2. Material and Methods

2.1 Chemicals

Table 2: Chemicals

Chemicals	Source
100 bp Standard Marker	Fermentas (USA)
3-Amino-1,2,4-Triazol	Sigma, München (Germany)
α-mouse antibodies linked to HRP	Clontech Laboratories GmbH, (Germany)
ß-Mercaptoethanol	Merck (Germany)
Acrylamide	Serva Heidelberg (Germany)
Agarose, Agar	Difco Lab. Detroit, Michigan (USA)
Amino acids	Sigma (Germany)
Ampicillin	Sigma (Germany)
Antibodies (polycolony, produced for labor)	Eurogentec (Belgien)
Anti-Protein A Antibody	Sigma, München (Germany)
Bacto Peptone	New England Biolabs (UK)
Bovine serum albumin (BSA)	Serva (Germany)
Bromphenolblue	Serva (Germany)
CIAP	New England Biolabs (UK)
Coomassie Brilliant Blue (Serva Blau R-250)	Serva Heidelberg (Germany)
DNA Gel-Elution Kit	Qiagen (Germany)
ECL-anti-rabbit/mouse antibodies	Sigma (Germany)
ECL-Hyperfilm	Amersham Buchler GmbH (Germany)
ECL-Western-Blotting-Detection System	Amersham Buchler GmbH (Germany)
Ethylenediaminetetraacetic acid (EDTA)	Merk (Germany)
Fish DNA	Sigma (Germany)
Glutathion Sepharose 4B	Amersham Biosciences (Germany)
IPTG	PEQLAB Biotech. GmbH, (Germany)
Lambda Marker (DNA)	Amersham Biosciences, (Germany)
Lysozyme	Sigma (Germany)
Nitrocellulose-Foil (0,45μm), Sterile filter (0,2μm)	Schleicher und Schuell, Dassel (Germany)
N,N-Methylenbisacrylamide	Serva Heidelberg (Germany)
Oleatic acid	Merk (Germany)
Oligonucleotides	MWG Biotech AG (Germany)
Pefabloc SC Serine Protease Inhibitor	Roche Diagnostics GmbH, (Germany)
Plasmid Miniprep Kit	PEQLAB Biotech. GmbH, (Germany)
Primers and Sequencing	MWG Biotech AG, Ebersberg, (Germany)
Protease Factor Xa	Novagen (USA) or New England biolabs (UK)
Protease Thrombin	Sigma (Germany)

Pwo DNA–Polymerase 10x Pwo-Polymerase- Buffer	Eurogentec (Belgium)
Restriction Endonucleases	Eurogentec (Belgium), New England Biolabs (UK)
S.cerevisiae Genomic DNA	Promega GmbH, (Germany)
SDS PAGE Molecular Weight Marker	Biorad (Germany)
Select Peptone, Select Agar	GIBCO Invitrogen Co., GmbH, (Germany)
Sodium dodecyl sulfate (SDS)	Serva Heidelberg (Germany)
T4-DNA-Ligase, Ligase Buffer (10x)	Eurogentec (Belgium)
Taq DNA Polymerase 10x Reaction Buffer, 25 mM $MgCl_2$	ABgene® House, Surrey (UK)
Temed	Serva (Germany)
TOPO-TA Cloning Kit	Invitrogen (Invitrogen, (CA) USA)
Triton X-100	Boehringer Ingelheim (Germany)
Tween 20, Tween 40	Serva (Germany)
$Vent_R$-DNA–Polymerase und Puffer (10x)	New England Biolabs (UK)
Whatman 3 MM	Whatman, Maidstone (UK)
Yeast Extract	Difco Lab., Detroit, Michigan (USA)
Yeast Nitrogen-base without amino acids	Difco Lab., Detroit, Michigan (USA)

