold purification. Similar results were obtained using a membrane with a MWCO of 50 kD. Furthermore, this ultrafiltration experiment confirmed former results of size exclusion chromatography of Crude Bovine Thrombin and partially purified fraction Hep3, showing that under native conditions the activity behaves as a large protein of more than 100 kD.
Figure 2.13 - Ultrafiltration of Hep3 on a membrane with a MWCO of 100 kD
Hep3 was pre-concentrated on a 10 kD membrane about 200-fold. Subsequently, 500 µl were transferred to a filter device with a 100 kD membrane. Low molecular weight proteins were removed by five cycles of 10-fold dilution with PBS and re-concentration. Little or no activity was found in the flow through fractions (1 to 5) and approximately 90% of SPRF activity was recovered in the retenate (Hep3-Conc).

2.2.9 Gel Filtration Of Partially Purified Fractions
2.2.9.1 Native Gel filtration (starting with Hep3-Conc)

The initial characterization of the Crude Bovine Thrombin starting material had shown, that under native conditions the SPRF activity acts as a molecular weight protein of 200 kD or higher. Moreover, the activity eluted from a Superdex 200 gel filtration column with the major bulk of proteins and was not suited as an early purification step. We therefore reinvestigated gel filtration under native conditions at a later step of the purification in order to see, if the purification both had separated proteins from the SPRF high molecular weight complex as well as reduced the quantity of high molecular weigh proteins in the sample.

For the experiment we used a partially purified fraction after cation exchange chromatography, heparin affinity chromatography followed by ultrafiltration on a 10 kD membrane. This Hep3-Conc fraction was then subjected to size exclusion chromatography under native condition. For a simplification of the assay, gel filtration was performed in serum free medium (SF-MEM) and the fractions after addition of FCS (final 0.5%) directly assayed on cells.
As for the Crude Bovine Thrombin we found again most of the activity migrating at 200 kD along with approximately 50% of the total protein in the sample. Only little activity was found at lower molecular weight. This finding is consistent with ultrafiltration of the same Hep3-Conc fraction on a 100 kD membrane, where almost full activity was recovered in the retentate and no activity found in the flow through. In order to separate the low molecular weight form and to include a separation step based on size we now decided to study the behavior of SPRF on a size exclusion column using denaturing conditions such as in SDS or at high pH.

2.2.9.2 Gel Filtration In 0.1% SDS (starting with Hep3-Conc) 

As depicted in the characterization of SPRF activity in Crude Bovine Thrombin, under native conditions SPRF activity eluted from a Superdex-200 gel filtration column at a volume corresponding to a molecular weight of ~ 200,000 Dalton or greater. In contrast, when gel filtration was performed under denaturing condition such as at pH 11 or in 0.1% SDS, SPRF was found at a molecular weight of either 45 or 20 kD respectively (Figure 2.3). We now used these features of the activity in a purification procedure and combined ultrafiltration on a 100 kD membrane for removal of low molecular weight proteins with size exclusion chromatography in SDS to dissociate the low molecular weight form of SPRF from high molecular weight proteins. An isolation of low molecular weight proteins, including SPRF, from high molecular weight contaminants would facilitate further purification of SPRF under extreme conditions such as low pH or organic solvents, where proteins with a higher molecular weight usually denature and precipitate.

For removal of low molecular weight proteins, pooled fractions (Hep3), after cation exchange chromatography and Heparin affinity chromatography, were concentrated on a membrane with a 100 kD molecular weight cut off. When concentrated Hep3 (Hep3-Conc) was afterwards subjected to gel filtration in 0.1% SDS on a Superdex-200 column, SPRF migrated as a protein with a molecular weight of approximately 25 kD. Furthermore, it eluted in a region of low protein concentration after the major protein peak (Figure 2.14). Subsequent reconstitution of denatured proteins by removal of SDS recovered 50% of the activity and gave 3.4-fold purification for this step by pooling the most active fractions (Table 2.13). As found also for starting material Crude Bovine Thrombin when fractionated under denaturing conditions, SPRF activity was exclusively detected in fractions of low molecular weight. Moreover SPRF activity ran in SDS as a protein with a homogenous size distribution.
Results

Figure 2.14 - Size exclusion chromatography of Hep3-Conc on Superdex-200 under denaturing conditions in PBS, 0.1% SDS

(A) Chromatogram: Hep3-Conc was mixed with SDS (final 2%) and for complete unfolding of proteins incubated at 37°C for 30 - 60 min. Subsequently 250 µl was run on a Superdex-200 size exclusion column. Collected fractions (0.4 ml) were assayed after dialysis with BSA on cells. Yellow bars represent the percentage of BrdU-positive myotubes, found for each fraction. (B) Silver stained gel across the gel filtration run: In each lane the same volume of fractions was loaded.

2.2.9.3 Gel Filtration At pH 11
2.2.9.3.1 Gel Filtration At pH 11 – Preparation Of The Starting Material

Gel filtration of the starting material, Crude Bovine Thrombin, at pH 11 displayed a shift of the activity from high molecular weight complexes towards a lower molecular weight of less than 90 kD (Figure 2.3). In order to establish a purification step that separates this low molecular weight form of SPRF from other proteins we investigated the fractionation
of partially purified SPRF by size exclusion chromatography at pH 11. An advantage of using high pH compared to gel filtration in other denaturants such SDS or urea would be, that fractions containing activity could be directly used for further purification steps afterwards simply by lowering the pH without the necessity of extensive dialysis.

Partially purified starting material was obtained from a four-step purification that included cation exchange chromatography (CMFF), hydrophobic interaction chromatography (Butyl) and heparin affinity chromatography using a step gradient (Hep-1M) or a linear gradient of NaCl (Hep3/4-Conc). Finally, the material was concentrated on a 100 kD ultrafiltration membrane with simultaneous desalting into 20 mM Tris (pH 7.0), 200 mM NaCl (for Hep-1M-Conc, after step gradient on heparin) or PBS (for Hep3/4-Conc, after linear gradient on heparin). After incubation for 2 h in 100 mM CAPS (pH 11), proteins were separated by size exclusion chromatography on a Superdex 200 column in 20 mM CAPS (pH 11), 200 mM NaCl. Fractions across the whole gel filtration run were assayed on myotubes to determine the migration pattern of SPRF under these conditions.

### 2.2.9.3.2 Gel Filtration At pH 11 Starting With Hep-1M-Conc

We first tested gel filtration at pH 11 on a 34-fold purified pool after heparin affinity chromatography where all proteins specifically bound to heparin were eluted with 1 M NaCl (Hep-1M) and then concentrated (Hep-1M-Conc). In agreement with previous results where Crude Bovine Thrombin was fractionated at pH 11 on gel filtration column, activity was again exclusively found in fractions of low molecular weight proteins with a peak around 45 kD (Figure 2.15A).Pooling the six most active fractions we got 200-fold purification with a total recovery of 14% after all five steps. For the individual step we calculated almost 6-fold purification with 44% recovery. The most purified fraction in this run was fraction 32 with 235-fold purification and 2.9% yield (Table 2.14). Native PAGE analysis of all fractions across the gel filtration at pH 11 run showed a clear separation of proteins or protein complexes according to their size with the assumption that all proteins left in the starting material have a very similar isoelectric point (Figure 2.15B). In contrast, when run on non-reducing SDS-PAGE we observed the appearance of numerous bands in all fractions across the gel filtration run indicating that the denaturing effect at pH 11 does not lead to complete dissociation of proteins from each other (Figure 2.15C). However, this only partial denaturation at pH 11 is even advantageous, since some low molecular weight proteins found in the high molecular weight fractions are separated from proteins including SPRF which elute at 45 kD from the Superdex 200 column. Furthermore, it is not clear, whether the SPRF activity migrates as a single
protein at pH 11 or is still associated to another protein. Non-reducing SDS-PAGE, however, suggests a single protein, as bands in the region between 28 and 39 kD (where SPRF migrates under these conditions) correlate with the activity profile of the gel filtration run. After this promising result we now tested the more purified fraction Hep3/4-Conc after heparin affinity using a linear gradient of sodium chloride for elution for gel filtration at pH 11.

Table 2.14 - Gel filtration at pH 11 after heparin affinity chromatography with step gradient elution (Hep-1M-Conc)

After cation exchange (CMFF-FT), hydrophobic interaction chromatography (Butyl-20%EtOH) and heparin affinity using a step gradient of NaCl (Hep-1M) the concentrated pool after ultrafiltration on a 100 kD membrane, Hep-1M-Conc, was applied to gel filtration at pH 11 on a Superdex 200 column. Activity was recovered in a molecular weight region of 45 kD (fractions 30-35). For each fraction yield (%) and purification factor (fold) is shown.

<table>
<thead>
<tr>
<th>Purification Step (Column)</th>
<th>Fraction</th>
<th>Overall Purification (fold)</th>
<th>Overall Yield (%)</th>
<th>Step Purification (fold)</th>
<th>Step Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Starting material</td>
<td>CB-Thrombin</td>
<td>1.0</td>
<td>100</td>
<td>1.0</td>
<td>100</td>
</tr>
<tr>
<td>Crude Bovine Thrombin</td>
<td>CMFT-FT</td>
<td>1.3</td>
<td>100</td>
<td>1.3</td>
<td>100</td>
</tr>
<tr>
<td>Cation exchange (HiPrep CMFF)</td>
<td>Butyl-20% EtOH</td>
<td>19</td>
<td>68</td>
<td>14.7</td>
<td>68</td>
</tr>
<tr>
<td>Hydrophobic interaction (HiPrep Butyl)</td>
<td>Hep-1M</td>
<td>30</td>
<td>44</td>
<td>1.6</td>
<td>65</td>
</tr>
<tr>
<td>Heparin affinity (HiTrap Heparin)</td>
<td>Hep-1M-Conc</td>
<td>34</td>
<td>31</td>
<td>1.1</td>
<td>70</td>
</tr>
<tr>
<td>Ultrafiltration (NMWL 100 kD)</td>
<td>30-35</td>
<td>197</td>
<td>13.7</td>
<td>5.7</td>
<td>44.2</td>
</tr>
<tr>
<td>Gef filtration (pH 11) (Superdex 200)</td>
<td>30</td>
<td>178</td>
<td>3.1</td>
<td>5.2</td>
<td>10.0</td>
</tr>
<tr>
<td></td>
<td>31</td>
<td>201</td>
<td>2.9</td>
<td>5.8</td>
<td>9.2</td>
</tr>
<tr>
<td></td>
<td>32</td>
<td>235</td>
<td>2.9</td>
<td>6.8</td>
<td>9.4</td>
</tr>
<tr>
<td></td>
<td>33</td>
<td>157</td>
<td>1.7</td>
<td>4.6</td>
<td>5.5</td>
</tr>
<tr>
<td></td>
<td>34</td>
<td>227</td>
<td>1.9</td>
<td>6.6</td>
<td>6.1</td>
</tr>
<tr>
<td></td>
<td>35</td>
<td>191</td>
<td>1.3</td>
<td>5.5</td>
<td>4.0</td>
</tr>
</tbody>
</table>
Results

Figure 2.15 - Gel filtration at pH 11 of Hep-1M-Conc

(A) Chromatogram: Proteins, eluted with a step gradient of 1 M NaCl from the heparin column were concentrated on a 100 kD membrane (Hep-1M-Conc) and in order to denature proteins for 2 h incubated with 100 mM CAPS (pH 11). Afterwards, gel filtration on a Superdex-200 column was performed in 20 mM CAPS (pH 11), 200 mM NaCl. All fractions across the run were assayed. Orange bars represent the percentage of BrdU for each fraction, when same volumes were assayed.

(B) Native PAGE: All fractions of the chromatography run at pH 11 were subjected to Native PAGE analysis using Tris-glycine buffer (pH 8.8). In each lane the same sample volume was loaded. Native PAGE clearly shows a separation of proteins or protein complexes according to their size, assuming that these proteins had a very similar isoelectric point.

(C) Non-reducing SDS-PAGE: When the same fractions were subjected to non-reducing SDS-PAGE, the proteins complexes were dissociated into a large number of bands. In each lane the same volume was loaded.
2.2.9.3.3 Gel Filtration At pH 11 Starting With Hep3/4-Conc

After the successful establishment of gel filtration at pH 11 we now examined the most purified fraction so far (Hep3/4-Conc) in order to include it into the overall purification scheme. We started with the 200-fold purified fraction Hep3/4-Conc after a four-step purification protocol including heparin affinity chromatography with a linear salt gradient (Hep3/4-Pool, Figure 2.10). After gel filtration at pH 11 we obtained a 1250-fold overall purification compared to Crude Bovine Thrombin, by pooling the six most active fractions around 45 kD. For the gel filtration step we calculated 6-fold purification with 48% yield, what is an excellent value for size exclusion chromatography. Furthermore, in the peak fraction number 33 we even obtained almost 2000-fold purification with a total yield of 1.9% (Table 2.15). This fraction number 33 is the most purified fraction we have found with all purification schemes so far. Interestingly, the chromatogram as well as the analysis on non-reducing SDS-PAGE displayed a very similar protein composition and distribution across the run compared to, when started with the less purified material Hep-1M-Conc (Figure 2.16). This confirms again the fact that a linear gradient on heparin does not separate some contaminating proteins away, but dilutes out major contaminating protein at high concentrations of NaCl, what then leads to a more purified fraction compared to step gradient elution on heparin.

In conclusion, gel filtration at pH 11 is a very powerful step in the overall purification scheme as it firstly separates the activity from the bulk of proteins and provides a good yield. Secondly, it isolates the low molecular weight form of SPRF (around 45 kD) without the necessity of any renaturation afterwards and finally allows the further purification of SPRF based on the nature of the low molecular weight form. This issue will be argued in more detail in the final discussion part.
Table 2.15 - Gel filtration at pH 11 after heparin affinity chromatography using a linear gradient of NaCl (Hep-3/4-Conc)

After cation exchange (CMFF-FT), hydrophobic interaction chromatography (Butyl-20%EtOH) and heparin affinity using a linear gradient of NaCl (Hep-3/4-Pool) the concentrated pool after ultrafiltration on a 100 kD membrane Hep-3/4-Conc was applied to gel filtration at pH 11 on a Superdex-200 column. Activity was recovered in molecular weight region around 45 kD (fractions 31-36). For each fraction yield (%) and purification factor (fold) is shown.

<table>
<thead>
<tr>
<th>Purification Step (Column)</th>
<th>Fraction</th>
<th>Overall Purification (fold)</th>
<th>Overall Yield (%)</th>
<th>Step Purification (fold)</th>
<th>Step Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Starting material</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Crude Bovine Thrombin</td>
<td>CB-Thrombin</td>
<td>1.0</td>
<td>100</td>
<td>1.0</td>
<td>100</td>
</tr>
<tr>
<td>Cation exchange</td>
<td>CMFF-FT</td>
<td>1.4</td>
<td>93</td>
<td>1.4</td>
<td>93</td>
</tr>
<tr>
<td>(HiPrep CMFF)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hydrophobic interaction</td>
<td>Butyl-20% EtOH</td>
<td>23</td>
<td>63</td>
<td>16.6</td>
<td>68</td>
</tr>
<tr>
<td>(HiPrep Butyl)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heparin affinity</td>
<td>Hep3/4-Pool</td>
<td>183</td>
<td>23</td>
<td>7.9</td>
<td>36</td>
</tr>
<tr>
<td>(HiTrap Heparin)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ultrafiltration</td>
<td>Hep3/4-Conc</td>
<td>209</td>
<td>14</td>
<td>1.1</td>
<td>61</td>
</tr>
<tr>
<td>(NMWL 100 kD)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gel filtration (pH 11)</td>
<td>31-36</td>
<td>1253</td>
<td>6.6</td>
<td>6.0</td>
<td>48</td>
</tr>
<tr>
<td>(Superdex 200)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>31</td>
<td>646</td>
<td>0.9</td>
<td>3.1</td>
<td>6.2</td>
</tr>
<tr>
<td></td>
<td>32</td>
<td>1438</td>
<td>1.6</td>
<td>6.9</td>
<td>11.5</td>
</tr>
<tr>
<td></td>
<td>33</td>
<td>1937</td>
<td>1.9</td>
<td>9.3</td>
<td>13.8</td>
</tr>
<tr>
<td></td>
<td>34</td>
<td>1531</td>
<td>1.2</td>
<td>7.3</td>
<td>8.5</td>
</tr>
<tr>
<td></td>
<td>35</td>
<td>1055</td>
<td>0.6</td>
<td>5.1</td>
<td>4.4</td>
</tr>
<tr>
<td></td>
<td>36</td>
<td>926</td>
<td>0.5</td>
<td>4.4</td>
<td>3.4</td>
</tr>
</tbody>
</table>
Figure 2.16 - Gel filtration at pH 11 starting with a 200-fold purified fraction (Hep3/4-Conc)

(A) Chromatogram: Proteins, eluted with a linear gradient of NaCl from the heparin column were concentrated on a 100 kD membrane (Hep3/4-Conc) and in order to denature proteins for 2 h incubated with 100 mM CAPS (pH 11). Afterwards, gel filtration on a Superdex 200 column was performed in 20 mM CAPS (pH 11), 200 mM NaCl. All fractions across the run were assayed. Orange bars represent the percentage of BrdU for each fraction, when same volumes were assayed. (B) Non-reducing SDS-PAGE: All fractions of the chromatography run at pH 11 were subjected to non-reducing SDS-PAGE. In each lane the same sample volume was loaded.
2.2.10 **Preparative PAGE And Isoelectric Focusing**

2.2.10.1 **Preparative Non-reducing SDS-PAGE**

In addition to gel filtration experiments in SDS we wanted to determine the molecular weight of SPRF by preparative SDS-PAGE in the absence of disulfide reducing agents. Knowing where the activity ran on SDS-PAGE gels would greatly facilitate the purification because it would allow us to correlate bands with activity across the purification. For preparative SDS-PAGE we used pre-cast Bis-Tris Gels. They provide excellent separation and resolution for small- to medium-sized proteins by utilizing a neutral pH environment, which minimizes protein modifications (NuPAGE Novex Bis-Tris gels, Invitrogen; (Laemmli, 1970)).

In initial experiments, using partially purified Hep3-Conc, the complete molecular weight range of gels was analyzed and it was ascertained that activity could be recovered in regions of less than 50 kD. We then further refined the localization of SPRF. Figure 2.17 shows an experiment in which Hep3 was analyzed on a 4-12% SDS gel, using a MOPS buffer system. Five lanes of the gel were sliced into segments and the proteins were extracted. All the SPRF activity was detectable in the region between 28 and 39 kD with an overall yield of 50%. Quantification of gels revealed that 1/10th of total protein runs in this region.

In experiments, where proteins between 28 and 39 kD were electro-eluted from the gel, we were able to recover between 80 to 100% of the loaded activity after reconstitution with a 10-fold purification, indicating that all of activity exists in this low molecular weight form.

As a way of testing whether it was possible to correlate major protein bands with activity, we performed preparative SDS-PAGE followed by a modified staining protocol with a Coomassie® G-250 stain (SimplyBlue™ SafeStain, Invitrogen). Similar levels of activity were recovered from regions between 28 and 39 kD compared to a non-stained control.