2.2 Microorganisms

2.2.1 *Escherichia coli*

Strain	Genotype	Source
DH5α	Φ80d*lac*ZΔM15, *rec*A1, *end*A1, *gyr*A96, *thi*-1, *hsd*R17, (r_k-, m_{k+}), *sup*E44, *rel*A1, *deo*R, Δ(*lac*ZYA-*arg*F)U169	lab
TG1	Δ(lac, pro), supE, thi, hsdD5, F'[traD36 pro^+ lac^{q1} lacZΔM15]	lab
BL21(DE3)	F., *omp*T, *hsd*S_B, (r_B-, m_B-), *dcm, gal,* λ(DE3)	Invitrogen

2.2.2 *Saccharomyces cerevisiae*

Strain	Genotype	Source	
HF7c	MATa, ura3-52, his3-200, lys2-801, ade2-101, trp1-901, leu2-3/112, gal4-542, gal80-538, LYS::GAL1-HIS3, URA::(GAL417-mers)$_3$-CYC1-LacZ	Clontech, USA	(Feilotter et al., 1994)
PCY2	MATa, Dgal4, Dgal80, URA3::GAL1-LacZ, lys2-801,his3-D200, trp1-D63, leu2, ade2-101	lab	
UTL-7A	MATα leu2-3, 112 ura3-52 trp1		(Erdmann et al., 1989)

2.3 Media

2.3.1 Media for *E. coli*

LB Medium

2% Peptone
2% NaCl
1% Yeast Extract

M9 Medium

50 mg/l EDTA	5 mg/l $FeSO_4$	0.5 mg/l $ZnCl_2$
0.1 mg/l $CuSO_4$	1 mM $MgSO_4$	0.3 mM $CaCl_2$
7.281 g/l Na_2HPO_4	2 g/l KH_2PO_4	0.5 g/l NaCl
4g/l glucose	1.5 mg/l thiamine-HCl	1.5 mg/l biotin
500 mg/l NH_4Cl	100 mg/l ampicillin (if needed)	

2.3.2 Media for yeast

YPD-Medium

2 % (w/v) glucose
2 % (w/v) Select Peptone
1 % (w/v) yeast extract
pH 6.0

SD-Medium

0.3 % - 2 % (w/v) glucose
0.17 % YNB
0.5 % (w/v) $(NH_4)_2SO_4$ ammonium sulfate
10 ml/l amino acids (100x)
pH 6.0

YNO-Medium

0.1 % (w/v) glucose
0.67 % YNB
0.5 % (w/v) ammonium sulfate
10 ml/l amino acid mixture (100x)
0.1 % (w/v) yeast extract
0.1 % oleic acid
0.05 % Tween 40
pH 6.0

100x amino acid mixture for YNO

L-adenine	400 mg/100 ml
L-histidine	200 mg/100 ml
L-leucine	600 mg/100 ml
L-lysine	300 mg/100 ml
L-tryptophan	400 mg/100 ml
uracil	200 mg/100 ml

100x amino acid mixture for SD

L-isoleucine	30 mg/100 ml
L-valine	150 mg/100 ml
L-adenine	20 mg/100 ml
L-arginine	20 mg/100 ml
L-Histidine	20 mg/100 ml
L-leucine	100 mg/100 ml
L-lysine	30 mg/100 ml
L-methionine	20 mg/100 ml
L-phenylalanine	50 mg/100 ml

L-threonine	200 mg/100 ml
L-tryptophan	20 mg/ml
Uracil	20 mg/ml

2.4 Antibodies

Antibody again	Concentration	Source
His_6-Pex7p	1:1000	This work
MBP-Pex13p-SH3	1:10000	AG R. Erdmann
His_6-Pex14p	1:5000	This work
His_6-Pex5p	1:5000	AG R. Erdmann