In addition, a protocol of reversed staining with ZnCl$_2$ (Fernandez-Patron et al., 1995) also allowed us to retrieve activity, but it was more difficult to visualize the bands afterwards compared to Simply Blue staining.
Figure 2.17 - Identification of SPRF activity after SDS-polyacrylamide gel electrophoresis

Samples were solubilized (without disulfide reduction) and electrophoresed as described under Material and Methods, then sample lanes were excised and sliced, as indicated, into regions bounded by the molecular weight markers. After elution of proteins into PBS followed by dialysis, samples were assayed. SPRF activity was only detected in the region between 28 and 39 kD, with the bulk of the activity found between 28 and 35 kD. Purification factor is calculated based on the fraction of protein in the region determined by scanning after fixation and silver staining. Yellow bars represent the percentage of BrdU-positive myotubes, found, when equal volumes of each fraction were assayed.

2.2.10.2 Preparative Native PAGE

After obtaining high recovery and good purification of the 30 kD low molecular weight form of SPRF by preparative SDS-PAGE we investigated, if native gel electrophoresis could be used to separate the high molecular weight form of SPRF from all other low molecular weight proteins. A subsequent denaturing step with SDS would then provide an even more powerful purification. Non-denaturing gel electrophoresis, also referred as native gel electrophoresis, separates proteins based on their molecular weight and their surface charge. In contrast to SDS polyacrylamide gel electrophoresis, the conditions for non-denaturing gel electrophoresis minimize protein denaturation and permit the separation of protein complexes. Native electrophoresis was carried out in Tris-Glycine buffer at pH 8.8, where most proteins are negatively charged (Bollag and Edelstein, 1991). The starting material for preparative native PAGE, Hep-1M-Conc, was a partially
purified product after four steps that included cation exchange chromatography (CMFF), hydrophobic interaction chromatography (Butyl), heparin affinity chromatography (step gradient elution with 1 M NaCl) and finally concentrating the material on a 100 kD membrane with simultaneous desalting into 20 mM Tris (pH 7.0), 100 mM NaCl (Table 2.14).

Initial tests of this starting material on analytical native gels revealed that proteins of the purified fraction were almost exclusively high molecular weight aggregates that did not even enter the stacking gel. Incubation of the partially purified material with 0.1% Triton X-100, 1% CHAPS or in 100 mM CAPS (pH 11) for 2 h dissociated high molecular weight proteins to some extent with incubation at pH 11 having the strongest effect. Therefore, the partially purified starting fraction (Hep-1M-Conc) was incubated in 100 mM CAPS (pH 11) in order to dissolve aggregates or protein complexes and to facilitate entry into the stacking gel. Preparative native PAGE was performed with Tris-glycine gels using a Tris-glycine buffer (pH 8.8). Electrophoresis was run at 4°C to reduce loss of protein activity by denaturation and to minimize attack by proteolysis. After electrophoresis the gel was washed in PBS, lanes sliced into eight sections followed by homogenization of gel pieces and extraction of proteins by passive diffusion. After desalting, samples were assayed for SPRF activity on myotubes. SPRF activity was found to migrate in all the sections from the upper half of the native gel in the region where 80% of the total protein was visualized on Coomassie stained gels. No peak of activity was observed with the activity uniformly spreading throughout this region (Figure 2.18). Moreover, none of the gel slices with activity provided a considerable purification compared to the starting material. The overall recovery of SPRF activity from the native gel was 47%.

In summary, preparative native PAGE did not succeed in separating the SPRF activity under native conditions from the bulk of proteins based on size and charge. Although in contrast to native gel filtration the surface charge of the protein is also essential for its running behavior, the activity still migrated with the mass of proteins. This finding demonstrated again heterogeneity in size and/or in charge of the high molecular weight SPRF complex, probably due to the variety of other bound proteins. Furthermore it is consistent with other chromatographic modes such as anion exchange or hydroxyapatite, where we also observed a charge diversity of high molecular weight SPRF under native conditions. We did not further investigate native PAGE as a potential purification step but decided to use a size-separating step under denaturing conditions such as gel filtration at pH 11 or preparative SDS-PAGE, where the 30 kD of SPRF was isolated.
Results

Figure 2.18 - Preparative native PAGE of a partially purified fraction (Hep-1M-Conc)

(A) Comparison of Molecular Standard Proteins (Sigma) without (control) and with 2 h incubation in 100 mM CAPS (pH 11) showing that migration of proteins was not impaired after incubation at pH 11. Arrows mark selected standard proteins that have a similar isoelectric point (less than 6) as supposed for the SPRF high molecular weight complex.

(B) The samples were solubilized (including 2 h incubation at pH 11) and electrophoresed as described under Material and Methods, then sample lanes were excised and sliced into eight regions. After elution of proteins into PBS followed by desalting into Low Serum MEM, samples were assayed. SPRF activity was detected in the upper region of the gel, with the bulk of other proteins. Purification factor is calculated based on the fraction of protein in the region determined by scanning after fixation and Coomassie staining. Yellow bars correspond to the percentage of BrdU-positive myotubes, found, when equal volumes of each fraction were assayed.
2.2.10.3 Preparative Isoelectric Focusing On IPG Strips

Size exclusion chromatography in 0.1% SDS as well as preparative SDS-PAGE have demonstrated that SPRF activity is a homogenous protein in terms of size with a molecular weight of about 30 kD. In order to further characterize the low molecular weight form of SPRF we have developed a semi-preparative isoelectric focusing procedure using immobilized pH gradient gel strips (IPG strips; (Berkelmann and Stenstedt, 2002; Gorg et al., 2000)). In this protocol a partially purified fraction, after cation exchange chromatography, heparin affinity chromatography (Hep3) and ultrafiltration on a 100 kD membrane (Hep3-Conc), was subjected to isoelectric focusing on IPG strips under denaturing conditions in 8 M urea, 2% CHAPS and then extracted from the strip and assayed for activity.

For proper focusing of proteins any salt contaminants present in the Hep3 fraction were removed by repeated concentration and dilution with Anion Buffer on a 100 kD membrane and subsequent desalting over NAP-5 columns into 8 M urea, 2% CHAPS. Following passive rehydration of the IPG strips, proteins were focused on strips with a linear pH gradient ranging from 3 to 10. Afterwards, the IPG strips were sliced into pieces, and then the proteins were extracted and re-natured by dialysis. SPRF activity could be recovered with a peak of stimulation from strip pieces in a pH range from 5.5 to 7 (Figure 2.19A) along with a total yield of 20% after isoelectric focusing. Analysis of IPG strips, focused in parallel and applied to non-reducing SDS-PAGE as second dimension with subsequent silver staining of proteins showed, that proteins were properly focused over the entire pH-range (Figure 2.19B). However, quantification of these stained gels did not reveal any additional purification in active fractions after isoelectric focusing compared to the Hep3-Conc starting material. Further experiments on IPG strips with a narrower pH-range, from 4 to 7, did not improve the resolution of the multiple forms of SPRF from each other.

In summary, isoelectric focusing of SPRF under denaturing conditions in 8 M urea, 2% CHAPS characterized the low molecular weight form of SPRF as a slightly acidic protein with an isoelectric between 5.5 and 7, but could not separate SPRF from other proteins and therefore did not succeed as a purification step.
Figure 2.19 - Isoelectric focusing of SPRF (Hep3-Conc) in 8 M urea on IPG-strips (with a pH-range from 3 to 10)

(A) Concentrated Hep3, desalted into 8 M urea, 2% CHAPS was focused, and the IPG-strip afterwards cut into pieces 1.5 cm in length. Proteins were extracted from strips by overnight incubation in PBS and then dialyzed against medium. Refolded proteins were finally assayed on myotubes. Substantial activity was recovered in a range from pH 5.5 to 7.0. Yellow bars represent the percentage of BrdU-positive myotubes, found, when equal volumes of each fraction were assayed.

(B) Silver stained gel: Non-reducing SDS-PAGE of an identical IPG-strip, focused in parallel and subjected to the second dimension,
2.2.10.4 2D-PAGE Preparative 2D-PAGE Including Staining With Simply Blue Safestain

With the knowledge that SPRF remains active after isoelectric focusing in urea or SDS-PAGE as well as after staining with colloidal Coomassie, it was now of interest to combine all methods and test whether it is possible to assign activity to certain proteins spot in a 2D gel. Therefore, we performed preparative isoelectric focussing followed by non-reducing SDS-PAGE (Berkelmann and Stenstedt, 2002; Gorg et al., 2000). After colloidal Coomassie staining, the gel region between 28 and 39 kD was subdivided and gel pieces assayed for SPRF activity.

Partially purified SPRF (Hep3-Conc; Table 2.13) was concentrated and desalted into Anion Buffer by ultrafiltration on a 100 kD membrane, further desalted over NAP-5 columns into 8 M urea, 2% CHAPS and focused on IPG strip with a pH range from 4 to 7. Subsequently, the strip was incubated in SDS-PAGE Equilibration Buffer, cut into three equal pieces and each piece put on top of a SDS gel respectively. For maximum resolution 10% gels were used. Running conditions and the staining procedure with Simply Blue were the same as for preparative SDS-PAGE. After staining, the region between 28 and 39 kD was selected and divided into 21 gel slices according to major protein spots or series of spots. Gel slices were minced and containing proteins extracted by passive diffusion, dialyzed and assayed on myotubes. Several gel slices, varying in isoelectric point and molecular weight contained activity and revealed a micro-heterogeneity of the low molecular weight form of SPRF (Figure 2.20). From visible protein spots, however, it can be excluded that improper focusing or migration in the gel occurred. The overall yield of SPRF activity after protein extraction was calculated to be 4.2 % starting from Hep3-Conc. Based on the quantification of stained SDS-PAGE gels no purification was obtained in any gel slices containing activity.

In conclusion, after preparative 2D-PAGE of a 40-fold purified fraction with subsequent Coomassie staining, we were not able to correlate major protein spots or bands in the 2D gel with the found activity profile. We therefore decided to subject gel slices with SPRF activity to mass spectrometry and try to correlate identified proteins with the distribution of the activity (see below).
Figure 2.20 - Preparative 2D-PAGE of Hep3-Conc with Simply Blue staining for correlation of protein spots with activity

After focusing proteins on IPG-strips, the strips were applied to SDS-PAGE, followed by staining with colloidal coomassie. The gel region between 28 and 39 kD was cut into slices based on visible protein spots, proteins extracted and assayed on myotubes for BrdU incorporation. Distinct regions of the 2D gel, with proteins varying in isoelectric point and molecular weight contained SPRF activity. Numbers shown, represent the percentage of BrdU-positive myotubes found, when equal volumes of each fraction were assayed.

2.2.11 Mass Spectrometry Of Fractions From The Purification
2.2.11.1 Mass Spectrometry Of Gel Slices After Preparative 2D-PAGE:
Correlation Of Activity With Protein Identity

As presented above we were able to recover activity from several regions after preparative 2D-PAGE of Hep3-Conc, a 40-fold purified fraction over Crude Bovine Thrombin. We therefore wanted to test whether it is possible to correlate one protein with activity by mass spectrometry analysis of all gel regions that contained activity.

An IPG strip was rehydrated and focused, then subjected to SDS-PAGE and the gels afterwards stained with Simply Blue as described for preparative 2D-PAGE above (Figure 2.20). We then cut out regions of the gel that contained activity when assayed on myotubes and subjected them to mass spectrometry. Data, obtained from LC MS/MS
analysis, were searched against all available bovine proteins sequences in the NCBI database and 45 proteins identified, many of them in multiple gel slices (Table 2.16). Nevertheless, we were not able to clearly identify one candidate for SPRF with this approach. Moreover, we cannot be sure that the activity was detected, due to the limited sensitivity of the mass spectrometer, the protein complexity of the gel slices as well as the significant loss of peptides using trypsin in-gel digestion.

2.2.11.2 Mass Spectrometry After SDS-PAGE Of Hep3-Butyl

A second attempt for mass spectrometry identification was performed with a more purified fraction, Hep3-Butyl (240-fold over Crude Bovine Thrombin). After cation exchange chromatography on CMFF, hydrophobic interaction chromatography on butyl and heparin affinity chromatography 3 ml of Hep3-Butyl (15 µg total protein) were precipitated by the Wessel-Fluegge-protocol (Wessel and Flugge, 1984) and run on a non-reducing SDS-PAGE gel. After Coomassie staining, visible protein bands as well as regions in between in the molecular weight region from 28 to 39 kD (about 1.5 µg protein) were analyzed separately using LC MS/MS. A total of 47 proteins were identified in the molecular weight region between 28 and 39 kD, searching against all bovine proteins in the NCBI database (Table 2.17). In addition, for many of the identified proteins, the peptide fragments gave good coverage for distinct domains of large polypeptides. We are currently cloning all interesting identified proteins and intend to test them on newt A1 myotubes after recombinant expression (see discussion).
When searching the LC MS/MS data against all available bovine protein sequences in the NCBI database, 45 proteins were identified with many of them in multiple gel slices. The proteins were classified according to Adkins et al. (2002). Only proteins with high score are shown.

| (1) Common circulating blood proteins       | (5) Proteases                              |
|                                         | carboxypeptidase N catalytic chain precursor |
| fibrinogen alpha chain                  |                                           |
| fibrinogen beta chain precursor         |                                           |
| fibronectin                             |                                           |
| fibronectin 1 isoform 3 preproprotein   |                                           |
| thrombospondin                          |                                           |

| (2) Coagulation and complement factors    | (6) Other enzymes                         |
| coagulation factor II [thrombin]         |                                           |
| coagulation factor IX, partial           |                                           |
| coagulation factor V (proaccelerin, labile factor) |   |
| coagulation factor X precursor (Stuart factor) | |
| complement component 4A preproprotein   |                                           |
| complement component C4                  |                                           |

| (3) Blood transport and binding proteins  | (7) Cytokines and hormones                |
| ceruloplasmin                            |                                           |

| (4) Protease inhibitors                   | (8) Channel and receptor-derived peptides |
| alpha-2-macroglobulin precursor (Alpha-2-M) |                                           |
| inter-alpha (globulin) inhibitor H3       |                                           |
| inter-alpha (globulin) inhibitor H4       |                                           |
| inter-alpha-trypsin inhibitor (protein HC), light |   |
| inter-alpha-trypsin inhibitor heavy chain2 |                                           |
| inter-alpha-trypsin inhibitor heavy-chain H1 |                                          |

| (9) Miscellaneous (structural, nuclear, etc.) | |
| 78 kDa glucose-regulated protein precursor  | |
| actin, cytoplasmic 2 (Gamma-actin)          | |
| cytkeratin                                 | |
| epithelial keratin 1                       | |
| eukaryotic translation elongation factor 1  | |
| alpha K15 intermediate filament type I keratin | |
| keratin 10 (epidermolytic hyperkeratosis)   | |
| keratin 4                                  | |
| keratin 6A                                 | |
| keratin 6L                                 | |
| keratin, type I cytoskeletal 14 (Cytokeratin 14) |  |
| keratin, type I cytoskeletal 18 (Cytokeratin 18) |  |
| keratin, type II cytoskeletal 6A (Cytokeratin 6A) |  |
Results

Table 2.17 - Identified proteins after SDS-PAGE of Hep3-Butyl in the region between 28 and 39 kD.
When searching the LC MS/MS data against all available bovine protein sequences in the NCBI database, 47 proteins were identified. The proteins were classified according to Adkins et al. (2002). Only proteins with high score are shown.

<table>
<thead>
<tr>
<th>(1) Common circulating blood proteins</th>
<th>(5) Proteases</th>
</tr>
</thead>
<tbody>
<tr>
<td>66K glycoprotein precursor (Vitronectin)</td>
<td>Carboxypeptidase N catalytic chain precursor</td>
</tr>
<tr>
<td>albumin</td>
<td>protein C prepropeptide</td>
</tr>
<tr>
<td>apolipoprotein A1</td>
<td></td>
</tr>
<tr>
<td>Fibrinogen alpha chain</td>
<td></td>
</tr>
<tr>
<td>Fibrinogen beta chain precursor</td>
<td></td>
</tr>
<tr>
<td>fibronectin 1 isoform 3 prepropeptide, partial</td>
<td></td>
</tr>
<tr>
<td>fibronectin precursor, partial</td>
<td></td>
</tr>
<tr>
<td>thrombospondin</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>(2) Coagulation and complement factors</th>
<th>(6) Other enzymes</th>
</tr>
</thead>
<tbody>
<tr>
<td>coagulation factor II [thrombin]</td>
<td></td>
</tr>
<tr>
<td>Coagulation factor IX (Christmas factor), partial</td>
<td></td>
</tr>
<tr>
<td>Coagulation factor V (proaccelerin, labile factor)</td>
<td></td>
</tr>
<tr>
<td>Coagulation factor X precursor (Stuart factor)</td>
<td></td>
</tr>
<tr>
<td>complement component 4A prepropeptide</td>
<td></td>
</tr>
<tr>
<td>complement component C3, partial</td>
<td></td>
</tr>
<tr>
<td>complement component C4</td>
<td></td>
</tr>
<tr>
<td>mannan-binding lectin serine protease 1 isoform</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>(3) Blood transport and binding proteins</th>
<th>(7) Cytokines and hormones</th>
</tr>
</thead>
<tbody>
<tr>
<td>ceruloplasmin, partial</td>
<td>bone morphogenetic protein 4</td>
</tr>
<tr>
<td>hemoglobin beta chain - domestic ferret</td>
<td>Bone morphogenetic protein 7, partial</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>(4) Protease inhibitors</th>
<th>(8) Channel and receptor-derived peptides</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heparin cofactor II precursor (HC-II) (Protease</td>
<td></td>
</tr>
<tr>
<td>inter-alpha (globulin) inhibitor H3</td>
<td></td>
</tr>
<tr>
<td>inter-alpha (globulin) inhibitor H4 (plasma Kall</td>
<td></td>
</tr>
<tr>
<td>inter-alpha-trypsin inhibitor (protein HC), light</td>
<td></td>
</tr>
<tr>
<td>inter-alpha-trypsin inhibitor heavy chain2, part</td>
<td></td>
</tr>
<tr>
<td>inter-alpha-trypsin inhibitor heavy-chain H1, pa</td>
<td></td>
</tr>
<tr>
<td>keratin 5 (epidermolysis bullosa simplex…</td>
<td></td>
</tr>
<tr>
<td>Keratin, type I cytoskeletal 14 (Cytokeratin 14)</td>
<td></td>
</tr>
<tr>
<td>keratin 24</td>
<td></td>
</tr>
<tr>
<td>Keratin, type II cytoskeletal 6A (Cytokeratin 6A</td>
<td></td>
</tr>
<tr>
<td>keratin 25B</td>
<td></td>
</tr>
<tr>
<td>keratin 4, partial</td>
<td></td>
</tr>
<tr>
<td>Lumican</td>
<td></td>
</tr>
<tr>
<td>NEDD8 ultimate buster-1, partial</td>
<td></td>
</tr>
<tr>
<td>myoglobin</td>
<td></td>
</tr>
<tr>
<td>type I keratin KA22</td>
<td></td>
</tr>
</tbody>
</table>
2.2.12 Raising Blocking Monoclonal Antibodies Against SPRF

In a completely different approach to identify SPRF we tried to raise mouse monoclonal antibodies that blocked the response of myotubes to re-enter S-Phase in response to partially purified SPRF. Antigen was prepared from 1 g Crude Bovine Thrombin by cation exchange chromatography, heparin affinity chromatography, concentration of the Heparin pool on a 100 kD membrane (Hep3-Conc) and finally preparative SDS-PAGE under non-reducing conditions. In the final step the region between 28 and 39 kD was excised, the gel homogenized and the proteins electro-eluted. The total protein recovery after electro-elution was ~50% with 80 to 100% activity recovery. Therefore, purification for the preparative SDS-PAGE/electro-elution was calculated to be 20-fold over Hep3-Conc. A total sample of 200-300 µg protein was obtained for immunization starting from 1 g Crude Bovine Thrombin (total purification was about 400 to 800 fold with 20% total yield).

The lyophilized samples were sent to Alan Sawyer, EMBL-Monterotondo, who tried to generate monoclonal antibodies in mice. The first series of immunizations, however, failed to generate a sufficient immune response for generation of hybridoma cells, likely due to insufficient antigen injection or the close evolutionary relationship of bovine and mouse plasma proteins. We therefore intend to repeat this experiment with a more purified fraction, higher amounts of antigen per injection, as well as different protocols for immunization of the mice.
3 Discussion

In contrast to human beings, the urodele amphibians, such as newt, are in the enviable position to replace complete body parts after serious damage. An early step in this amazing process is the mobilization of cells next to the site of injury, which undergo cellular dedifferentiation and form a mass of proliferating stem cell-like progenitor cells referred as the blastema (Brockes and Kumar, 2005; Tanaka, 2003). How differentiated cells re-enter the cell cycle and then contribute to the blastema is a crucial question in studying amphibian regeneration. Previously, Tanaka et al. reported that serum of various animal species contains a factor, termed S-Phase Re-entry Factor (SPRF) that stimulates DNA synthesis in differentiated newt myotubes (Tanaka et al., 1997). This initiation of DNA synthesis in a differentiated non-proliferating cell is thought to be one of the initial events in dedifferentiation. Interestingly, the serum factor becomes activated by two blood proteases, thrombin or plasmin, that are central players in the course of blood coagulation and fibrinolysis (Tanaka et al., 1999). Therefore, SPRF might represent a link between blood coagulation and the local activation of factors that stimulate newt cells to proliferate again and replace lost cells and tissue. The main focus of the PhD Thesis presented here was the biochemical characterization and purification of this S—phase re-entry factor.

3.1 Crude Bovine Thrombin – The Starting Material For The Purification

The starting material for the purification, Crude Bovine Thrombin, is a commercially available, partially purified bovine plasma fraction where coagulation was activated by addition of an extract of bovine lung tissue. This preparation is the best starting material for the purification found so far with 10 to 20-fold higher specific activity than bovine serum. It contains high concentrations of active thrombin that in turn has probably activated all or most of the latent form of the myotube stimulating activity. SDS-PAGE analysis of the starting material revealed the disappearance or significant reduction of some major protein bands in Crude Bovine Thrombin compared to bovine serum. However, the use of this commercial bovine serum preparation also raised several issues: firstly, the purification from a serum versus a plasma product; secondly, the constant availability of the starting material; and finally the coverage of bovine protein sequences in databases.
Generally, the purification of proteins from serum should be more difficult than from plasma because serum contains a much more complex pattern of proteins and proteolytic fragments after initiation of blood coagulation in concert with the activation of numerous blood proteases. However, the latent form of S-phase re-entry factor in plasma requires activation with thrombin or plasmin, both members of the blood coagulation process. Therefore a purification of SPRF from plasma would entail the subsequent activation of all fractions from the purification with thrombin and the inhibition of thrombin afterwards. This did not seem to be a very practical approach when screening a large number of fractions. We further do not know whether thrombin and plasmin are sufficient for the complete activation of the factor or other components of serum are also required. The purification from Crude Bovine Thrombin allows us to directly purify and assay the active form of SPRF without the need of any prior proteolytic processing. Furthermore, Crude Bovine Thrombin is already 10 to 20-fold enriched in SPRF providing another great advantage over unfractionated plasma or serum.

A second concern for the purification process from this starting material was its availability and cost upon the need to scale up in order to finally identify the activity. We are now in direct contact with the original manufacturer and able to order bulk amounts of this preparation for a reasonable price, less than 50 Euros per gram. Therefore, the amount of starting material is not considered to be a bottleneck anymore and the estimated amounts required for identification will be discussed in more detail below.

Thirdly, one major drawback in the purification scheme, with respect to the final identification by mass spectrometry, was the utilization of bovine material. For this reason, in order to facilitate the final mass spectrometry analysis we were extensively searching for a starting material from a human source. In the course of this work we have tested, after activation with thrombin, human plasma and several commercially available human plasma concentrates but never obtained comparable specific activity compared to Crude Bovine Thrombin. We therefore decided to continue with Crude Bovine Thrombin as the starting material with the objective of obtaining enough material for micro-sequencing approaches or mass spectrometry de novo sequencing after scaling up the purification process. However, an ongoing effort to sequence the entire bovine genome (Andersson and Georges, 2004; Fadiel et al., 2005; Lewin, 2003; Sonstegard and van Tassell, 2004) led to the release of a high-quality draft genome sequence in 2005 and this should greatly facilitate the identification of the serum factor by mass spectrometry.
3.2 Characterization Of The Serum Factor In The Starting Material

For the establishment of the best possible purification procedure we have carefully characterized the SPRF activity in the starting material, Crude Bovine Thrombin. We have demonstrated that the activity, driving cell-cycle re-entry in newt A1 myotubes, is a heat-labile, pH- and Protease K-sensitive glycoprotein that has a specific affinity to heparin. Furthermore, the activity is a very robust molecule as it can be recovered from strong denaturing conditions such as 8 M urea, 2% SDS, 60% acetonitrile or ethanol but also from pure water.

Depletion of the activity by immobilized lectins with specificity for glucose, mannose, galactose, sialic acid and N-acetylglucosamine suggested glycosylation of the target protein. Furthermore, deglycosylation of N-linked sugar chains with PNGase F diminished but did not abolish activity. It is important to note that at later stages in the purification, when lectin affinity chromatography was again tested, the activity was only retained by wheat germ agglutinin (affinity to N-acetylglucosamine) but not by Concanavalin A or Lentil lectin indicating that the proteins binding these lectins were presumably fractionated away.

As demonstrated by size exclusion chromatography of both Crude Bovine Thrombin and fetal bovine serum, the serum factor behaves as a high molecular weight complex of 200 000 Dalton or larger under native conditions (Tanaka et al., 1997). In contrast, gel filtration of Crude Bovine Thrombin under denaturing conditions, such as with SDS or at pH 11, revealed that the activity acts as a low molecular weight protein of about 30 kD. Subsequently, we have studied the chromatographic behavior of the activity under denaturing conditions in SDS, at pH 11 and in urea, starting with a more purified fraction. We found again that activity was present in this low molecular weight form. Gel filtration in SDS as well as at pH 11 revealed a relatively homogeneous size distribution of the activity centered at 30 kD. In contrast to results under native conditions, no activity was detected at higher molecular weights. These findings were validated by preparative SDS-PAGE in the absence of disulfide-reducing agents, where most of the SPRF activity was recovered from the gel between 28 and 35 kD.

In order to determine the isoelectric point of the 30 kD form of SPRF we performed semi-preparative isoelectric focusing under denaturing conditions on IPG strips. After focusing in 8 M urea SPRF activity was recovered within a pH-range from 5.5 to 7, indicating a micro-heterogeneity of the 30 kD SPRF with respect to charge. It is unclear whether this heterogeneity derives from glycosylation, proteolytic processing or another posttranslational modification.
3.3 Investigation Of Chromatography Techniques For The Purification

In order to develop a purification scheme we have investigated various chromatographic separation modes. Cation exchange chromatography on CMFF at pH 6.5, where SPRF is not retained on the column, was established as the first step in the purification (Straube et al., 2004). It does not provide a significant purification however efficiently removes thrombin from the preparation that otherwise would be toxic to the myotubes at high concentrations and also causes a background activation of latent SPRF in the serum containing culture media.

By testing various chromatographic modes we found that hydrophobic interaction chromatography, heparin affinity chromatography, ultrafiltration on a 100 kD membrane, size exclusion chromatography under denaturing conditions (SDS or at pH 11) and preparative SDS-PAGE gave acceptable yield and significant purification (Table 3.1). On the other hand size exclusion chromatography under native conditions, anion exchange chromatography, chromatography on hydroxyapatite, chromatofocusing, lectin affinity chromatography as well as preparative native PAGE could not fractionate the activity from the major bulk of proteins with high yield and therefore were not included in a purification scheme (Table 3.1). In an initial trial we also failed to produce a mouse monoclonal antibody against SPRF that blocks the S-phase re-entry response in newt A1 myotubes, presumably due to insufficient amounts of antigen injection.

Many of the unsuccessful purification approaches revealed an intrinsic heterogeneity in charge and size of the high molecular weight serum factor. In order to circumvent these difficulties when purifying under native conditions, our ongoing purification strategies involve fractionation of the low molecular weight form.
Table 3.1 - Overview of all investigated chromatography modes

The table summarizes all tested purification modes starting with Crude Bovine Thrombin (CBT) or a more purified fraction (PF). Denaturing conditions such as SDS, urea, acetonitrile or pH 11 were used for the purification of the low molecular weight SPRF.

<table>
<thead>
<tr>
<th>Successful modes</th>
<th>Starting material</th>
<th>Unsuccessful modes</th>
<th>Starting material</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native conditions</td>
<td></td>
<td>Native conditions</td>
<td></td>
</tr>
<tr>
<td>Cation exchange – CMFF</td>
<td>CBT</td>
<td>Gel filtration</td>
<td>CBT, PF</td>
</tr>
<tr>
<td>Hydrophobic interaction - Butyl</td>
<td>PF</td>
<td>Anion exchange – HiTrap Q / Mono Q</td>
<td>CBT, PF</td>
</tr>
<tr>
<td>Heparin affinity (step / linear)</td>
<td>PF</td>
<td>Hydroxyapatite</td>
<td>PF</td>
</tr>
<tr>
<td>Ultrafiltration - MWCO 100 kD</td>
<td>PF</td>
<td>Chromatofocusing</td>
<td>PF</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Lectin affinity (WGA, ConA, Lentil lectin)</td>
<td>PF</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Affinity to Cibacron Blue F3G-A</td>
<td>PF</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Native PAGE</td>
<td>PF</td>
</tr>
<tr>
<td>Denaturing conditions</td>
<td></td>
<td>Denaturing conditions</td>
<td></td>
</tr>
<tr>
<td>Gel filtration – SDS / pH 11</td>
<td>PF</td>
<td>Gel filtration – SDS / pH 11</td>
<td>CBT</td>
</tr>
<tr>
<td>Preparative SDS-PAGE</td>
<td>PF</td>
<td>Isoelectric focusing (urea)</td>
<td>PF</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reversed phase chromatography</td>
<td>PF</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Generation of a monoclonal antibody</td>
<td>PF</td>
</tr>
</tbody>
</table>

3.4 Heterogeneity Of SPRF

A basic characteristic of the serum factor in the purification described here is that, in denaturing buffers, SPRF behaves as a protein of low molecular weight whereas under native conditions, it fractionates predominantly as a higher molecular weight complex. This is not an unexpected property for plasma proteins as they often bind to each other and affect the function of their binding partner. Furthermore many plasma and serum proteins form high molecular weight complexes because they are otherwise too small to persist as monomers and would be removed by the kidney filtration apparatus with a cutoff of approximately 45 kD (Anderson and Anderson, 2002). We do not know whether the high molecular weight of SPRF reflects a natural complex or derives from nonspecific aggregation of proteins during the purification. However, one explanation for the heterogeneity of the high molecular weight SPRF in chromatography modes, where proteins are separated based on charge, might be the association with different combinations of binding partners.
In addition, plasma proteins undergo extensive post-translational modifications, including proteolytic processing, disulfide bonding, addition and processing of N- and O-linked glycans, gamma-carboxylation of glutamic acid residues, beta-hydroxylation of aspartic acid residues, sulfation of tyrosine residues, and phosphorylation of serine residues (White et al., 1998). This leads to an added complexity estimated to be up to 500 different variants per gene product (Anderson and Anderson, 2002). Of these potential modifications we have only addressed the issue of glycosylation where charge heterogeneity is thought to be due to the extent of sialic acid incorporation.

A further aspect of the diversity of SPRF might be the combination of several bovine blood sources for the preparation of Bovine Crude Thrombin. This might lead to a further increase in heterogeneity of the sample composition due to different isoforms of serum proteins. Whatever the cause, this charge heterogeneity is apparent during preparative isoelectric focusing experiments under denaturing conditions where the low molecular form of SPRF showed an isoelectric point ranging from 5.5 to 7.0.

These considerations of the heterogeneous behavior of SPRF in the native state suggest a number of purification strategies if additional purification steps are required. After chromatographic separation of thrombin and inhibition of all remaining proteolytic activity in the starting material (in order to stabilize the preparation) we could first isolate the high molecular weight complex with high yield. Following removal of low molecular weight proteins we could then dissociate the high molecular weight SPRF complex by gel filtration under denaturing conditions and separate the active, low molecular weight form from the bulk of proteins. This may increase the resolution of subsequent chromatographic fractionations as the behavior of 30 kD form of SPRF may no longer be influenced by formation of the high molecular weight complex. In addition we intend to test whether deglycosylation of N-linked sugar chains with immobilized PNGase F and the removal of sialic acid with Neuraminidase (Sialidase) is able to reduce charge heterogeneity and can provide superior chromatographic fractionation (Davies et al., 1994; Maley et al., 1989; O'Neill, 1996).
3.5 Summary Of The Final Purification Scheme

In this PhD thesis we have presented a new five-step purification scheme starting with a lyophilized Crude Bovine Thrombin preparation dissolved in Buffer at pH 6.5 (Table 3.2). The first step, cation exchange chromatography, serves for the removal of thrombin and unstable components. After cation exchange chromatography on Sepharose-CM-FF at pH 6.5, the flow through fractions were pooled (CMFF-FT) and remaining thrombin inactivated with the irreversible inhibitor PPACK (Bode et al., 1989). In order to prevent further proteolytic processing, other proteases in the preparation were inhibited by addition of an inhibitor mix. Afterwards the flow through was adjusted to 0.1 M ammonium sulfate and subjected to hydrophobic interaction chromatography on a Butyl-Sepharose column. Most of SPRF activity was eluted in 50 mM phosphate buffer (pH 7.0), containing 20% ethanol (Butyl-20%EtOH). After adjustment to 200 mM sodium chloride the active pool was loaded onto a heparin-Sepharose column and bound proteins eluted with a linear gradient of sodium chloride. Activity was found to elute between 200 mM NaCl and 800 mM across the gradient and pooling fractions between 430 and 680 mM NaCl gave 36% recovery and 8-fold purification for this step (Hep3/4-Pool). In order to re-concentrate proteins and to remove low molecular weight proteins we used ultrafiltration on a 100 kD membrane. We lost in this step almost 50% of the activity, probably due to the very low protein concentration and non-specific protein adsorption. Finally, the concentrated material (Hep3/4-Conc) was incubated for four hours at pH 11 and then applied to a size exclusion column at pH 11. This gel filtration step denatures protein complexes in the preparation allowing the isolation of the low molecular weight form of SPRF. With the purification procedure described, we have achieved about 2000-fold purification with 2% activity yield in the most purified fraction (GF11-33) compared to the starting material Bovine Crude Thrombin (Table 3.2). Considering that the starting material is already 20-fold enriched over bovine serum (Tanaka et al., 1999) we have obtained in combination a 40 000-fold purification of the S-phase re-entry factor over serum. Non-reducing SDS-PAGE revealed about 10 major visible bands in the most purified fraction GF11-33 (Figure 3.1).
The current purification scheme of SPRF starting from Crude Bovine Thrombin.

Our best purification scheme comprises a five-step protocol starting with cation exchange chromatography on CMFF in order to remove thrombin. Afterwards the flow through (CMFF-FT) is subjected to hydrophobic interaction chromatography, where the activity almost exclusively eluted in 20% ethanol from the Butyl column (Butyl-20%EtOH). In the next step Butyl-20%EtOH was loaded onto a heparin column and bound proteins eluted with a linear gradient of sodium chloride. Fractions, that gave good yield and purification were pooled (Hep3/4-Conc) and concentrated on a 100 kD membrane (Hep3/4-Conc). This ultrafiltration step also serves the removal low molecular weight proteins. Finally, the concentrated pool was applied to gel filtration under denaturing conditions at pH 11. For this step the pool of the six most active fractions as well as single fractions are given.

* In the purification chart presented here, all purification steps were normalized to 1 g of the starting material Crude Bovine Thrombin (77% protein).

** One unit is defined as the amount of SPRF-activity that stimulates 1% of myotubes to undergo S-phase re-entry (BrdU-positive myotubes in 150 µl medium volume)

<table>
<thead>
<tr>
<th>Column</th>
<th>Fraction</th>
<th>Purification (fold)</th>
<th>Yield (%)</th>
<th>Total Protein * (µg)</th>
<th>Total Activity ** (Units)</th>
<th>Specific Activity ** (Units/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Starting material</td>
<td>CB-Thrombin</td>
<td>1.0</td>
<td>100</td>
<td>777000</td>
<td>210567</td>
<td>271</td>
</tr>
<tr>
<td>Cation exchange</td>
<td>CMFF-FT</td>
<td>1.4</td>
<td>93</td>
<td>518000</td>
<td>194960</td>
<td>376</td>
</tr>
<tr>
<td>Hydrophobic interaction</td>
<td>Butyl-20% EtOH</td>
<td>23</td>
<td>63</td>
<td>21170</td>
<td>132471</td>
<td>6257</td>
</tr>
<tr>
<td>Heparin affinity</td>
<td>Hep3/4-Pool</td>
<td>183</td>
<td>23</td>
<td>959</td>
<td>47483</td>
<td>49487</td>
</tr>
<tr>
<td>Ultrafiltration (NMWL 100 kD)</td>
<td>Hep3/4-Conc</td>
<td>209</td>
<td>14</td>
<td>515</td>
<td>29166</td>
<td>56591</td>
</tr>
<tr>
<td>Gel filtration (pH 11)</td>
<td>Pool (31-36)</td>
<td>1253</td>
<td>6.6</td>
<td>41</td>
<td>13983</td>
<td>339630</td>
</tr>
<tr>
<td></td>
<td>31</td>
<td>646</td>
<td>0.9</td>
<td>10.4</td>
<td>1820</td>
<td>175022</td>
</tr>
<tr>
<td></td>
<td>32</td>
<td>1438</td>
<td>1.6</td>
<td>8.6</td>
<td>3362</td>
<td>389671</td>
</tr>
<tr>
<td></td>
<td>33</td>
<td>1937</td>
<td>1.9</td>
<td>7.7</td>
<td>4034</td>
<td>524872</td>
</tr>
<tr>
<td></td>
<td>34</td>
<td>1531</td>
<td>1.2</td>
<td>6.0</td>
<td>2480</td>
<td>414881</td>
</tr>
<tr>
<td></td>
<td>35</td>
<td>1055</td>
<td>0.6</td>
<td>4.5</td>
<td>1297</td>
<td>285894</td>
</tr>
<tr>
<td></td>
<td>36</td>
<td>926</td>
<td>0.5</td>
<td>3.9</td>
<td>990</td>
<td>250968</td>
</tr>
</tbody>
</table>
Figure 3.1 - Non-reducing SDS-PAGE of fractions across the best purification scheme so far

In each lane the same amount of total protein (0.5 µg) was loaded based on the calculation of protein concentration from the chromatograms using the absorbance at 280 nm. Note that in the most purified fraction GF11-33 the protein content is only half of expected when quantified on BSA standard and this is likely due to the difficulties in calculating the concentrations at low levels of protein from the absorbance at 280 nm.