2.5 DNA and Vector plasmids

DNA/Plasmid Name	Description	Primers	Restriction Sites	From
S.c. genomic DNA				Promega
pBluescript SK+				lab
pGEX-4T2				lab
pPC86				lab
pPC97				lab
pRSET				Invitro Gen
pQE31, pQE41				Qiagen
pQE31ScPex13SH3-I	ScPex13p (309–370)	RE162, RE163	KpnI, HindIII	this work
pQE41ScPex13SH3-I	ScPex13p (309–370)	RE162, RE163	KpnI, HindIII	this work
pGEX-4T2-ScPex5	ScPex5p (full)	RE248, RE249	BamHI, EcoRI	this work
pGEX-4T2-ScPex13SH3-I	ScPex13p (309–370)	RE162, RE163	SalI, NotI	this work
GST-6xHis-SH3	ScPex13p (309–370)	RE481, RE482	BamHI, EcoRI	this work
pQE31ScPEX7	ScPex7p (full)	RE79, RE80	SphI, PstI	this work
pRSETScPex14	ScPex14p (full)	RE87, RE88	NheI, EcoRI	this work
pRSETScPex5	ScPex5p (full)	RE77, RE78	BamHI, HindIII	this work
pPC97-SH3-1	ScPex13p (286-386)	RE160, RE161	BamHI, SalI	this work
pPC97-SH3-2	ScPex13p (309-370)	RE162, RE163	BamHI, SalI	this work
pPC86ScPEX5	ScPex5p (full)			(Albertini et al., 1997)
pPC86ScPEX5F1	ScPex5p (1-312)	RE191, RE194	EcoRI, SpeI	this work
pPC86ScPEX5F2	ScPex5p (1-180)	RE191, RE192	EcoRI, SpeI	this work
pPC86ScPEX5F3	ScPex5p (181-312)	RE193, RE194	EcoRI, SpeI	this work
pPC86ScPEX5F4	ScPex5p (181-240)	RE319, RE320	EcoRI, SpeI	this work
pPC86ScPEX5F5	ScPex5p (241-312)	RE321, RE322	EcoRI, SpeI	this work
pPC86ScPEX5F6	ScPex5p (201-226)	RE323, RE324	EcoRI, SpeI	this work
pPC86ScPEX14	ScPex14p (full)			(Albertini et al., 1997)

2.6 Primers for PCR

```
RE160:   GGGGTACCGTCGACAATCGAAGGTCGTCTACAGACCTCTGGAAC
RE161:   GCGGATCCAAGCTTCTAGTGTGTACGCGTTTC
RE162:   GGGGTACCGTCGACAATCGAAGGTCGTTTTGCAAGAGCGTTATATG
RE163:   GCGGATCCAAGCTTTCATATGATCTCAATATAGTTATACG
RE191:   GGAATTCAAATGGACGTAGGAAGTTGC
RE192:   GCTCTAGAACTAGTTCACGATTGGAGCCTTGATCC
RE193:   GGAATTCAACCTGCTTTCATGAATCAAC
RE194:   TCTAGAACTAGTTCAATTATTAAAATATTCGTTG
RE248:   CGGGATCCATGGACGTAGGAAGTTGC
RE249:   CGGAATTCTCAAAACGAAAATTCTCCTTTAAATC
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RE320:   GCTCTAGAACTAGTTCATTCTGGTTTGTTTTGTTCTAC
RE321:   GGAATTCAAACTGTTGAGAAGGAAGAAG
RE323:   GGAATTCAACAACAACCCTGGACAGAT
RE324:   GCTCTAGAACTAGTTCACTCTATTTCATCATTTATGTCC
RE481:   GCGGATCCCATCATCATCATCATCATTTTGCAAGAGCGTTATATG
RE482:   CGGAATTCTCATATGATCTCAATATAGTTATACG
```

2.7 Molecular Biological Methods

2.7.1 Standard PCR Protocols

Polymerase chain reaction was used for producing high quantities of copies of DNA molecules from a given DNA template. The main components necessary to make new strands of DNA in the PCR are: 1x reaction buffer, 200mM deoxynucleotides (equal amount of each NTP), 0.2-1.0 mM of primers and 0.01-1 ng of plasmid or phage DNA and 0.1-1 mg of genomic DNA. Primers are usually 18-25 nucleotides in length and their GC content should be 40-60%. PWO polymerase has proofreading activity, which guarantees little mutation in the PCR product, therefore it was used for preparation PCR

PCR reaction components (for 50 µl volume):

1x PCR-buffer (10x)
0,2 mM dNTP
25 pmol oligo nucleotide (sense/antisense)
20-100 ng DNA template
1-2 U Pwo bzw. Taq-DNA-Polymerase
Adjust to 50 µl with Millipore-Water

General program for PCR:

Step	Reaction	Time	Temperature
1	denaturation of DNA	5 min	94°C
2	denaturation of DNA	1 min	94°C
3	Anealing	90 sec	40-60 °C according to the primers
4	elongation	90-120 sec	72°C
5	elongation	10 min	72°C
6	Ende		4°C
	Cycling of step 2-4 for 30 times.		