3.6 How Close Are We To Identify Candidates

Many growth factors act at the low to sub-nanomolar range typically between 0.1-10 nM (Gospodarowicz and Moran, 1976). For a protein with an apparent molecular weight of 30 000 Da this would represent a concentration of 3 to 300 ng/ml. Assuming that 10 % serum is sufficient for maximal stimulation of newt A1 myotubes, SPRF would constitute between 0.5 and 50 ppm protein in serum. Therefore, in order to obtain the pure protein from serum, if SPRF acts at this low nM range we would require between 20 000 and 2 000 000-fold total purification.

In our best purification so far, we have obtained approximately 2000-fold higher specific activity in the most purified fraction, GF11-33, compared to Crude Bovine Thrombin. Depending on the assay and the batch of the starting material we have also shown that the Crude Bovine Thrombin is 10 to 20-fold purified over serum and therefore GF11-33 would present a total purification of up to 40 000 over serum.
In order to test whether it is possible to correlate defined molecular weight regions or distinct bands with the activity profile, we have quantified a silver stained gel after non-reducing SDS-PAGE across the gel filtration run at pH 11, where a 200-fold purified fraction was applied to the column (Hep3/4-Conc, Table 3.1). We noted a good correlation for a band around 30 kD with the activity profile from the myotube assay (indicated with an arrow in Figure 3.2). Moreover, protein bands above or below this candidate band did not correlate strongly with the activity profile. From our calculations (below), however, it is clear that if SPRF acts at the low to sub nanomolar range it likely represents one of the minor bands that might easily be obscured by contaminants.

We then quantified a silver stained gel across the purification where we loaded equal amounts of protein (Figure 3.1). We also determined the amount of protein for the “candidate” band in the lane for GF11-33 (Figure 3.1) and found that it represents 10 ng or 4% of the total protein in the lane. Thus we would in effect obtain 25-fold purification by isolating this single band prior to processing for mass spectrometry. Moreover, when calculating the concentration of the “candidate” band in the S-phase re-entry assays on myotubes, for a saturating response the protein would act at a concentration of 3 ng/ml or 0.1 nM, towards the low end of the typical range for growth factors. Taking together all assumed and experimentally determined purification factors from bovine serum to Crude Bovine Thrombin (20-fold), Bovine Crude Thrombin to GF11-33 (2000-fold) and GF11-33 to “candidate” band (25-fold) the isolation of this single band would represent a 1000000-fold purification from bovine serum.

Current mass spectrometry devices are easily capable of identifying proteins, if 100 fmol or even less is present. This would require 3 ng of a 30 kD protein. Even if post-translational modifications such as glycosylation blocked the identification of a third of the tryptics fragments, 10-fold more protein (30 ng) should be sufficient to match at least some peptides in the database. We are therefore very confident that mass spectrometry (MS) is sensitive enough to identity the S-phase re-entry factor. As noted above, if SPRF acts at 0.1 nM in the myotube assay, this would reflect 10 ng of a 30 kD protein in the lane GF11-33 in the silver stained gel of Figure 3.1. Considering the activity yield of the most purified fraction GF11-33 (1.9%, Table 3.2) and starting with 1 g Crude Bovine Thrombin (77.7% protein content), we would obtain approximately 150 ng or 5000 fmol of SPRF, an amount easily detectable by MS. This suggests that sensitivity will not stand in the way of identifying SPRF by MS and that this will be so even after losses stemming from additional purification steps. In the end we will always be much more limited by the sensitivity of the myotube assay than by mass spectrometry.
3.7 Quantitative Mass Spectrometry

Although we are convinced that SPRF can be identified by mass spectrometry, it is equally certain that we will obtain multiple hits for even from our most purified sample. Due to the sensitivity of the mass spectrometry methods and based on our last attempts we expect peptides from at least 10 to 20 proteins, all of which would have to be tested as possible “candidates”. In order to eliminate false positive protein hits we have now started a collaboration with the laboratory of Ruedi Aebersold in Zuerich and want to establish quantitative mass spectrometry as a tool to facilitate the purification. The basic concept of this method is to correlate SPRF activity in single fractions across a purification run with the peptide pattern of the fraction after digestion with trypsin. In the first round of mass spectrometry the tryptic digest is subjected a LC-MS run, where the mass, elution time, as well as the quantity of all detected tryptic peptides is ascertained. In the following computational analysis, all peptides that correlate with the activity profile are identified. Subsequently, in a second mass spectrometry experiment only the interesting peptides are analyzed by LC-MS/MS, where the amino acid sequence is determined. This approach was already shown to work successfully on crude serum samples (Li et al., 2005). In an initial attempt we are currently analyzing samples across the size exclusion chromatography runs at pH 11, where a 34-fold purified (Hep-1M-Conc, Table 2.14, Figure 2.15) or a 200-fold (Hep3/4-Conc, Table 2.15, Figure 2.16) fraction was subjected to gel filtration. Furthermore, we will analyze fractions from different stages across the purification, which differ about 10-fold in their specific activity (e.g. CMFF-FT, Butyl-20%EtOH, Hep3/4-Conc and GF11-33, see Table 3.1, Figure 3.1). The mass spectrometry analysis of equal amounts of total protein for each fraction should then reveal peptides, which are 10-fold enriched between different fractions.
Figure 3.2 - Quantitative mass spectrometry of fraction across gel filtration at pH 11

(A) Silver stained SDS-PAGE and activity profile of fractions from the gel filtration run at pH 11, starting with 200-fold purified Hep3/4-Conc: In each lane the same volume of a fraction was loaded. Activity data from several myotube assays were averaged and normalized to fraction 33, the most purified fraction so far.

(B) Correlation of SPRF activity with the “candidate” band across the gel filtration run: For the protein band the absorbance units were normalized to fraction 33 and compared to normalized activity data from (A). A correlation between activity and amount of protein is clearly recognizable. The candidate band is located in the gel region that contains most of the activity after preparative SDS-PAGE.
3.8 Future Purification Strategy

As presented above our current purification strategy first isolates the high molecular weight complex by cation exchange, hydrophobic interaction and heparin affinity chromatography using a linear or stepwise gradient of sodium chloride followed by protein concentration on a 100 kD membrane. Finally we isolate the low molecular SPRF by means of size exclusion chromatography at pH 11. An enormous benefit of gel filtration at pH 11 in contrast to gel filtration in SDS or preparative SDS-PAGE is that the low molecular weight factor can be directly subjected to another chromatographic mode without elaborate steps for removal of SDS. Moreover, this fraction is active on newt myotubes after simply adjusting the buffer back to pH 7. We have not tested yet whether this 30 kD SPRF exists in a monomeric form at physiological pH or oligomerizes and/or associates with another protein in the preparation. However, the removal of the contaminating large proteins, that may bind the activity and complicate the fractionation, offers the hope for a worthwhile re-investigation of several chromatographic separation techniques, that use harsh conditions and have failed so far in the purification of the high molecular weight complex (Table 3.1).

Our focus lies on high-resolution techniques such as anion exchange chromatography on Mono Q (with or without 20% ethanol), reversed phase chromatography on C4 using phosphate buffer and n-propanol, or chromatofocusing, all in combination with quantitative mass spectrometry analysis. Alternatively we could investigate hydrophilic interaction chromatography (HILIC) after gel filtration at pH 11. HILIC separates compounds by eluting proteins bound to a neutral hydrophilic stationary phase with a descending hydrophobic mobile phase gradient causing solutes to come off in order of increasing hydrophilicity – the inverse of reversed phase chromatography. This new method has been shown to be successful in the separation of phospho- and glycopeptides (Alpert, 1990; Zhu et al., 1992).

If the above separation modes display heterogeneity of SPRF with respect to charge, we will examine deglycosylation with recombinant PNGase F and Neuraminidase for the removal of sialic acid residues prior to chromatographic fractionation. We also have the opportunity to repeat affinity chromatography, in particular on wheat germ agglutinin (WGA) or heparin, and test whether the small SPRF protein binds directly as a ligand. Finally, we intend to repeat our current protocol and include a final preparative SDS-PAGE step followed by electro-elution of distinct regions or bands that, we calculated, would likely give a 50 000 to 100 000-fold purification, starting from Crude Bovine Thrombin.
However, the desired reduction of total protein in the course of the purification causes some technical challenges as more and more care has to be taken during the concentration of samples, already less than 10 µg/ml total protein. In previous purifications we have often used a carrier mix of insulin and aprotinin to reduce non-specific interactions; however, this is not preferable if we want to proceed with mass spectrometry analysis. In addition to minimizing intermediate steps to reduce losses, we are now testing activity recovery after passivation of the plastic surface of tubes and ultrafiltration devices with non-ionic detergents such as Triton X-100 and Tween-20 or with polyethyleneglycol (PEG). Furthermore, we will use commercially available low binding tubes and material.

3.9 Identification And Validation Of SPRF Candidates

Once we have identified interesting proteins by mass spectrometry we have to test and assess these candidates. A very quick way is the assay of commercially available purified or recombinant proteins upon proteolytic activation with thrombin or plasmin. In the case that thrombin does not act directly on SPRF, subthreshold amounts of serum, used during incubations with protease, may facilitate activation. However, most of these protein preparations are not very pure and therefore one cannot exclude contaminating proteins, specifically proteases that in turn might lead to a deceptive background response. A second approach is monoclonal or polyclonal antibodies against the candidate protein used to immunodeplete the activity. A third way to test candidates is the investigation of deficient plasma, which is available for many plasma proteins.

We are currently using these three approaches to test candidate proteins, that were identified by mass spectrometry from in Hep3-Butyl and the 2D-PAGE gel between 28 and 39 kD, but have not obtained an encouraging finding so far (Table 2.16, Table 2.17). In parallel, we are cloning the full-length sequences of all candidate proteins (bovine and human) for recombinant expression in HEK 293 cell suspension cultures (Baldi et al., 2005; Graham et al., 1977; Harrison et al., 1977). This mammalian cell line is well suited for production of glycosylated and secreted proteins under serum free conditions with high yield (1 to 10 µg/ml). After activation with thrombin, the culture supernatants will be tested on newt myotubes. Finally, we plan to undertake a second attempt to find a mouse monoclonal blocking antibody using higher amounts of a more purified fraction for antigen this time.
3.10  Perspective

With the identification of the S-Phase re-entry factor one would have a first molecule in hand, allowing the analysis of dedifferentiation on the molecular level in vitro. So far, no other pure factor has been described that causes similar effects on differentiated newt cells. However, unlike in vivo, formation of mononucleated cells derived from newt A1 myotubes has never been observed in cell culture. Upon stimulation with serum or a partially purified serum fraction, newt myotubes undergo DNA-synthesis but afterwards arrest in the G2 phase of the cell cycle. Therefore, we speculate about the presence of other factors, which are required for the fragmentation of myotubes into mononucleated cells and their traverse through mitosis. In particular, we are interested to understand the interaction between muscle cells and extracellular matrix for dedifferentiation, as mechanical load or partial detachment seems to be necessary for fragmentation and cellularization of myotubes (Echeverri et al., 2001; Kumar et al., 2004; Thornton, 1953). On the other hand, possible sources for the mitogenic growth factor might extracellular matrix (Levesque et al., 1991) or nerves (Brockes, 1987; Stocum, 2004; Thornton, 1968). In the latter case, it has long been recognized that denervation prior to or simultaneously with amputation of newt or axolotl limbs prevents proliferation and outgrowth of the blastema but not dedifferentiation and DNA-synthesis of cells. While several candidates for this neurotrophic or mitogenic factor have been proposed, including transferrin, fibroblast growth factors, substance P, glial growth factor, neuregulin and insulin-like growth factors (Brockes, 1984; Mescher, 1996; Stocum, 2004), only transferrin has been shown to fulfill this function in vitro. The purification and identification of SPRF would allow testing these candidates for the mitogenic activity under defined serum free conditions.

Finally, the purification of SPRF gives us a molecular handle for comparing newt and mammalian cells and answering important questions in the regeneration field. Does the difference in response derive from a missing receptor on mouse myotubes or is there an intracellular block of the signaling cascade? Would ectopic expression of the SPRF-receptor in C2C12 mouse myotubes lead to stimulation and subsequent S-phase re-entry? What are the targets of the signaling pathway in the newt myotube? Is the identified serum activity a necessary signal for initiating the regenerative response after wounding in the living newt or axolotl? The final purification and characterization of the serum factor, we described here, could serve as a first footstep to solve the mystery of replacing complete body parts after loss and greatly facilitate the understanding of the process of cellular de-differentiation.
4 Materials And Methods

4.1 Cell Culture And Myotube Assay

4.1.1 Cell Culture Solutions

Amphibian-PBS (APBS)
100 ml PBS (Gibco) was mixed with 25 ml sterile ddwater and stored at RT.

Trypsin-EDTA (TE, 10X)
Frozen Trypsin-EDTA solution (Gibco 15400-054) was thawed in a 37°C water bath and 4 ml aliquots stored at -20°C. For trypzination of cells 4 ml Trypsin-EDTA was diluted in 36 ml APBS and stored at 4°C.

Fetal Bovine Serum (FBS)
FBS (Perbio CH30160.03) was thawed in a water bath at 37°C and then heat-inactivated at 56°C for 30 min. Aliquots of 40 ml were frozen and stored at -30°C. Smaller aliquots of 100 µl and 4 ml for the myotube assay were prepared in parallel and stored at -80°C or —30°C.

Insulin (100X, 1 mg/ml)
250 mg insulin (Sigma I-5500) was dissolved in 25 ml 0.1 M HCl with swirling. Afterwards while swirling 225 ml APBS were added (final solution should be clear). After sterile-filtration using a steri-top, stock aliquots of 40 ml were stored at -20°C. For direct use in media preparation aliquots of 4 ml were frozen.

Penicillin-Streptomycin (100X)
Frozen Penicillin/Streptomycin-solution (Gibco 15140-122) was thawed in a 37°C water bath and 4 ml aliquots stored at -20°C.

Glutamine (100X)
Frozen glutamine-solution (Gibco 25030-024) was thawed in a 37°C water bath and 4 ml aliquots stored at -20°C.
Fibronectin (1mg/ml)
Fibronectin from bovine plasma (Sigma F-4759) was dissolved in ddH₂O at 1 mg/ml at 37°C in a water bath and aliquots of 100 µl frozen in liquid nitrogen. Aliquots were stored at -80°C or -20°C.

**High Serum AMEM (contains 10% FBS)**
- 250 ml MEM, w/o L-Glutamine (Gibco 21090-022)
- 100 ml ddH₂O
- 4 ml insulin
- 4 ml penicillin-streptomycin
- 4 ml glutamine
- 40 ml FBS

**Low Serum AMEM (contains 0.5% FBS)**
- 230 ml MEM
- 20 ml High Serum AMEM
- 140 ml ddH₂O
- 4 ml insulin
- 4 ml penicillin-streptomycin
- 4 ml glutamine

**SF-AMEM (Serum Free AMEM)**
- 250 ml MEM
- 140 ml ddH₂O
- 4 ml insulin
- 4 ml penicillin-streptomycin
- 4 ml glutamine

**SF-BSA AMEM (SF-AMEM containing 0.01% BSA)**
For 400 ml SF-BSA AMEM 40 mg of BSA (Albumin fraction V, SERVA 11926) were dissolved in SF-AMEM.

**Freezing media (with 2% DMSO)**
18 ml High Serum AMEM was mixed with 2 ml DMSO (Sigma) and aliquots of 4 ml stored at -20°C.
Soy Bean Trypsin Inhibitor
Trypsin inhibitor from soybean (SERVA 37328) was dissolved in SF-AMEM at 10 mg/ml. Aliquots of 200 µl were frozen in liquid nitrogen and stored at -80°C.

Gelatin (0.75% Type A: from porcine skin, 300 bloom)
7.5 g of gelatin (Sigma G-2500) was dissolved in 1000 ml water at 65°C for 15 to 30 min. While gelatin solution was still hot, it was filtered through a 0.22 µm steri-top and then 40 ml aliquoted in 50 ml falcon tubes. Aliquots were stored at 4°C.

100 and 35 µm filter
Sheets of CellMicroSieves (Promochem) with a pore size of 100 µm and 35 µm were cut under the cell culture hood into pieces of 3 x 3 cm and placed in a 10 cm dish. Filters were then sterilized under UV-light in the cell culture hood overnight.

BrdU (10 mg/ml, 5-bromo-2’-deoxyuridine.)
BrdU (Sigma B-9285) was dissolved in ddwater (final concentration 10 mg/ml) and aliquots of 200 µl stored at -80°C or -20°C.

4.1.2 Growing And Maintenance Of Newt A1 Cells And A1 Myotubes
4.1.2.1 Freezing Of A1 Cells

Old High Serum AMEM from a flask of confluent A1 cells was aspirated, the flask washed with 7 ml APBS and the APBS aspirated. For detachment of cells, the flask was incubated with 6 ml TE until most of cells came off (usually between 1.5 and 2 minutes) and trypsinization was stopped by addition of 3 ml of High Serum AMEM. Cells were spun down in a 15 ml Falcon tube for 3 min at 1000 x g and the supernatant aspirated. After gentle resuspension with 1 ml freezing media, cells were immediately transferred into a cryotube and placed in an -80°C freezer. The next day the frozen cell aliquots were put into liquid nitrogen.
4.1.2.2 Thawing Of Frozen A1 Cells

A 164 cm$^2$ cell culture flask was coated with pre-warmed (at 37°C) gelatin. Then a cryotube with A1 cells was thawed in a water bath at 25°C. As soon as the last pieces of ice disappeared, cells were transferred into a 15 ml Falcon tube, mixed with 6 ml fresh High Serum AMEM and spun down in a 15 ml Falcon tube for 3 min at 1000 x g. The supernatant was aspirated and cells resuspended in 2 ml High Serum AMEM. Finally cells were put into the 164 cm$^2$ flask containing 25 ml High Serum AMEM.

4.1.2.3 Passage Of A1 Cells

Newt A1 cells were passaged once per week by splitting one confluent flask into three. The two remaining flasks are re-plated each on one 10 cm dish. To limit the size of forming myotubes 10 cm dishes are scratched with a scalpel into a set of horizontal and vertical lines about 3 mm apart. Using 10 ml gelatin the entire bottom of three fresh flasks and two scratched 10 cm dishes were coated by serial transfer. The old media of three flasks was aspirated, the flasks washed with 7 ml APBS and the APBS aspirated. For detachment of cells, each flask was incubated with 6 ml TE until most of cells came off (usually between 1.5 and 2 minutes) and trypsinization was stopped by addition of 3 ml of High Serum AMEM. Cells were spun down in 15 ml Falcon tubes for 3 min at 1000 x g and the supernatant aspirated. The three flasks were filled with 25 ml High Serum AMEM and the two 10 cm dishes with 12 ml. Each Falcon tube cell pellet was resuspended in 6 ml High Serum and 2 ml transferred into each flask. Cells from the two remaining tubes were resuspended in 4 ml High Serum AMEM and 2 ml added to each 10 cm dish.

4.1.2.4 Initiation Of Myogenesis

Lowering the serum concentration in the medium from 10% to 0.5% induces myogenesis of newt A1 myoblasts. Between 12 to 24 h after passage of A1 cells, High Serum AMEM from the confluent 10 cm dishes was aspirated, the cells were washed with 5 ml APBS and 12 ml Low Serum AMEM was added.
4.1.2.5  Myotube Purification

For the myotube S-phase re-entry assay newt A1 myotubes are re-plated on fibronectin-coated 96 well plates (Corning 3595). To coat plates with fibronectin a 100 µl aliquot was thawed on ice and diluted in 3.5 ml SF-AMEM (final concentration of 30 µg/ml). After applying 30 µl of this diluted stock to each well the 96 well plate was incubated for at least 2 hours (usually over night) in a humidified incubator before performing the myotube purification. After 4 days in Low Serum AMEM more than 90% of A1 cells have fused to form multinucleated cells (Lo et al., 1993). Therefore purification of myotubes was carried out between 4.5 and 4.75 days after starting Low Serum AMEM incubation. Two sets of filters, 100 µm and 35 µm pore size, were prepared by mounting precut pieces of CellMicroSieves on 25 ml universal containers (NUNC 364246). In order to get a sufficient number of myotubes per well in the myotube S-Phase re-entry assay, two 10 cm dishes were used to seed one 96 well plate. Low Serum AMEM was aspirated, the cells washed with 5 ml APBS and then briefly trypsinized with 2 ml TE. As soon as first clumps of cells detach trypsin was inhibited by addition of 200 µl Soy Bean Trypsin Inhibitor and subsequently cells were gently resuspended in 6 ml Low Serum AMEM by pipetting up and down five to seven times. For removal of cell clumps the cell suspension was dripped through the 100 µm filter and subsequently the 10 cm dish and the filter was washed with 4 ml of Low Serum AMEM. In order to separate myotubes from the majority of myoblasts the flow through of 100 µm filter was dripped through a 35 µm filter where myotubes were retained and myoblasts passed through. Myotubes on the filter were washed with 4 ml of Low Serum AMEM and afterwards the filter was transferred into a 6 cm dish containing 4 ml Low Serum AMEM. The 6 cm dish was gently shaken to detach all myotubes from the filter and after removal from the dish the filter washed with an additional 4 ml of Low Serum AMEM. The procedure was then repeated with the second 10 cm dish. Purified myotubes from both 10 cm dishes were combined and transferred into a universal tube. The final volume of the cell suspension was 16 ml. The supernatant of the fibronectin pre-coated 96 well plate was aspirated, 150 µl of purified myotubes added with a multipipetter to each well and the plate placed into the incubator.
Notes:
For serum-free myotube purifications all steps were performed using SF-BSA AMEM in place of Low Serum AMEM. In Low Serum AMEM purifications addition of Soy Bean Trypsin Inhibitor was sometimes omitted and trypsin inhibited only by addition of 6 ml Low Serum AMEM.

4.1.2.6 Sample Application For Myotube S-Phase Re-Entry Assay

Fractions from the purification were assayed by addition to myotubes in a 96 well plate between 12 to 36 h after myotube purification when most of the cells had attached and spread in the wells. Using an 8-channel pipette 100 µl of medium was removed from each well. Sample volumes from 5 to 100 µl were then added to cells and all samples were assayed in triplicates, such that 32 different samples could be assayed on one 96 well plate. One positive control (30 µl FCS) and a negative control (100 µl Low Serum AMEM or SF-BSA AMEM) were included in each plate. When all samples were applied, medium to a final sample volume of 100 µl was added to each well (e.g. if sample volume was 30 µl, 70 µl of medium was applied to a well).

4.1.2.7 BrdU-Addition

For assay of S-phase re-entry by incorporation of BrdU, myotubes were incubated for 12 h with BrdU between day 3.5 and 4.5 after sample application. An aliquot of BrdU was thawed in a water bath at 37°C and 40 µl mixed with 2 ml SF-AMEM. With a multipipette 10 µl of the mixture was added to each well (final concentration in well 13 µg/ml).

4.1.2.8 Fixation And Staining Of Myotubes

4.1.2.8.1 Buffers For Fixation And Staining Of Myotubes

1X PBS
sterile filtered and stored at 4°C

20% Sodium Azide (NaN₃)
20 g NaN₃ (Merck 1.06688) was dissolved in 100 ml ddwater, sterile filtered and stored at 4°C.
**Materials And Methods**

**Goat Serum**
Goat Serum (Gibco 16210-072) was thawed in a water bath at 37°C and then heat-inactivated at 56°C for 30 min. Aliquots of 20 ml were frozen and stored at -30°C.

**1X PBS with 0.01% BSA, 0.1% NaN₃ (0.01% BSA/PBS)**
100 mg BSA (Bovine albumin fraction V, SERVA 11926) and 5 ml 20% NaN₃ were dissolved in 1 l PBS, sterile-filtered and stored at 4°C.

**6% PFA (paraformaldehyde)**
24 g paraformaldehyde (Riedel-de Haen 16005) was dissolved in 360 ml ddwater by addition of 8 drops of 5 N NaOH, swirling the solution and heating to 60°C. When dissolved the solution was cooled down on ice and 40 ml 10X PBS was added to bring the final pH to 8. Aliquots of 25 ml were frozen and stored at -20°C.

**2 N HCl**
40 ml 12 N HCl (37% HCl) was diluted with 200 ml ddwater and stored at RT.

**10% Goat Serum in 1X TBS, 0.1% NaN₃**
A 20 ml aliquot of Goat Serum was diluted in 180 ml 1X TBS and 1 ml 20% NaN₃ added. The solution was sterile filtered and stored at 4°C.

**1X TBS, 0.1% Tween-20 (TBS/Tween)**
10X TBS was diluted to 1X TBS and sterile filtered. Then 1 g Tween-20 (or Triton X—100) was added and stirred. The solution was stored at RT.

**Cold 100% Methanol**
stored at -20°C

**Hoechst 33342 (1 mg/ml)**
Hoechst 33342 (Bisbenzimide H 33342, Sigma B-2261) was dissolved in water at 1 mg/ml and kept in the dark at 4°C.
4.1.2.8.2 Fixation Of Myotubes

Before fixation of cells an aliquot of 6% PFA was thawed at 37°C then cooled on ice. In a tray PFA was diluted with an equal volume of cold PBS (final 3% PFA). A second tray was filled with cold 1X PBS (containing 0.01% BSA, 0.1% NaN₃) and a third tray filled with ice-cold methanol. In order to fix myotubes 50 µl 3% PFA was added to one column of the 96 well plate using an 8-channel multipipette. After 15 s medium was aspirated quickly with a multiwell plate washer/dispenser manifold (Sigma M-2656) connected to a vacuum pump and the wells washed twice with 100 µl 0.01% BSA/PBS. Cells were then further fixed by addition of 75 µl methanol for 5 min or until staining (up to one week).

4.1.2.8.3 Staining Of Myotubes

Methanol was aspirated from the 96 well plate and the myotubes were rehydrated by incubation for 5 min with 75 µl TBS/Tween. Following aspiration of TBS/Tween, cells in all wells were incubated with 75 µl 2 N HCl for 12 min to denature DNA in the nucleus and subsequently washed 4 times with 75 µl TBS/Tween using a multiwell plate washer manifold. FITC-labeled antibody against muscle specific myosin heavy chain and rhodamine-labeled antibody against BrdU were diluted in a tray with 3.5 ml 10% Goat Serum /1X TBS and 30 µl of the mix applied to each well. Myotubes were stained at 4°C in the dark for 12 h up to one week. Non-specifically bound antibody was washed 4 times with 75 µl TBS/Tween. For staining of nuclear DNA 7 µl of Hoechst stock was diluted in 3.5 ml TBS/Tween and all wells incubated for 5 min with 30 µl. Finally wells were washed 3 times with 75 µl TBS/Tween and stored in 75 µl methanol at 4°C in the dark.

4.1.2.9 Counting Of Myotubes

In order to follow SPRF activity across the purification, the percentage of stimulated myotubes in a 96 well plate was ascertained by counting myotubes with nuclear BrdU incorporation and the total number of myotubes in each well. A myotube was counted as positive if two or more nuclei of a myotube had incorporated BrdU. All assays in 96 well plates were counted on an Axioplan 2 imaging microscope (ZEISS) with a 10X objective in a dark room using a FITC/TRITC dual band filter set (CHROMA 51004v2). Myotubes were identified by green cytoplasmic staining of muscle-specific myosin heavy chain (FITC). Positive nuclei were recognized by red nuclear staining of BrdU (rhodamine).
Materials And Methods

Staining of BrdU-incorporation into nuclei was confirmed by blue Hoechst staining using filter set 01 (ZEISS). The number of positive and total myotubes were entered into a microsoft excel spreadsheet and the average percentage of positive myotubes calculated for the triplicate samples.

4.1.2.10 Preparation Of Labeled Antibodies

Monoclonal mouse anti-muscle-specific myosin heavy chain antibody
The hybridoma cell line for monoclonal mouse anti-muscle-specific myosin heavy chain IgG1 antibody (clone 4A1025), a kind gift from Dr. Simon Hughes (Randall Institute, Kings College London, UK) was grown and maintained as described (Harlow and Lane, 1988), and antibodies from the supernatant were purified over a Protein G column. Later antibodies were obtained from the Antibody Facility at the Max Planck Institute of Molecular Cell Biology and Genetics Dresden and were also purified as described (Harlow and Lane, 1988).

Monoclonal mouse anti-Bromodeoxyuridine (BrdU) antibody
Monoclonal mouse anti-Bromodeoxyuridine (clone BU20a) antibody was obtained as culture supernatant from DAKOCytomation (M0744) and purified over a Protein G column.

Fluorescein-succinimydyl ester
5-(and-6)-carboxyfluorescein, succinimidyl ester (5(6)-FAM, SE) *mixed isomers* (Molecular Probes C-1311) was freshly dissolved in DMSO at 1.5 mg/ml just before use and stored at -20°C.

Tetramethylrhodamine-succinimydyl ester
5-(and-6)-carboxytetramethylrhodamine, succinimidyl ester (5(6)-TAMRA, SE) *mixed isomers* (Molecular Probes C-1171) was freshly dissolved in DMSO at 1.5 mg/ml just before use and stored at -20°C.

0.2 M Borate Buffer (pH 8.0)
sterile-filtered and stored at RT

100 mM Glycine (pH 7.0)
sterile-filtered and stored at 4°C
Materials And Methods

1% BSA in 1 X PBS/ 0.1% NaN₃
sterile-filtered and stored at 4°C

1 X PBS/ 0.1% NaN₃
sterile-filtered and stored at 4°C

100% Glycerol
autoclaved and stored at RT

Purified antibody was concentrated to 2 – 5 mg/ml and then 1 ml desalted over a NAP—10 column into 1.5 ml 0.2 M Borate Buffer (pH 8.0). Labeling of antibodies was started by addition of 25 µl of Tetramethylrhodamine-succinimydyl ester or Fluorescein-succinimydyl ester per 500 µl antibody and the mix incubated on a rotation shaker for 4 h at RT. After quenching the labeling reaction with 30 µl 100 mM Glycine (pH 7.0) per 500 µl mix, antibodies were desalted over NAP-5 and NAP-10 columns into 1X PBS/0.1% NaN₃. In order to stabilize the antibody for long-term storage 1/10th volume of 1% BSA /1X PBS and an equal volume of 100% Glycerol (final 50%) was added. The working concentration of labeled antibody was determined by applying different dilutions to myotubes. The antibody solution was stored at -20°.
4.2 General Methods For Purification

4.2.1 FPLC System For Chromatography

All column chromatography was performed on a BioCad SPRINT FPLC System (Perseptive Biosystems, Inc., Framingham, MA, USA) at room temperature. This instrument is equipped with inline flow cells for monitoring protein concentration by absorbance at 280 nm and 254 nm, as well as conductivity and pH-value.

4.2.2 Calculation Of Protein Concentration

The concentration of proteins in fractions across the purification was determined by BCA-Assay, integration of absorbance at 280 nm and quantification of stained SDS-PAGE gels.

For protein determination using the BCA-Assay (BCA Protein Assay Kit, Pierce) all steps were performed according to manufacturer’s instruction.

For calculation of the protein concentration by integration of the absorbance at 280 nm, the data file for each purification run was exported into an excel spreadsheet (1 data point per second). Following baseline drift correction, the protein concentration was calculated for each fraction across the run after taking into account the flow cell path length (3 mm) of the UV/Vis detector ($A_{280}$ of 1 corresponds to 1 mg/ml).

SDS-PAGE gels of the purification were quantified using the software Science Lab 99 Image Gauge Version 3.3 (Fuji Photo Film, LTD). The gels were scanned (Umax Powerlook 1100, Umax Systems GmbH Willich Germany) using SilverFast Ai (LaserSoft Imaging AG Germany). The scanned images were modified and saved as Grayscale TIFF files (8 bits per channel) using Adobe Photoshop (Adobe Systems Incorporated). All Tiff files were then imported into Science Lab 99 Image Gauge (Fuji Photo Film CO, LTD) and the intensity for regions of interest determined. Following export of the quantification chart into an excel spread sheet, the amount of protein was calculated based on known amounts of standards (BSA or ovalbumin) run on the same gel.

4.2.3 Cap Dialysis

Lids and upper ends (about 2 mm from the edge) of 2 ml Eppendorf tubes were cut off and autoclaved. For dialysis, pieces of Spectra/Por 1 membrane tubings (MWCO 6000 - 8000, Roth 1955.1) were soaked in water for at least 2 hours before use and then cut
into 2 cm x 2 cm squares. The lid was filled with up to 280 µl sample, then covered with a piece of membrane and finally the upper end of an Eppendorf tube was snapped into place. Dialysis was performed in 1 liter glass bottles or 500 ml cell culture steri-cups.

4.2.4 Desalting On Nap-5 Columns

Nap-5 columns were purchased from APBiotech. Columns were washed three times with 1X PBS and then three times with the desired medium. 500 µl of sample was applied and then desalted with 1 ml medium. For sample volumes less than 500 µl, sample was applied and after the sample entered the gel bed completely, medium was applied to the column, so that the combined volume of sample and medium was equal to 500 µl. Finally all columns were cleaned by washing three times with 10X PBS and then stored in 10X PBS at RT.

4.2.5 Concentrating And Desalting Of Diluted Protein Samples

Concentration and Desalting was performed according to manufacturer’s instruction using one of the following devices:

- Centricon Plus-20 Centrifugal Filter Device PL-10, PL-30 and PL-100 (Millipore)
- Centriplus YM-50, YM-100 (Millipore)
- Vivaspin 20 MWCO 100 kD (Vivascience AG)

All centrifugation was carried out at 20°C in a swinging bucket to reduce concentration times. For cleaning filter devices were rinsed and spun briefly in 20% Ethanol and afterwards in ddwater. In order to minimize protein loss due to unspecific adsorption filter devices were incubated for 2 hours in 1X PBS containing Carrier-Mix (insulin and aprotinin each 40 µg/ml final) and finally spun for 3 min. Retenate and flow through of Carrier-Mix solution were poured off and sample concentration started. Buffer changes were achieved by repeated dilution and concentration of proteins with the desired buffer. Finally, for removal of aggregates, retenates were filtered using Spin-X Centrifuge Tube Filters (0.22 µm, Corning Costar 8160).
4.2.6 Thrombin Inhibition Assay

**Thrombin Assay Buffer**
1X TBS containing 0.01% BSA, 0.1% NaN₃, sterile-filtered and stored at 4°C

In order to confirm that thrombin was completely inhibited by PPACK, a kinetic thrombin assay using the chromogenic thrombin substrate Chromozym TH (Roche 206849) was performed. Chromozym TH was dissolved in ddwater at 1 mg/ml and aliquots of 1.5 ml stored at -80°C. Aliquots of frozen Chromozyme TH were thawed, diluted 1:10 into Thrombin Assay Buffer and stored at 4°C. Before use the buffer was warmed up to room temperature.

For thrombin assay a 10 µl sample was mixed with 200 µl of Thrombin Assay Buffer containing Chromozym TH in a flat well 96-well plate. All samples were assayed in triplicate. As a control Crude Bovine Thrombin was diluted 1:100, 1:200 and 1:400 and 10 µl assayed, respectively. Activity of thrombin was calculated as the slope (ΔOD 405 nm/min) of absorption at 405 nm over 5 minutes by measuring absorption every 20 s.

4.2.7 Preparation of Carrier-Mix

In order to minimize protein loss due to non-specific adsorption of proteins to tubes for very dilute protein samples (usually less than 50 µg/ml) a Carrier-Mix containing insulin and aprotinin was added to a final concentration of 50 µg/ml each. Insulin and aprotinin are low molecular weight proteins and migrate close to the dye front when run on SDS-PAGE, where they do not interfere with the detection of other proteins. Aprotinin (Trasylol, a kind gift from Bayer AG) was dissolved in water at 20 mg/ml, 500 µl aliquots frozen in liquid nitrogen and stored at -80°C. Insulin (1 mg/ml) was prepared as described above. The Carrier-Mix was prepared by mixing the compounds in the following order:

**Carrier-Mix (12 ml)**
- 10 ml 1 mg/ml insulin (see cell culture)
- 1 ml 100 mM anion buffer (50 mM Tris, 50 mM Bis-Tris-Propane, pH 9.0)
- 0.5 ml ddwater
- 0.5 ml 20 mg/ml aprotinin
The Carrier-Mix was sterile-filtered and if not used directly aliquots were frozen in liquid nitrogen and stored at -20°C. Just before use aliquots were then thawed in a 37°C water bath.

4.2.8 Analytical SDS-PAGE

NuPAGE MOPS SDS Running Buffer (pH 7.7)

- 50 mM MOPS
- 50 mM Tris Base
- 1 mM EDTA
- 0.1% SDS

Analytical SDS-PAGE was performed with NuPAGE Novex Bis-Tris gels (Invitrogen) using a NuPAGE MOPS SDS Running Buffer (Invitrogen NP-0001). All gels were run at 130 V at room temperature until the dye front reached the end of the gel (80 min).

5X SDS Sample Buffer (without reducing agent)

- 400 mM Tris (pH 6.8)
- 10% SDS (Serva 20760)
- 50% glycerol
- 0.1% Bromphenol Blue (Sigma B-5525)

5X SDS Sample Buffer is heated up to 50°C and sterile-filtered.

4.2.9 Staining Of Protein Gels

4.2.9.1 SimplyBlue Safestain (Invitrogen)

SimplyBlue Safestain, a ready-to-use Coomassie G-250 stain, was purchased from Invitrogen (LC6060). It does not contain any methanol or acetic acid for fixation of proteins. After electrophoresis gels were washed three times for 5 minutes each with 100 ml ddwater and then stained for 1 to 3 hours with Simply Blue stain. Finally de-staining of gels was performed by two incubations in 100 ml water for 1 h.
4.2.9.2 Coomassie Staining (Coomassie Brilliant Blue R-250)

**Staining Solution (0.5% Coomassie Brilliant Blue R-250)**

- 1 g Coomassie Brilliant Blue R-250 (Sigma B-7920)
- 250 ml methanol (50%)
- 50 ml acetic acid (10%)
- 200 ml water

**Destaining solution**

- 200 ml methanol (20%)
- 75 ml acetic acid (7.5%)
- 725 ml water

After electrophoresis gels were quickly rinsed with water and then incubated for at least one hour in Staining Solution. Afterwards gels were destained with two changes of Destaining Solution.

4.2.9.3 Silver Staining

**Silver Stain Fix**

- 600 ml ddH₂O
- 300 ml ethanol
- 100 ml glacial acetic acid

**Reducer**

- 67 ml ddH₂O
- 30 ml ethanol
- 3.3 ml 3M sodium acetate (pH 5.5)
- 100 mg sodium thiosulfate (Sigma S-1648)

**Silver Nitrate Solution**

- 100 ml ddH₂O
- 100 mg AgNO₃ (0.1%, Merck 1.01512)
- 25 µl 37% formaldehyde
Materials And Methods

119

Developer

160 ml ddH₂O
40 ml 12.5% Na₂CO₃
100 µl 37% formaldehyde

Stocks of Silver Stain Fix, 3 M sodium acetate (pH 5.5), 12.5% Na₂CO₃, 37% formaldehyde and glacial acetic acid were stored at RT. Reducer, Silver Nitrate Solution and Developer were always prepared fresh.