Estimation of annealing and melting temperature is calculated using the following formula:

$Tm = 4(G + C) + 2(A + T)$

Annealing temperature should be approximately 5°C lower than the melting temperature.

Solutions and Reagents for Standard PCR

2 mM dNTP's solution in sterile water, 10x reaction buffer for *Pwo* polymerase-incomplete, 25 mM $MgSO_4$, 214 mg/ml *S. cerevisiae* genomic DNA (Promega), *Pwo* polymerase (2.5 U/ml)

2.7.2 Restriction of DNA

The restriction of DNA by restriction endonucleases was carried out according to the optimized condition of the endonucleases described by the providers. Ribonuclease was also added into the reaction mixture containing RNA, for example, if the substrate DNA to be digested was isolated from *E. coli* cells by miniprep without treatment with RNAse.

2.7.3 Ligation of DNA Fragments

DNA ligations include DNA fragments and cloning vectors with appropriate sticky or blunt ends, which are incubated in the presence of ligation buffer (containing ATP) and of 1 U of T4 DNA ligase. For a successful ligation the ratio of insert to vector of 3:1 was maintained. If needed, vectors were previously treated with 1U of CIAP for 25 min at room temperature to prevent their religation. After dephosphorylation, vectors were purified by ethanol precipitation to remove remaining phosphatase, which could interfere with the ligation. The best ligation efficiency was achieved by incubation of samples overnight at 16°C in a thermocycler in a total reaction volume of 20 μl. After ligation, the reaction mixture could be directly used for transformation in *E.coli*.

2.7.4 Preparation of $CaCl_2$ Competent Cells

Competent cells were prepared as suggested in (Ausubel M. Frederick, 1987). LB medium was inoculated a colony of *E. coli* (C41) and the culture was left to grow overnight at 37°C. 1/100 volume of the overnight culture was inoculated to LB medium. The cells were grown at 37°C, shaking at 250 rpm, until OD_{590} reached 0.4. After cell density reached OD_{590} of 0.4-0.5, the cells were left on ice for 5-10 minutes. The cells were centrifuged for 7 minutes at 1600 x g at 4°C. The supernatant was discarded and the pellets were resuspended in 10 ml ice-cold $CaCl_2$ solution. The cells were centrifuged for 5 minutes at 1100 x g, 4°C and the pellets were resuspended in 10 ml of ice-cold $CaCl_2$ solution. The cell suspension was kept on ice

for 30 min before centrifugation of 5 min at 1100g, 4°C. Supernatant was discarded and the pellets were resuspended in 2 ml ice-cold $CaCl_2$ solution.

Solutions and Reagents

L-Broth (autoclaved):	**$CaCl_2$ solution- pH =7 (autoclaved):**
1% tryptone 0.5% yeast extract 1% NaCl (pH = 7-8)	15% glycerol 60 mM $CaCl_2$ 10 mM PIPES*

*PIPES - [piperazine-N,N'-bis(2-hydroxypropanesulfonic acid)].

2.7.5 Transformation in E.coli

The $CaCl_2$ method was used to make *E.coli* competent cell. 1-10 ng of plasmid DNA was added to 30-100 µl aliquots of $CaCl_2$ competent cells. The cells were gently mixed with the plasmid DNA and left on ice for 10 - 30 min. A heat shock was performed at 42°C for 90 s. Cells were placed on ice for 1-2 min before they were plated on antibiotic selective LB plates.