After electrophoresis gels were rinsed twice with water and subsequently put twice in Silver Stain Fix for 15 min. Gels were then incubated for 20 min in 50 ml Reducer and washed three times over 5 minutes with 50 ml ddH₂O. Afterwards gels were incubated in 50 ml Silver Nitrate Solution for 20 min. For development gels were quickly rinsed once with ddH₂O and twice with Developer. Gels were then incubated in 50 ml Developer on a rocker until bands appeared. When sufficient staining was reached, development was stopped by adding 500 µl glacial acetic acid and further incubation for 5 min. Finally gels were washed three times with water for 5 min.

4.2.10 Protein Recovery From Dilute Solution - Precipitation

4.2.10.1 Trichloroacetic Acid (TCA) Precipitation For SDS-PAGE

Resuspension Buffer

10 parts 200 mM Tris base, 20 mM EDTA
12 parts 2X SDS Sample Buffer (from 5X SDS Sample Buffer)
3 parts 1 M DTT (or water for non-reducing conditions)

In an Eppendorf-tube a protein sample was mixed with an equal volume of 20% (w/v) trichloroacetic acid (TCA, final concentration 10%). After incubation for 15 min on ice the sample was spun for 5 min at 25 000 x g and the supernatant was aspirated. Remaining TCA was removed after a second spin. Then the pellet was washed with acetone, the sample spun again and the acetone discarded. After drying the pellet was dissolved in 25 µl of Resuspension Buffer. If the sample was yellow at this point 1 M Tris base in 1 µl increments was added until the sample turned blue. In order to redissolve the pellet, the sample was incubated at 50°C. For complete denaturation of proteins the sample was boiled for 5 min. After spinning down the sample an equal volume of 2X SDS Sample Buffer was added and the sample run immediately on SDS-PAGE.
4.2.10.2 Protein Precipitation After Wessel And Fluegge

For mass spectrometry analysis, proteins from very diluted solutions were precipitated after Wessel and Fluegge (Wessel and Flugge, 1984). In an Eppendorf-tube 0.4 ml methanol was added to 0.1 ml protein sample, the tube vortexed and centrifuged 10 s at 9000 x g. Then 0.1 ml chloroform was added and the sample vortexed. After centrifugation for 10 s at 9000 x g, 0.3 ml ddH₂O was added, the tube vortexed vigorously and centrifuged for 1 min at 9000 x g. Now the upper phase was carefully removed and discarded as proteins are at the interface. The interface and lower phase were mixed with 0.3 ml methanol and the sample was vortexed vigorously and centrifuged for 2 min at 9000 x g. Subsequently all the supernatant was removed and the pellet was dried. Finally the pellet was dissolved in a mixture of 5 X SDS Sample Buffer and an equal volume ddwater (final 5% SDS). For recovery of proteins from sample volumes of 1 ml, precipitation were carried out in 14 ml Polypropylene Round-Bottom Tubes (Falcon 352059) and 10-fold volumes of chemicals were used.
4.3 Characterization And Purification Of S-Phase Re-Entry Factor

4.3.1 Common Buffers Used Across The Purification

For chromatography all buffers and solutions were prepared from deionized water (conductivity 0.055 µS) and sterile-filtered before use through 0.22 µm Steritop Filter (Millipore) or 0.22 µm Bottle Top Filter (Corning).

100 mM anion buffer (pH 6 and pH 9)
- 50 mM Tris (Roth 4855.2)
- 50 mM Bis-Tris-Propane (Sigma B-6755)

adjusted to the appropriate pH with HCl or NaOH.

100 mM cation buffer (pH 4.5 and pH 7.5)
- 33.3 mM HEPES (Roth 9105.3)
- 33.3 mM MES (Calbiochem 475893)
- 33.3 mM NaAcetate (Merck 1.06268)

adjusted to the appropriate pH with HCl or NaOH.

3 M NaCl (Merck 1.06404)

1 M di-sodium hydrogen phosphate (Na$_2$HPO$_4$) (Merck 1.06586)

1 M sodium dihydrogen phosphate (NaH$_2$PO$_4$) (Merck 1.06346)

500 mM phosphate buffer (pH 7.0)
prepared by mixing of 1 M di-sodium hydrogen phosphate (Na$_2$HPO$_4$) and 1 M sodium dihydrogen phosphate (NaH$_2$PO$_4$)

70% Ethanol

20% Ethanol

Cation Buffer (pH 6.5)
7 mM HEPES, 7 mM MES, 7 mM NaAcetate (pH 6.5),
prepared by mixing 6.7% 100 mM cation buffer (pH 4.5), 13.3% 100 mM cation buffer (pH 7.5), 80% ddwater
**Materials And Methods**

**Anion Buffer (pH 7.0)**
10 mM Tris, 10 mM Bis-Tris-Propane (pH 7.0),
prepared by mixing 15.7% 100 mM anion buffer (pH 6.0), 4.3% 100 mM anion buffer (pH 9.0), 80% ddwater

**50 mM Phosphate Buffer (pH 7.0)**
50 mM phosphate buffer (pH 7.0), prepared by dilution of 500 mM phosphate buffer (pH 7.0)

**Phosphate buffered saline pH 7.4 (PBS, 1X PBS)**
- 137 mM NaCl
- 2.7 mM KCl
- 10 mM NaH2PO4
- 2 mM KH2PO4

**pH 11 Gelfiltration Buffer**
- 20 mM CAPS (pH 11) (Sigma C-6070)
- 200 mM NaCl

**SDS Gelfiltration Buffer**
- PBS
- 0.1% SDS (pH 7.4) (Serva 20760)
4.3.2 Important Fractions Across The Purification

**Crude Bovine Thrombin**
Starting material for the purification,
Dissolved, if not otherwise stated, at a concentration of 8 mg/ml in Cation Buffer (pH 6.5); incubated at RT on a rotation shaker for 30 min and afterwards sterile-filtered

**CMFF-FT**
Pooled fractions of flow through after cation exchange chromatography on HiTrap CMFF or HiPrep CMFF, treated with PPACK for complete inhibition of remaining thrombin;
The starting material for cation exchange chromatography was Crude Bovine Thrombin.

**CMFF-1.5M**
Pooled fractions of bound proteins on HiTrap CMFF or HiPrep CMFF, that were eluted with 1.5 M NaCl, contains high concentrations of thrombin

**Butyl-20%EtOH**
Pooled fractions after hydrophobic interaction chromatography on HiTrap Butyl or HiPrep Butyl, that were eluted in 50 mM Phosphate Buffer, containing 20% ethanol;
The starting material for the butyl column was CMFF-FT, containing 1 M ammonium sulfate.

**Hep3**
Pooled fractions between 430 and 590 mM NaCl after heparin affinity chromatography on HiTrap Heparin using a linear gradient of NaCl;
The starting material for HiTrap Heparin was CMFF-FT.

**Hep3-Conc**
Between 100 and 200-fold concentrated Hep3; Hep3 from several runs of heparin affinity chromatography was concentrated on membranes with a MWCO of either 10 kD or 100 kD.

**Hep3-Butyl**
Pooled fractions between 430 and 590 mM NaCl after heparin affinity chromatography on HiTrap Heparin with a linear gradient of sodium chloride;
The starting material for HiTrap Heparin was Butyl-20% EtOH.

**Hep3/4-Pool**
Pooled fractions between 430 and 680 mM NaCl after heparin affinity
chromatography on HiTrap Heparin with a linear gradient of sodium chloride;  
The starting material for HiTrap Heparin was Butyl-20%EtOH.

Hep3/4-Conc  67-fold concentrated Hep3/4-Pool; Hep3/4 from two runs of heparin affinity chromatography with a linear gradient of sodium chloride was concentrated by ultrafiltration on membranes with a MWCO of 100 kD.

Hep-1M  Pooled fractions after heparin affinity chromatography on HiTrap Heparin, where bound proteins were eluted with a step of 10 CV 1 M NaCl, in 20 mM Tris (pH 7.0);  
The starting material for HiTrap Heparin was Butyl-20%EtOH.

Hep-1M-Conc  Concentrated Hep-1M; Hep-1M was concentrated by ultrafiltration on a membrane with a MWCO of 100 kD.

SD200-Pool  Pooled active fractions after size exclusion chromatography on Superdex-200 column in SDS Gel Filtration Buffer;  
The starting material for the Superdex-200 column was Hep3—Conc.
### Table 4.1 - Overview of all purifications and important fractions across the purifications

<table>
<thead>
<tr>
<th>Step 1</th>
<th>Step 2</th>
<th>Step 3</th>
<th>Step 4</th>
<th>Step 5</th>
<th>Step 6</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gel Filtration (Native)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Superdex 200</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Gel Filtration (pH 11)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Superdex 200</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Gel Filtration (SDS)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Superdex 200</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>AXC</strong></td>
<td><strong>CMFF-FT</strong></td>
<td><strong>Step gradient</strong></td>
<td><strong>Linear gradient</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HiTrap Q</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>CXC</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hitrap CMFF</td>
<td><strong>Hep3</strong></td>
<td><strong>Step gradient</strong></td>
<td><strong>Linear gradient</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>HiTrap Q</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>AC</strong></td>
<td><strong>Hep3</strong></td>
<td><strong>Step gradient</strong></td>
<td><strong>Linear gradient</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HiTrap Heparin</td>
<td>Mono Q</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>Gel Filtration (Native)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Superdex 200</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Ultrafiltration</strong></td>
<td><strong>Hep3-Conc</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MWCO 10 or 100 kD</td>
<td>(40x / 20%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Prep. SDS-PAGE</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Dialysis</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Electroelution</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Preparation of blocking monoclonal antibodies</td>
</tr>
<tr>
<td><strong>Gel Filtration (SDS)</strong></td>
<td><strong>SD200-Pool</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Superdex 200</td>
<td>(130x / 11%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>HAP</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CHT-II</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
### Materials And Methods

<table>
<thead>
<tr>
<th>Step</th>
<th>Method</th>
<th>Details</th>
<th>Trituration</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prep. Isoelectric Focusing</td>
<td>Dialysis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prep. 2D-PAGE</td>
<td>Dialysis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HIC</td>
<td>Butyl-20%EtOH</td>
<td>(20x / 60%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HAP</td>
<td>CHT-II</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chromatofocusing</td>
<td>Mono P</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AC</td>
<td>Hep3-Butyl</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Hep3/4-Pool</td>
<td>(180x / 23%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Hep-1M</td>
<td>(30x / 44%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Hitrap Heparin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ultrafiltration</td>
<td>MWCO 100 kD</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Hep3/4-Conc</td>
<td>(210x / 14%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Hep1-M-Conc</td>
<td>(34x / 31%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gel Filtration (pH 11)</td>
<td>Superdex 200</td>
<td></td>
<td></td>
<td>LC-MS/MS ongoing</td>
</tr>
<tr>
<td>Native PAGE</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lectin Affinity Chr.</td>
<td>ConA, Lentil Lectin, WGA,</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**AXC:** anion exchange chromatography; **CXC:** cation exchange chromatography; **AC:** affinity chromatography; **HAP:** hydroxyapatite chromatography; **HIC:** hydrophobic interaction chromatography; **LC-MS/MS:** Liquid Chromatography mass spectrometry

**Blue:** important fractions; 
(χ / %) represents purification (fold) and activity yield for respective fraction

**Yellow Background:** chromatography steps in the best purification scheme so far
4.3.3 Crude Bovine Thrombin - The Starting Material For The Purification

Crude Bovine Thrombin was purchased in bulk amounts from Calbiochem (605157) and later from the original manufacturer Celliance Corp (82-036). Based on Celliance Corp., it is a partially purified plasma fraction, where blood coagulation was subsequently activated with bovine lung tissue. It contains active thrombin, but also 10-fold higher amount of SPRF-activity per total protein compared to bovine serum. Crude Bovine Thrombin is supplied as a lyophilized powder with the following specifications:

<table>
<thead>
<tr>
<th>Specifikation</th>
<th>Wert</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thrombin specific activity (by calculation)</td>
<td>73 NIH units per mg protein</td>
</tr>
<tr>
<td>Thrombin activity (by substrate coagulation)</td>
<td>59 NIH units per anhydrous</td>
</tr>
<tr>
<td>Protein (by Biuret)</td>
<td>77.7%</td>
</tr>
<tr>
<td>pH (7% solution)</td>
<td>6.4</td>
</tr>
<tr>
<td>Preservative (Benzethonium Chloride)</td>
<td>0.02 mg/1000 NIH units</td>
</tr>
<tr>
<td>Moisture (by Karl Fischer)</td>
<td>&lt;5%</td>
</tr>
</tbody>
</table>

For purification lyophilized Crude Bovine Thrombin was dissolved at 8 mg/ml in 20 mM Cation Buffer (pH 6.5), incubated for 30 to 60 min on a rotation shaker and sterile-filtered through a 0.22 µm filter. Due to low salt concentration in Crude Bovine Thrombin preparation, proteins start to precipitate within a few hours. Therefore the first step of purification must be performed directly after ultrafiltration. After treatment with PPACK 1/10th volume of 10X PBS can be added to Crude Bovine Thrombin, which prevents precipitation and also dissolves already precipitated proteins.

4.3.4 Inactivation Of Thrombin And Other Contaminating Proteases (PPACK Treatment)

PPACK (D-Phe-Pro-Arg-chloromethylketone, HCl)

PPACK is an extremely potent and selective irreversible inhibitor of thrombin. Stocks of 10 mM PPACK (Calbiochem 520222) were prepared by dissolving 5 mg PPACK in 950 µl 10 mM HCl. Afterwards 50 µl aliquots were frozen and stored at -80°C. Aliquots were thawed directly before use.
1000X Protease-Inhibitor-Mix (PI's)
Inhibitor mix (all Sigma) was prepared as 1000X stock solution in DMSO and aliquots stored at -20°C. The mix contains chymostatin (6 mg/ml), leupeptin (0.5 mg/ml), antipain-HCl (10 mg/ml), aprotinin (2 mg/ml), pepstatin (0.7 mg/ml) and APMSF (10 mg/ml).

In order to completely inhibit remaining thrombin activity after cation exchange chromatography on HiTrap CMFF or HiPrep CMFF the flow through fractions (CMFF-FT) were treated three times with PPACK. Hereby 50 µl PPACK were used per 15 ml Crude Bovine Thrombin starting material (100 mg). Finally for inhibition of other proteases 5 µl 1000X Protease-Inhibitor-Mix was added.
The thrombin activity in 1.5 ml Crude Bovine Thrombin or 1.5 ml CMFF-1.5M was inhibited by adding three times 10 µl PPACK and finally 2 µl 1000X Protease-Inhibitor-Mix. The inhibition of thrombin was then confirmed using the Thrombin Inhibition Assay.

4.3.5 Characterization Of S-Phase Re-Entry Factor
A lyophilized Crude Bovine Thrombin preparation (Calbiochem) was dissolved at 8 mg/ml (80% protein content) in Cation Buffer (pH 6.5) and treated with PPACK. Subsequent to complete inhibition of intrinsic thrombin activity with PPACK Crude Bovine Thrombin preparation was desalted over NAP-25 columns into 20 mM Anion Buffer (pH 7.6), 100 mM NaCl and diluted with the same buffer to a final protein concentration of 1.92 mg/ml. From this preparation usually 700 µl was then used for characterization experiments. Before assaying on cells all samples were centrifuged for two minutes at 25 000 x g at 20°C in a microfuge, then the supernatant was removed and desalted over NAP-5 columns into Low Serum AMEM. Crude Bovine Thrombin was typically added to cells at a concentration of 0.2-0.6 mg/ml (100 and 30 µl of desalted samples).

For Proteinase K treatment, PPACK-treated Crude Bovine Thrombin was treated with 14 µg/ml ProteinaseK (Roche) for 4 hours at room temperature. For protease inactivation controls, proteinase K was incubated with 4 mM Pefabloc SC (AEBSF, Roche), or boiled for 30 minutes before adding to Crude Bovine Thrombin.

In thermal lability tests, Crude Bovine Thrombin was incubated for 4 hours at the indicated temperatures.
For pH stability tests, Crude Bovine Thrombin was mixed with the following buffers at the indicated concentration: pH 2 and 3 (100 mM phosphate), 4 (100 mM succinic acid), 5 (100 mM citrate), 6 (100 mM MES), 7 (100 mM MOPS), 8 (100 mM Tris), 9 (50 mM CHES), 10 and 11 (50 mM CAPS), 12 and 13 (100 mM phosphate) and incubated for 12 hours at room temperature.

Sensitivity to reducing agents was tested by incubation of Crude Bovine Thrombin with 10 mM or 100 mM dithiothreitol (DTT) for 12 hours at RT.

To check whether more SPRF activity can be generated in Crude Bovine Thrombin preparations by addition of purified thrombin, 220 µl of crude thrombin in Cation Buffer (pH 6.5) was diluted with 450 µl Anion Buffer (pH 7.6), 100 mM NaCl. Afterwards samples were mixed with 50 µl of CMFF-1.5M fraction (contains high concentrations of purified thrombin) and incubated for 0, 8, 24 or 48 hours at room temperature. Before desalting into Low Serum AMEM all thrombin activity was inhibited by addition of PPACK.

In order to reduce total amount of proteins in the preparation, for lectin binding assays Hep3–Conc after heparin affinity chromatography was used. Hep3-Conc was desalted into Lectin Buffer and if required Ca\(^{2+}\) and/or Mn\(^{2+}\) added. Then Hep3-Conc was incubated with the indicated lectin resins (Table 4.2) for 12 hours at 4°C with rocking. The preparation was centrifuged for two minutes at 5000 x g in a microfuge then the supernatant was removed and desalted on NAP-5 desalting columns into Low Serum AMEM.

**Lectin-Buffer:**
10 mM HEPES (pH 7.5), 0.15 M NaCl (+ 0.1 mM Ca\(^{2+}\) and/or 0.01 mM Mn\(^{2+}\) if required)
Table 4.2 - Immobilized lectins used for the characterization of the glycosylation pattern of SPRF

<table>
<thead>
<tr>
<th>Lectin</th>
<th>Cat.-No. (Vector Laboratories)</th>
<th>Divalent Cation requirements *</th>
<th>Primary Sugar Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Con A</td>
<td>Concanavalin A VC-AL-1003-L010</td>
<td>0.1 mM Ca$^{2+}$, 0.01 mM Mn$^{2+}$</td>
<td>Glucose, Mannose</td>
</tr>
<tr>
<td>SBA</td>
<td>Soybean Agglutinin VC-AL-1013-L002</td>
<td>0.1 mM Ca$^{2+}$, 0.01 mM Mn$^{2+}$</td>
<td>N-Acetyl-Galactosamine, Galactose</td>
</tr>
<tr>
<td>PNA</td>
<td>Peanut Agglutinin VC-AL-1073-L002</td>
<td>0.1 mM Ca$^{2+}$, 0.01 mM Mn$^{2+}$</td>
<td>Galactose</td>
</tr>
<tr>
<td>WGA</td>
<td>Wheat Germ Agglutinin VC-AL-1023-L002</td>
<td></td>
<td>N-Acetyl-Glucoseamine</td>
</tr>
<tr>
<td>UEA I</td>
<td>Ulex Europaeus Agglutinin I VC-AL-1063-L002</td>
<td>0.1 mM Ca$^{2+}$</td>
<td>Fucose</td>
</tr>
<tr>
<td>Jacalin</td>
<td>Jacalin VC-AL-1153-L002</td>
<td>0.1 mM Ca$^{2+}$</td>
<td>Galactose (specific for O-linked oligosaccharides)</td>
</tr>
<tr>
<td>SNA (EBL)</td>
<td>Elderberry Bark (Sambucus Nigra) Lectin VC-AL-1303-L002</td>
<td>0.1 mM Ca$^{2+}$</td>
<td>Sialic acid</td>
</tr>
<tr>
<td>Sepharose CI-4B</td>
<td>Control Fluka 84 963</td>
<td></td>
<td>–</td>
</tr>
</tbody>
</table>

For treatment with Peptide: N-Glycosidase F (PNGase F, NEB), the enzyme was first treated with Protease-Inhibitor-Mix (Roche) to inhibit intrinsic protease activity. Crude Bovine Thrombin was then incubated with PNGase F (6000 units/mg crude thrombin) for 4 hours at 37°C before desalting into Low Serum AMEM.

Precipitation of proteins in Crude Bovine Thrombin with ethanol and acetone was performed as follows. Eppendorf-tubes containing 700 µl Crude Bovine Thrombin were chilled to 0°C (on ice). Following addition of ice-cold acetone or ethanol to the respective final concentration (20, 30, 40, 50 and 60%), samples were kept on ice for 15 min and then precipitated proteins pelleted at 5000 x g for 10 min at 0°C. The supernatant was carefully removed and pellets resuspended in ice-cold Anion Buffer (pH 7.6), 100 mM NaCl.

For determination of the molecular weight of SPRF in the starting material, Crude Bovine Thrombin was dissolved in Cation Buffer (pH 6.5) at a concentration of 26.4 mg/ml (corresponding to 21 mg/ml protein). After complete thrombin inhibition with PPACK Crude Bovine Thrombin was desalted over NAP-10 columns into either PBS (pH 7.4) or pH 11 Gel Filtration Buffer. For SDS gel filtration, 20% SDS was added to Crude Bovine
Materials And Methods

Thrombin in PBS to a final concentration of 1%. All samples were incubated for at least 2 h at room temperature, before application to the gel filtration column.

Size exclusion chromatography was performed at RT using a 24 ml Superdex-200 gel filtration column with a flow rate of 0.25 ml/min. The following buffers were used: for native gel filtration, PBS (pH 7.4); for gel filtration at pH 11, pH 11 Gel Filtration Buffer; and for gel filtration in SDS, SDS Gel Filtration Buffer. Before each run, the gel filtration column was equilibrated with 5 CV (native, pH 11) or 10 CV (SDS) of degassed buffer. Then 250 µl of crude thrombin was injected onto the Superdex-200 column and fractions (0.4 ml) collected. Subsequently fractions were desalted into Low Serum AMEM (native, pH 11). After gel filtration in SDS, in order to remove SDS, 200 µl of each fraction was dialyzed three times against PBS and then once against SF-AMEM for 12 h respectively. Afterwards dialyzed samples were diluted with the same volume of SF-AMEM containing 1% FCS (final 0.5% FCS). All fractions were assayed in triplicate by adding 100 µl to cells.
4.3.6 Chromatographic Fractionation Of The Serum Factor

4.3.6.1 Cation Exchange Chromatography

4.3.6.1.1 Charging Of Cation Exchange Columns

All columns were charged with the following sequence: 5 CV ddwater, 5 CV 1.5 M NaCl, 5 CV ddwater and afterwards equilibrated in 5 CV starting buffer (usually 20 mM Cation Buffer, pH 6.5).

4.3.6.1.2 Screening Of Cation Exchange Columns at pH 6.5

All cation exchange chromatography was performed in Cation Buffer (pH 6.5). The starting material, Crude Bovine Thrombin, was loaded onto cation exchange columns (Table 4.3), pre-equilibrated with Cation Buffer. Bound proteins were washed with 5 CV Cation Buffer and then eluted with a stepwise gradient with 5 CV 100 mM, 5 CV 200 mM, 5 CV 500 mM and 5 CV 1500 mM NaCl. Fractions were pooled, treated with PPACK, desalted into Low Serum AMEM and assayed on cells.

Table 4.3 - Characteristics of cation exchange columns

<table>
<thead>
<tr>
<th>Column chemistry</th>
<th>Column</th>
<th>Column Characteristics</th>
<th>Flow Rate (ml/min)</th>
<th>CB-Thrombin loaded</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carboxymethyl (CM) / weak</td>
<td>HiTrap CM FF (5 ml)</td>
<td>1.6 x 2.5 cm</td>
<td>5</td>
<td>5 ml</td>
</tr>
<tr>
<td>Sulphopropyl (SP) / strong</td>
<td>HiTrap SP FF (5 ml)</td>
<td>1.6 x 2.5 cm</td>
<td>5</td>
<td>5 ml</td>
</tr>
<tr>
<td>Methyl sulphonate (S) / strong</td>
<td>Resource S (6 ml)</td>
<td>1.6 x 3.0 cm</td>
<td>5</td>
<td>5 ml</td>
</tr>
<tr>
<td>SO3−-group (S) / strong</td>
<td>Fractogel EMD SO3−(S)</td>
<td>0.5 x 10 cm</td>
<td>2</td>
<td>2 ml</td>
</tr>
<tr>
<td>Sulphopropyl / strong</td>
<td>Poros 20 HS (2 ml) *</td>
<td>0.46 x 10 cm</td>
<td>5</td>
<td>2 ml</td>
</tr>
</tbody>
</table>

* These columns were poured by ourselves.

4.3.6.1.3 Test Of Different pHs On Cation Exchange Columns

Crude Bovine Thrombin was dissolved at 8 mg/ml in 20 mM Cation Buffer at pH 4.5 or 5.5. Precipitated or non-dissolved proteins were spun down and the supernatant sterile-filtered. Afterwards 5 ml Crude Bovine Thrombin was loaded onto cation exchange columns (5 ml HiTrap CMFF and 5 ml HiTrap SPFF), which were equilibrated
with Cation Buffer at respective pH. Bound proteins were washed with 5 CV Cation Buffer and then eluted with a stepwise gradient with 5 CV 100 mM, 5 CV 200 mM and 5 CV 1000 mM NaCl. Fractions were pooled, treated with PPACK, desalted into Low Serum AMEM and assayed on cells.

4.3.6.1.4 Cation Exchange Chromatography On HiTrap CMFF As First Step Of Purification (Preparation of CMFF-FT)

For preparation of CMFF-FT either a 5 ml HiTrap CMFF or a 20 ml HiPrep CMFF, pre-equilibrated with Cation Buffer was used. Lyophilized Crude Bovine Thrombin was dissolved at 8 mg/ml in Cation Buffer and sterile-filtered. Afterwards 15 ml Crude Bovine Thrombin was loaded onto a HiTrap CMFF column or 90 ml onto a HiPrep CMFF. The columns were washed with 10 CV Cation Buffer and bound proteins eluted with 10 CV 1500 mM NaCl (CMFF-1.5 M). The flow through (CMFF-FT) was pooled (2.5 fold volume of loaded volume), treated with PPACK to completely inhibit thrombin and used as starting material for all subsequent chromatographic steps.

4.3.6.2 Anion Exchange Chromatography
4.3.6.2.1 Charging Of Anion Exchange Columns

All columns were charged with the following sequence: 5 CV ddwater, 5 CV 1.5 M NaCl, 5 CV ddwater and afterwards equilibrated in 5 CV starting buffer (usually 20 mM Anion Buffer, pH 7.0).

4.3.6.2.2 Screening Of Anion Columns At pH 7.0 With Or Without 20% Ethanol

For this experiment, Crude Bovine Thrombin was dissolved in Anion Buffer and sterile-filtered. Afterwards 4 ml Crude Bovine Thrombin were loaded onto anion exchange columns, the columns washed with 5 CV Anion Buffer and bound proteins eluted in a stepwise gradient with 5 CV 200 mM, 5 CV 400 mM, 5 CV 800 mM and 5 CV 1600 mM NaCl. Fractions were pooled, treated with PPACK, desalted into Low Serum AMEM and assayed on cells. For comparison all columns were also run in the presence of 20% ethanol.
4.3.6.2.3 Optimization Of HiTrap Q

The starting material for HiTrap Q was CMFF-FT. A 5 ml HiTrap Q column was equilibrated with Anion Buffer (pH 7.0), then loaded with 17 ml CMFF-FT and the column washed with 3 CV Anion Buffer (pH 7.0). Adsorbed proteins were eluted either with a linear gradient of 0 to 1.6 M NaCl over 20 CV or with a step gradient of 5 CV 200 mM, 5 CV 400 mM, 5 CV 600 mM, 5 CV 800 mM and 1600 mM NaCl. Fractions (1.8 ml) were pooled, desalted into Low Serum AMEM and assayed on cells.

4.3.6.2.4 Anion Exchange Chromatography On MonoQ After Heparin Affinity Chromatography

The starting material for the Mono Q column was Hep3 desalted into Anion Buffer, 50 mM NaCl by concentration and dilution on 10 kD membrane. A Mono Q column (1 ml) was equilibrated with Anion Buffer (pH 7.0), 50 mM NaCl and 22 ml of desalted Hep3 pool was loaded. After washing with 5 CV Anion Buffer, 50 mM NaCl, adsorbed proteins were eluted with a linear gradient of 0.05 to 1.5 M NaCl over 30 CV in Anion Buffer. Fractions (0.5 ml) were pooled (1.5 ml), desalted into Low Serum AMEM and assayed on cells.

4.3.6.3 Hydrophobic Interaction Chromatography (HIC)
4.3.6.3.1 Screening Of 1 ml HiTrap Butyl, HiTrap Phenyl And Valine Sepharose Columns

For an initial test three 1 ml HIC-columns with distinctive chemistry were examined. HiTrap Butyl and HiTrap Phenyl were purchased from APBiotech. Valine-Sepharose was prepared by incubating 10 mM valine with an NHS-activated 1 ml HiTrap column (APBiotech) in 0.1 M NaHCO₃ (pH 8.0). All HIC-columns were equilibrated in 50 mM Phosphate Buffer (pH 7.0), 1 M ammonium sulfate (AS) before run. The flow rate for all columns was 1 ml/min.

The starting material, CMFF-FT, was desalted over NAP-25 columns (PD-10) into 50 mM Phosphate Buffer (pH 7.0), 1 M ammonium sulfate (AS) and 8.5 ml (corresponds to 6 ml CMFF-FT) loaded onto HIC-columns. All columns were washed with 10 CV 50 mM Phosphate Buffer, 1 M AS and bound proteins eluted with 10 CV
50 mM Phosphate Buffer and with 10 CV 50 mM Phosphate Buffer, 20% ethanol. Fractions (0.5 ml) were pooled, desalted into Low Serum AMEM and assayed on cells.

4.3.6.3.2  **Comparison Of 5 ml HiTrap Butyl And HiTrap Octyl Columns**

In a second screen two 5 ml HIC-columns, HiTrap Butyl and HiTrap Octyl (both APBiotech), were directly loaded with CMFF-FT and proteins eluted with a more detailed step gradient. The flow rate for these columns was 5 ml/min.

The starting material, CMFF-FT, was mixed 1:1 with 100 mM phosphate buffer (pH 7.0), 2 M AS and 38 ml (19 ml CMFF-FT) loaded onto the HiTrap Butyl or the HiTrap Octyl column. For elution of bound proteins the columns were developed with a step gradient in 50 mM Phosphate Buffer (pH 7.0), 10 CV for each step, containing 1 M AS, 0.1 M AS, 0 M AS, 20% ethanol and 40% ethanol. Fractions of 0 M, 20% EtOH and 40% EtOH step (first 5 CV) were pooled, desalted into Low Serum AMEM and assayed on cells.

4.3.6.3.3  **Preparation Of Butyl-20% EtOH**

The starting material for the preparation of Butyl-20%EtOH was CMFF-FT. A procedure as described above for the 5 ml HiTrap Butyl column (APBiotech) was used, and the first five column volumes of the elution step in 50 mM Phosphate Buffer (pH 7.0), containing 20% ethanol (EtOH), were pooled. This pool is referred as Butyl-20%EtOH. Later this purification procedure was adapted to a 20 ml HiPrep Butyl column (APBiotech) using a flow rate of 5 ml/min. In a recent protocol the 20 ml HiPrep Butyl column was equilibrated with 50 mM Phosphate Buffer, 0.1 M ammonium sulfate. The starting material, CMFF-FT, was mixed with 1/10th 333 mM phosphate buffer (pH 7.0), 1 M AS and incubated for 30 min at RT. Then, CMFF-FT in 33 mM phosphate buffer, 0.1 M AS was loaded onto the HiPrep Butyl column. The elution of bound proteins was performed in 50 mM Phosphate Buffer, 10 CV for each step, containing 0.1 M AS, 0 M AS, 20% ethanol and 40% ethanol.
4.3.6.4 Affinity Chromatography
4.3.6.4.1 Heparin Affinity Chromatography
4.3.6.4.1.1 Step Gradient On HiTrap Heparin

The starting material, CMFF-FT was diluted threefold with Anion Buffer, 600 mM NaCl. A 5 ml HiTrap Heparin column was equilibrated with Anion Buffer, 200 mM NaCl and 12.5 ml of CMFF-FT in 200 mM NaCl was loaded. For a step gradient elution after protein loading the column was developed with a step gradient in Anion Buffer, 3 CV for each step, containing 0, 200, 250, 300, 350, 400, 450, 500, 750 and 1500 mM NaCl. Fractions (1.8 ml) were pooled, desalted into Low Serum AMEM and assayed on cells.

4.3.6.4.1.2 Linear Gradient On HiTrap Heparin - Preparation Of Hep3

The starting material, CMFF-FT was diluted threefold with Anion Buffer, 600 mM NaCl. A 5 ml HiTrap Heparin column was equilibrated with Anion Buffer, 200 mM NaCl and 12.5 ml of CMFF-FT in 200 mM NaCl was loaded. The heparin column was washed with 5 CV Anion Buffer (pH 7.0), and bound proteins eluted with a linear gradient of 0 to 1 M NaCl over 20 CV. Remaining proteins were eluted with 5 CV 1.5 M NaCl. For obtaining Hep3, fractions between 430 and 590 mM NaCl were pooled.

4.3.6.4.1.3 Step Gradient On HiTrap Heparin After Hydrophobic Interaction Chromatography - Preparation of Hep-1M

The starting material, Butyl-20%EtOH, was obtained as described for hydrophobic interaction chromatography on HiTrap Butyl. A 5 ml HiTrap Heparin column was equilibrated with 20 mM Tris (pH 7.0) and 55 ml Butyl-20% EtOH was loaded. Non-specifically bound proteins were removed from the column with 10 CV 200 mM NaCl and bound proteins eluted with 5 CV 1 M NaCl (Hep-1M). Remaining proteins were eluted with 5 CV 1.5 M NaCl. Fractions (1.8 ml) were pooled, desalted into Low Serum AMEM and assayed on cells.
4.3.6.4.1.4  Linear Gradient On HiTrap Heparin After Hydrophobic Interaction Chromatography - Preparation of Hep3-Butyl And Hep3/4-Pool

For heparin affinity chromatography after hydrophobic interaction chromatography a purification procedure with HiTrap CMFF (5 ml) and HiTrap Butyl (5 ml) and later with HiPrep CMFF (20 ml) and HiPrep Butyl (20 ml) was used as described for preparation of Butyl-20%EtOH. From two butyl runs Butyl-20%EtOH fractions were pooled and 43.2 ml loaded onto a 5 ml HiTrap Heparin column, pre-equilibrated in Anion Buffer. The heparin column was washed with 5 CV Anion Buffer (pH 7.0), and bound proteins were eluted with a linear gradient of 0 to 1 M NaCl over 20 CV. Remaining proteins were eluted with 5 CV 1500 mM NaCl. For obtaining Hep3-Butyl, fractions (1.8 ml) between 430 and 590 mM NaCl and for Hep3/4-Pool, fractions between 430 and 680 mM NaCl were pooled desalted into Low Serum AMEM and assayed on cells.

4.3.6.4.2  Affinity Chromatography On Cibacron Blue F3G-A (HiTrap Blue)

The starting material for affinity chromatography on Cibacron Blue F3G-A (HiTrap Blue) was CMFF-FT. A HiTrap Blue column (5 ml) was equilibrated with Anion Buffer (pH 7.0), loaded with 10 ml of CMFF-FT and washed with 5 CV Anion Buffer (pH 7.0). Elution of adsorbed proteins was done with a step gradient in Anion Buffer, 5 CV each step, containing 200, 500, 1000 and 2000 mM NaCl. Fractions (1.8 ml) were pooled, desalted into Low Serum AMEM and assayed on cells.

4.3.6.4.3  Lectin Affinity Chromatography

4.3.6.4.3.1  Buffer For Wheat Germ Agglutinin (WGA) Lectin Affinity Chromatography

WGA Binding buffer
20 mM Tris-HCl (pH 7.0), 0.5 M NaCl

WGA Elution buffer
20 mM Tris-HCl (pH 7.0), 0.5 M NaCl, 0.5 M N-acetyl-D-glucosamine (Sigma A-8625)
Materials And Methods

WGA Storage buffer
20 mM Tris-HCl (pH 7.0), 0.5 M NaCl, 20% Ethanol

WGA Regeneration buffer
20 mM Tris-HCl (pH 8.5), 1 M NaCl

4.3.6.4.3.2 Buffer For Concanavalin A (ConA) And Lentil Lectin Affinity Chromatography

ConA Binding Buffer
20 mM Tris-HCl (pH 7.0), 0.5 M NaCl, 1 mM Ca$^{2+}$, 1 mM Mn$^{2+}$

ConA Elution Buffer
20 mM Tris-HCl (pH 7.0), 0.5 M NaCl, 0.5 M Methyl-α-D-glucopyranoside (Sigma M—9375), 1 mM Ca$^{2+}$, 1 mM Mn$^{2+}$

Lentil Lectin Elution Buffer
20 mM Tris-HCl (pH 7.0), 0.5 M NaCl, 0.3 M Methyl-α-D-mannopyranoside (Sigma M—6882), 1 mM Ca$^{2+}$, 1 mM Mn$^{2+}$

ConA Storage buffer
20 mM Tris-HCl (pH 7.0), 0.5 M NaCl, 20% Ethanol, 1 mM Ca$^{2+}$, 1 mM Mn$^{2+}$

ConA Regeneration Buffer
20 mM Tris-HCl (pH 8.5), 1 M NaCl, 1 mM Ca$^{2+}$, 1 mM Mn$^{2+}$

4.3.6.4.3.3 Screening Of Lectin Affinity Columns

The starting material for lectin affinity chromatography was Hep-1M, containing eluted proteins after heparin affinity chromatography in 20 mM Tris (pH 7.0), 1 M NaCl. Before lectin affinity chromatography proteins in 1 M pool were diluted twofold with 20 mM Tris (pH 7.0) to a final NaCl concentration of 500 mM. Lectin affinity was tested with 1 ml columns of HiTrap Lentil Lectin, HiTrap Wheat Germ Lectin (WGA, both APbiotech) and a 1.0 ml Concanavalin A column (ConA, Sigma C-9017, self-poured). The flow rate for all steps was 0.5 ml/min.
For the Lentil Lectin column the same buffers were used as for the ConA column, except proteins were eluted with Lentil Lectin Elution Buffer. Lectin columns were equilibrated with 10 CV Binding Buffer and 5 ml of diluted Hep-1M pool (in 500 mM NaCl) was injected. The Columns were washed with 10 CV Binding Buffer and bound proteins eluted with 10 CV Elution Buffer. Regeneration of the lectin columns was carried out with the sequence of 10 CV Binding Buffer, 10 CV Regeneration Buffer, 10 CV Binding Buffer and finally 10 CV Storage Buffer. Fractions (0.5 ml) were pooled, desalted into Low Serum AMEM and assayed on cells.