Solutions and Reagents

SOC Medium (autoclaved):	**LB- plates (pH = 7-8) -autoclaved**
2% Bacto tryptone 2.5 mM KCl 0,5% Bacto yeast extract 10 mM $MgSO_4$ 10 mM NaCl 20 mM glucose 10 mM $MgCl_2$	1% tryptone 2% agar 0.5% yeast extract 1% NaCl Antibiotic*

*appropriate antibiotics were added into LB after it was cooled to 55°C.

Ampicillin stock solution: 10 mg/ml – ampicillin in 1 M Tris, pH = 8.0 and filter sterilized.

Kanamycin stock solution: 5mg/ml – kanamycin in distilled water - filter sterilized.

-*E. coli* plates were incubated at 37°C for 1-2 days.

2.7.6 Plasmid Isolation from E.coli by NaOAc and Ethanol Precipitation

Pure plasmid DNA of samples selected for sequencing and for transformation in yeast was isolated from *E. coli* using peqlab kit. NaOAc and ethanol precipitation was used to isolate plasmids of newly made constructs for subsequent restriction test. Plasmid isolation by NaOAc and ethanol precipitation is described below:

A single clone was inoculated to 3 ml of LB medium and incubated overnight at 37°C, at 250 rpm. 1.5 ml of overnight culture was transferred into an eppendorf tube and the cells were centrifuged for 30 sec at 14,000 rpm. All supernatant was removed and the pellets were resuspended in 100 µl of Solution I. 200 µl of Solution II were added to the cell suspension and mixed gently until the suspension became clear. 150 µl of Solution III were added to the cell lysate and mixed well until white protein precipitate formed. The samples were centrifuged at 13,000 rpm for 10 min at 4°C. The supernatant was carefully transferred to a clean Eppi to which 1 ml of ice-cold ethanol was added. The sample was mixed and centrifuged at 14,000 rpm for 8 min at 4°C. The supernatant was aspirated and the pellet was mixed with 250-500 µl of 70% ice-cold ethanol. The sample was centrifuged for 5 min at 13,000 rpm, at 4°C. The supernatant was removed and the pellets were dried in Spin Vac. 1 µl of the plasmid prep. was used for restriction test.

Solutions and Reagents

Solution I:	**Solution II:** (prepared fresh)	**Solution III:**
10 mM Tris/HCl (pH = 7,9) 10 mM EDTA 50 mM glucose	2M NaOH, 01% (w/v) SDS	5 M KOAc, 10% (v/v) acetic acid (pH= 4,8 adjusted with HCl)

2.8 DNA Analysis Methods

2.8.1 Agarose Gel Electrophoresis

DNA fragments were separated using agarose gels. Agarose gels contained 0.8% to 2% agarose (depending on the size of DNA fragments) dissolved in 40 ml of TBE buffer, into which 20 l (0.5 g/ml) -mercaptoethanol was added. DNA samples were mixed with DNA-sample buffer and loaded on a gel covered with TBE buffer. Electrophoresis was performed at 100-120V. Detection of DNA bands was performed by UV light. Size of the DNA was determined using standard - and 100 bp Marker.

Solutions and Materials

10x TBE Buffer	**10x DNA-Sample buffer**	**Agarose-Gel**
900 mM Tris	50 % (w/v) glycerol	0.6-3% Agarose

900 mM boric acid 20 mM EDTA, pH = 8.0	0.1 M EDTA 0.5 % (w/v) bromphenolblue	1x TBE buffer 0.4 µg/ml ethidium bromide

2.8.2 Isolation of DNA-Fragments from Agarose Gels Using QIAEX II Kit

QIAEX II Agarose Gel Extraction Kit was used for isolation of DNA fragments, after PCR or after restriction digestions. DNA fragments were separated by electrophoresis, identified by UV light and excised from the for subsequent purification. After the ose gel was melted, DNA could adhere to special silica matrix that enabled separation of DNA from salts and other impurities contained in the solution. Elution of DNA was performed with 1xTE or with sterile water.