4.3.6.5  **Chromatography On Hydroxyapatite**

The starting material for chromatography on hydroxyapatite was Butyl-20%EtOH, except that elution of proteins from HiTrap Butyl column with 0 M AS, 20% EtOH and 40% EtOH was performed in 10 mM Phosphate Buffer (pH 7.0). Hydroxyapatite chromatography was performed on a 5 ml Econo-Pac CHT-II cartridge (Bio-Rad) with a flow rate of 1 ml/min. The 5 ml CHT-II column was equilibrated according to manufacturer instructions with 5 ml 10 mM phosphate buffer, 10 ml 500 mM phosphate buffer and 25 ml 10 mM Phosphate Buffer (pH 7.0). Afterwards 40 ml of Butyl-20% EtOH (in 10 mM phosphate) from HiTrap Butyl was loaded, the column was washed with 5 CV 10 mM phosphate and adsorbed proteins eluted with a linear gradient from 10 to 500 mM phosphate buffer (pH 7.0) over 10 CV. Finally the column was hold for 3 CV in 500 mM phosphate buffer. Fractions (1.8 ml) were desalted into Low Serum AMEM and assayed on cells.

4.3.6.6  **Chromatofocusing on Mono P**

The starting material for chromatofocusing was Butyl-20%EtOH, except that elution of proteins from HiTrap Butyl column with 0 M AS, 20% EtOH and 40% EtOH was performed in 10 mM phosphate buffer (pH 7.0). Chromatofocusing was performed on a 4 ml Mono P 5/200 GL column (APBiotech) with a flow rate of 1 ml/min. The Mono P column was charged by injection with 2 ml 5 M NaOH, washed with 5 CV 25 mM phosphate buffer (pH 7.0) followed by injection of 2 ml 2 M NaCl and a wash with 5 CV 25 mM phosphate buffer. Polybuffer 74 (APBiotech) was diluted 1:10 with ddH2O and the pH adjusted to 4.0 with HCl. In order to load proteins in 25 mM phosphate buffer onto Mono P column, 45 ml 20% EtOH pool
from HiTrap Butyl (in 10 mM phosphate buffer) were mixed with 1.35 ml 500 mM phosphate buffer (final 25 mM). Afterwards 40 ml were loaded, washed with 10 CV 25 mM phosphate and proteins eluted with 100 ml Polybuffer 74 (pH 4.0) forming a pH gradient from 7 to 4 over 50 ml. Non-eluted proteins were washed from the column by re-equilibration of MonoP column with 10 CV 25 mM phosphate buffer (pH 7.0) followed by a wash with 5 CV 2 M NaCl. Fractions (1.8 ml) were desalted into Low Serum AMEM and assayed on cells.

4.3.6.7 Reversed Phase Chromatography

The starting material for reversed phase chromatography was the SD200-Pool after gel filtration in 0.1% SDS.

Reversed phase chromatography was performed on C4 column (GraceVydac 214TP54, 4.6 mm inner diameter, 250 mm length) in 0.1% TFA (v/v) (Uvasol, Merck 1.08262.0100) with a flow rate of 1 ml/min. Proteins were eluted with a gradient of acetonitrile by mixing 0.1% TFA (v/v) / HPLC water (LiChrosolv, Merck) and 0.085% TFA (v/v) / acetonitrile (LiChrosolv, Merck). For removal of SDS, which interferes with reversed phase chromatography, a SDS removal guard column (SDS MacroTrap Michrom BioResources, Inc.) was placed before the C4 column. Bound SDS was removed and SDS removal trap column was regenerated after each run with 10 ml 0.1% TFA / 90% acetonitrile. Elution of proteins was observed by recording absorption at 215 nm and 280 nm.

For purification of SPRF by reversed phase chromatography the SD200-Pool from gel filtration in 0.1% SDS was mixed with 10% TFA to a final concentration of 0.1% and 1 ml injected onto a C4 column, equilibrated in 0.1% TFA / 4% acetonitrile. Proteins were eluted with a gradient from 4 to 70% acetonitrile over 60 min, followed by a wash of 10 min with 90% acetonitrile. Fractions (1 ml) or pools of 4 ml were mixed with 200 µl 1% BSA (Albumin bovine Fraction V, SERVA, dissolved in water), frozen in liquid nitrogen and lyophilized. Afterwards lyophilized proteins were re-dissolved in 1 ml PBS (containing now 0.2% BSA) and dialyzed three times 12 h against PBS and once against SF-AMEM. Before assaying on cells dialyzed fractions were mixed with 1/20th High Serum AMEM.

For the re-run on the C4 column, the 10 most active fractions (between 40 and 50% acetonitrile) from seven runs were pooled (70 ml) and diluted 1:1 with 0.1% TFA / water to a final acetonitrile concentration of 25%. The C4 column was equilibrated in 0.1%TFA / 20% acetonitrile and 100 ml of diluted pool of the first run was subjected to a
second run by 20 times injection of 5 ml. Afterwards a gradient was run from 20 to 40% acetonitrile over 10 ml, followed by a gradient from 40 to 60% acetonitrile over 40 ml and a gradient from 60 to 90% acetonitrile over 15 ml. Finally C4 column was washed with 10 ml 90% acetonitrile. For the region where proteins were detectable by absorption at 280 nm two fractions were pooled (0.5 ml each) and 200 µl 1% BSA added. For the rest of the run four fractions were pooled (0.5 ml each) and 200 µl 1% BSA added. All fractions and pools were treated and assayed as described above for first run on the reversed phase column.

4.3.6.8 Ultrafiltration and Concentration On A 100 kD Membrane

Ultrafiltration and Concentration of protein fractions was performed as described in concentration and desalting of diluted protein samples.

4.3.6.8.1 Preparation Of Hep3-Conc

The most purified pool from Heparin (Hep3) was concentrated 100 – 200-fold on a Centricon Plus-20 (Millipore) with a 10 kD MWCO. For removal of low molecular weight proteins the concentrated Hep3 pool was diluted 10-fold with PBS and concentrated on a Centricon Plus-20 (Milipore) with a 100 kD MWCO. This procedure was repeated four times. In later experiments, Hep3 was directly concentrated on a 100 kD membrane (Hep3-Conc).

4.3.6.8.2 Preparation Of Hep-1M-Conc And Hep3/4-Conc (For Gel Filtration At pH 11 And Native PAGE)

For preparation of Hep-1M-Conc, 7 ml Hep-1M (in 20 mM Tris, 1 M NaCl, pH 7.0) was concentrated on a Vivaspin 20 (MWCO 100 kD) to 1 ml, then 5-fold diluted with 20 mM Tris (pH 7.0) and re-concentrated. The final volume of Hep-1M-Conc (in 20 mM Tris, 200 mM NaCl, pH 7.0) for gel filtration at pH 11 was 0.850 ml (8.2-fold concentrated Hep-1M).

In order to further reduce the concentration of sodium chloride for native PAGE 0.85 ml Hep-1M-Conc (in 20 mM Tris, 200 mM NaCl, pH 7.0) was diluted twofold with
20 mM Tris (pH 7.0) and re-concentrated. The final volume of Hep-1M-Conc (in 20 mM Tris, 100 mM NaCl, pH 7.0) was 0.530 ml (13.2-fold concentrated Hep-1M).

For obtaining Hep3/4-Conc, 48.9 ml Hep3/4-Pool was concentrated to 1 ml on a Vivaspin 20 (MWCO 100 kD) and then 10-fold diluted with PBS and concentrated again. Concentration and dilution with PBS was repeated three times. The final volume of Hep3/4-Conc was 0.730 ml (67-fold concentrated Hep3/4-Pool).

### 4.3.6.9 Gel filtration Of Partially Purified Fraction

#### 4.3.6.9.1 Gel Filtration Marker And Running Conditions

**Gel filtration Marker**

Markers, used for gel filtration (gel filtration LMW and HMW calibration kit, APBiotech) were blue dextran 2000 (2000 kD), thyroglobulin (669 kD), ferritin (440 kD), catalase (232 kD), aldolase (158 kD), bovine serum albumin (67 kD), ovalbumin (43 kD), chymotrypsinogen A (25 kD) and ribonuclease A (13.7 kD).

All gel filtration experiments were performed using a 24 ml Superdex-200 HR 10/30 column (APBiotech) at RT. Before use all buffers were degassed. The column was equilibrated with 5 CV buffer (10 CV for PBS, containing 0.1% SDS) using a flow rate between 0.2 and 0.5 ml/min.

For purification a sample volume of 100 to 500 µl was injected and runs were performed with a flow rate of 0.25 ml/min and a fraction size of 0.4 ml. Molecular weight marker proteins (APbiotech) were dissolved in the respective buffer and injected in a volume of 100 µl.

#### 4.3.6.9.2 Native Gel Filtration Of Hep3-Conc

The starting material for gel filtration under native conditions was Hep3-Conc. A 24 ml Superdex-200 column (APBiotech) was equilibrated with 5 CV SF-AMEM (see cell culture solutions). Afterwards Hep3-Conc was applied to the gel filtration column, and fractions (0.4 ml) collected. Fractions were supplemented with 1/20th High Serum MEM and assayed on cells.
4.3.6.9.3 Gel Filtration Of Hep3-Conc Under Denaturing Conditions In 0.1% SDS

4.3.6.9.3.1 Analytical Gel Filtration In 0.1% SDS

The starting material for gel filtration in SDS was Hep3-Conc. A 24 ml Superdex-200 column (APBiotech) was equilibrated with 10 CV PBS, 0.1% SDS. Hep3-Conc was mixed with SDS and incubated at 37°C for 30 min (final 2%) to denature the proteins and then kept at RT until use. A 250 µl sample was applied to the gel filtration column, and fractions (0.4 ml) were collected. Afterwards 200 µl of each fraction was mixed with 50 µl 1% BSA (Serva) and dialyzed (cap-dialysis) three times for 12 h against a 100-fold volume of PBS and finally against SF-AMEM. Before assaying on cells 1/20th High Serum AMEM was added to each sample.

4.3.6.9.3.2 Preparation Of SD200-Pool

Gel filtration in 0.1% SDS was performed as described above for analytical gel filtration in 0.1% SDS. For preparation of SD200-Pool 500 µl 200-fold concentrated Hep3 (in 0.1% SDS) was applied to a Superdex-200 column. The six most active fractions (each 0.4 ml) were pooled and referred as SD200-Pool.

4.3.6.9.4 Gel Filtration At pH 11 (Starting With Hep-1M-Conc Or Hep3/4-Conc)

The starting material for gel filtration at pH 11 was Hep-1M-Conc or Hep3/4-Conc. A 24 ml Superdex-200 column (APBiotech) was equilibrated with 5 CV pH 11 Gel Filtration Buffer. Hep-1M-Conc or Hep3/4 Conc were mixed with one-fifth 0.5 M CAPS (pH 11, final 100 mM) and proteins incubated at RT for 2 h. Afterwards 400 µl were applied to gel filtration column, and fractions (0.4 ml) collected. In order to decrease the pH of samples before desalting 100 µl 500 mM Tris (pH 7.0) was added to each fraction (final 100 mM) and then 400 µl desalted over NAP-5 columns into Low Serum AMEM. Due to incompatibility of high concentrations of Tris buffer with the myotube assay, in following experiments the pH was lowered with 1/10th 1 M HEPES (pH 7.4).
4.3.6.10 Preparative PAGE And Isoelectric Focusing

4.3.6.10.1 Preparative SDS-PAGE (Starting With Hep3-Conc)

The starting material for preparative SDS-PAGE was Hep3-Conc. Hep3-Conc was mixed with 5X Sample Buffer w/o DTT, incubated at 37°C for 1 h and loaded onto NuPAGE Novex Bis-Tris Gels (1.5 mm / 10 well, Invitrogen). Electrophoresis was carried out in MOPS Buffer (Invitrogen) at RT, 65 V until the tracking dye had migrated to the end of the gel. After electrophoresis the gel was incubated for 20 min in 1% BSA / PBS on a rotation shaker and the gel sliced according to the SeeBlue Plus2 Pre-Stained Standard (Invitrogen). Single slices were minced in PBS containing 0.2% BSA and proteins extracted overnight on a rotation shaker. Samples were centrifuged, filtered and the supernatant was dialyzed as described for gel filtration in SDS.

For electro-elution the gel region between 28 and 39 kD was cut out, minced and proteins eluted with the Electro-Eluter Model 422 in 50 mM ammonium bicarbonate, 0.1% SDS (according to manufacturer's instruction, Bio-RAD). Electro-eluted fractions were subsequently lyophilized, reconstituted in PBS and aliquots dialyzed as described for gel filtration in SDS.

4.3.6.10.2 Preparative Native PAGE (Starting With Hep-1M-Conc)

10X Tris-glycine Buffer (pH 8.8)

- 30 g Tris base (250 mM)
- 144 g glycine (1.92 M)

dissolved in 1 l ddwater and sterile-filtered

1X Tris-glycine Buffer (pH 8.8)

10X Tris-glycine Buffer (pH 8.8) diluted 1:10 and stored at 4°C.

5X Native Sample Buffer

- 312.5 mM Tris (pH 6.8)
- 50% glycerol
- 0.1% Bromphenol Blue (Sigma B-5525)

5X Native Sample Buffer was sterile-filtered (0.22 µm)

Preparative native PAGE was performed with Novex Tris-Glycine Gels (Invitrogen) using a Tris-glycine buffer (pH 8.8). Electrophoresis was run at 4°C to reduce loss of protein
activity by denaturation and to minimize attack by proteolysis. Gels were run at 65 V for 30 min and then run at 130 V until the dye-front reached the end of the gel.

The starting material for native PAGE was Hep-1M-Conc in 100 mM NaCl. This concentrated Hep-1M was sterile-filtered using Spin-X Centrifuge Tube filter (Corning Costar 8160) and stored at 4°C before use. In order to separate aggregated proteins, the Hep-1M-Conc was supplemented with one-fifth volume 0.5 M CAPS (pH 11) and incubated for 2 h at RT on a shaker. Afterwards the sample was mixed with 5X Native Sample Buffer and loaded on a 1.5 mm 6% Novex Tris-Glycine Gel.

After electrophoresis the native gel was washed three times for 5 min in 100 ml 1X PBS and five lanes of the gel were sliced with a scalpel into eight 1 cm pieces. Gel slices were minced using a dounce homogenizer (Kontes) and transferred into an Eppendorf tube. Proteins were extracted by passive diffusion on a rotation shaker for about 24 h at RT and gel debris removed by centrifugation. Supernatant, containing extracted proteins, was filtered using Spin-X Centrifuge Tube Filters (0.22 µm), then desalted over NAP-5 columns into Low Serum AMEM and assayed on cells.

4.3.6.10.3 Isoelectric Focusing (Starting With Hep3-Conc)

IEF Rehydration Buffer

8 M urea (Merck 1.08487)
2% CHAPS (Sigma C9426)
+ 0.1% Bio-Lyte 3/10 Ampholyte (Bio-Rad) after desalting

The starting material for preparative isoelectric focusing was Hep3-Conc that was concentrated on a 10 kD membrane. For removal of NaCl, Hep3-Conc was diluted 10-fold in Anion Buffer (pH 7.0) and concentrated on either 10 kD or 100 kD MWCO Centricon Plus-20 centrifugal ultrafiltration units (Milipore). This procedure was repeated four times. The sample was then desalted into IEF Rehydration Buffer and then Bio-Lyte 3/10 Ampholyte added to a final concentration of 0.1%. Isoelectric focusing was performed using 17 cm ReadyStrip IPG strips with a linear pH gradient range from 4-7 and 4-10 (Bio-Rad). The IPG strips were re-hydrated in 450 µl Hep3-Conc in IEF Rehydration Buffer overnight and then focused on a PROTEAN IEF system (Bio-Rad) according to the manufacturer's instructions (40000 V-hr). Afterwards the IPG strips were washed for 10 min in 10 ml PBS, 1% BSA, then cut into 1.5 cm pieces and the proteins extracted by passive diffusion in 1 ml of PBS, 0.2% BSA overnight. The supernatant was
sterile-filtered (Spin-X, Corning Costar) and then dialysed as described for gel filtration in SDS.

4.3.6.10.4 2D-PAGE (Starting With Hep3-Conc)

SDS-PAGE Equilibration Buffer

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>6 M urea</td>
<td></td>
<td>(Merck 1.08487)</td>
</tr>
<tr>
<td>0.375 M Tris, pH 8.8</td>
<td></td>
<td>(Roth 4855.2)</td>
</tr>
<tr>
<td>2% SDS</td>
<td></td>
<td>(Serva 20760)</td>
</tr>
<tr>
<td>20% glycerol</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.1% Bromphenol Blue</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The starting material for gel filtration in SDS was Hep3-Conc. Samples of Hep3-Conc were focused as described above. Subsequently the IPG strips were equilibrated for 15 min in SDS-PAGE Equilibration Buffer, then cut into three pieces (each 5.7 cm) and placed on top of a 10% NuPAGE Novex Bis-Tris gel (1.0 mm / 2D well, Invitrogen). Electrophoresis in MOPS buffer was performed at 65 V until the dye front reached the bottom of the gel. The NuPAGE gels were washed three times with 150 mM NaCl, incubated with Simply Blue Safestain (Invitrogen) containing 150 mM NaCl and finally de-stained for 1 h in 150 mM NaCl. Gel regions between the 28 and 39 kDa markers were cut out and sliced into pieces based on visible major bands. Proteins were extracted and assayed on cells as described for preparative SDS-PAGE.

4.3.7 Mass Spectrometry Analysis

Mass spectrometry analysis on purified fractions was performed in the MPI-CBG Mass Spectrometry Facility. Briefly, protein bands were excised from the Coomassie stained gel and digested with trypsin as described previously (Shevchenko et al., 1996). Tryptic peptides were recovered from the gel matrix by extraction with 5% formic acid and acetonitrile. The extracts were pooled together, dried down in a vacuum centrifuge and re-dissolved in 10 µl of 0.04% trifluoroacetic acid (TFA). Peptides were analyzed by LC MS/MS method on a LTQ ion-trap mass spectrometer as described recently (Mayya et al., 2005). Database searching of tandem mass spectra was performed using MASCOT software, version 2.1 (Matrix Science, London, UK) installed on a local server, against all bovine proteins in the NCBI database.
Acknowledgments
6 References


References


7 Publications

Parts of this thesis have already been published:

8 Declaration According To §5.5

I herewith declare that I have produced this paper without the prohibited assistance of third parties and without making use of aids other than those specified; notions taken over directly or indirectly from other sources have been identified as such. This paper has not previously been presented in identical or similar form to any other German or foreign examination board.

The thesis work was conducted from 1.12.2001 to 31.01.2006 under the supervision of Dr. Elly Tanaka at the Max Planck Institute of Molecular Cell Biology and Genetics Dresden.

Dresden, 22.02.2006

Werner Straube