2.9 Protein Analysis Methods

2.9.1 SDS-PAGE

SDS –PAGE was used for proteins separation under denaturing conditions that ensured protein migration according to their molecular weight. A discontinuous buffer system was used in order to achieve better resolution of protein bands. The experimental procedure was performed according to Laemmli (1970) protocol. Samples were boiled for 10 min at 95°C in 1 x SDS-Sample Buffer before they were loaded on the gel. Gels were run at 20 mA/gel and at 200 V as maximal limit. Electrophoresis was performed for 45 min-1 hour. The gels were stained with Coomassie Brilliant Blue for 30 min at room temperature with agitation. Destaining was accomplished with methanol : water : acetic acid mixture, with agitation. Molecular weight of proteins was determined using Protein Marker (Low-Range): (kDa) 97, 66, 45, 31, 21, and 14.5

Solutions and Materials

SDS-Sample Buffer:	**Coomassie Brilliant Blue:**	**Destaining Solution**
62.6 mM Tris/HCl, pH = 6.8 1% (w/v) bromphenolblue 3% (w/v) SDS, 5% (v/v) -mercaptoethanol 10% (v/v) glycerol	0.1% (w/v) Coomassie-Blue R 250, 10% Acetic acid 30% Methanol	35% methanol 10% acetic acid

Running Buffer:	Stacking Gel:	Resolving Gel:
25 mM Tris, 0.19 M Glycine 0.1% (w/v) SDS	4% acrylamide 60 mM Tris pH = 6.8 0.1% SDS, 0.001% BPB 0.06 % APS, 0.1 % TEMED	10% or 12% acrylamide 320 mM Tris pH = 8.8 0.1% SDS 0.06% APS, 0.1% TEMED

2.9.2 Western Blot

Immunoblotting was employed for detection and identification of proteins using antibodies. Separation of proteins by SDS-PAGE was followed by their transfer from the gel onto a nitrocellulose membrane. The transfer cassette was assembled according to the Biorad instructions and protein transfer took place at 300 mA for 1-1.5 hours in a chilled transfer buffer. Protein transfer was confirmed by staining the nitrocellulose membrane with amido black. At this point, marker bands were marked with a pen to locate them during immunodetection. The membrane was incubated in blocking solution, 2 x 15 min with agitation. Immunodetection was performed with primary and secondary antibodies, according to the standard lab protocol. The antibodies bound to the membrane were detected by ECL- Western Blot detection system (Amersham) based on chemiluminescence. Exposing the membrane to photographic film could detect light produced at the site of the target protein. Films were developed using a Konica Developer (SRX 101A).

Antibodies

Primary Antibody	Dilution	Secondary Antibody	Dilution	Protein
Anti-ScPex14p	1:5000	ECL-anti rabbit- IgG	1:10000	GST-ScPex14p
Anti-ScPex7p	1:1000	ECL-anti rabbit- IgG	1:10000	His-ScPex7p
Anti-ScPex13p	1:5000	ECL-anti rabbit-IgG	1:10000	GST-SH3

Solutions and reagents

Transfer Buffer	Amido-Black	Wash Buffer	Blocking Solution
20 mM Tris (pH=9.0) 150 mM glycine 0.05% (w/v) SDS 20 (v/v) methanol	45% (v/v) methanol 10% (v/v) acetic acid 0.1% (w/v) amidoblack	20 x PBS 20% Triton-X100 10% (w/v) SDS	5% (w/v) nonfat dry milk in wash solution

2.9.3 Protein Concentration Assay

2.9.3.1 Spectroscopic Determination

The protein concentration has been determined by measuring absorbance at 280 nm (A_{280}) is based on the absorbance of UV light by aromatic amino acids in protein solutions – primarily due to tryptophan and tyrosine residues and to a lesser extent by phenylalanine residues. The measured absorbance of a protein sample solution was used to calculate the concentration by comparison with a calibration curve prepared from measurements with standard protein solutions. The protein concentration of a sample to be assayed with this method should be in range from 20 to 2000 µg/ml.

If necessary the protein sample to measure was diluted so that the absorbance A_{280} ranges from 0.2 to 1.5, then the concentration of the sample can be calculated as following:

$$\text{Concentration (mg/ml)} = A_{280} / (a_{280} \times b)$$

Where b is the path length in centimeter which the UV light travels through the sample, usually b is 1 cm, depending on the cuvette used.

2.9.3.2 Colorimetric Determination of Protein Concentration by Pierce BCA kit

This assay exploits the biuret reaction in which Cu^{2+} is reduced by proteins in an alkaline medium, then the cuprous cation (Cu^{1+}) produced by this reaction is detected colorimetrically by a UV photometer.

Protein concentration was measured with the standard protocol in the manual of Pierce BCA "Protein Assay Kit". BSA was used as the standard protein.

2.9.3.3 Colorimetric Determination of Protein Concentration by Bio-Rad Protein Assay

The Bio-Rad Protein Assay is a dye-binding assay in which a differential color change of a dye occurs in response to various concentrations of protein. The absorbance maximum for an acidic solution of Coomassie Brilliant Blue G250 dye shifts from 465 nm to 595 nm when binding to protein (especially arginine, basic and

aromatic amino acid residues); therefore the absorbance at 595 nm allows determining the concentration of the protein sample.

The standard protocol proposed by Bio-Rad was used with BSA as standard reference.

2.10 Protein Over-expression in E. coli and Solubility Test

Preparation of proteins fused to certain tags (for ex. GST) under native conditions was performed according to the QIAGEN protocol. Protein expression and solubility was tested using SDS-PAGE and immunoblot analysis.

2.10.1 Overexpression of Protein in *E. coli*

Plasmids were first transformed into BL21(DE3) *E. coli* strain and plated on selective LB plates with certain antibiotics. 5 ml of LB medium containing appropriate antibiotics was inoculated with a single clone and the culture was grown overnight at 37°C with agitation. The overnight culture was transferred into 50 ml of antibiotic selective LB medium to OD_{600} = 0.2. The cells were incubated at 37°C with shaking until they reached OD_{600} = 0.5-0.6 (approximately after 4 hours). At this point, 1 ml aliquot was taken for SDS-PAGE (non-induced; control). At OD_{600} = 0.5-0.6, cells were induced by adding IPTG to a final concentration of 0.6 mM. The culture was grown for additional 3-4 hours at room temperature and a second 1 ml sample was aliquoted for SDS-PAGE (induced). The cells were harvested by centrifugation at 4000 x g for 20 min.

2.10.2 Determination of Protein Solubility in E. coli

The cell pellet was resuspended in 5 ml of lysis buffer containing 1 mg/ml lysozyme and one tablet of protease inhibitors. Cells were incubated for 30 min at 4°C. Samples were sonicated 4 x 15 sec. The lysate was placed on ice during sonication and kept in liquid nitrogen for 10 sec between sonications. 50 µl of lysate (homogenate) representing total protein content was separated for SDS-PAGE total protein content. Lysates were centrifuged for 25 min at 10000 g, at 4°C (Sorvall, SLA3000). 50 µl of supernatant representing soluble protein fraction was removed and mixed with 50 µl of 2 x SDS- Sample Buffer. The pellets representing insoluble

protein fraction were resuspended in 5 ml of lysis buffer by sonication and 50 µl of suspension was mixed with 2 x SDS- Sample Buffer. 10 µl of each sample was tested by SDS-PAGE.

Solutions and Reagents

Lysis Buffer:

50 mM NaH_2PO_4, 300 mM NaCl, 5mM Imidazole, pH = 8.0, Complete- Protease Inhibitor Coctail (Roche)

LB medium (see section 2.3.1)

SDS PAGE running buffer (see section 2.9.1).

2.11 Isolate protein out of cell lysate

2.11.1 Purification of GST Fusion Protein

The *E. coli* cells expressing GST fusion SH3 domain were lysed by combination of freezing in liquid nitrogen and sonication. The soluble expressed GST fusion protein was isolated from the cell debris by centrifugation at 10 000 g for 10 minutes. The solution containing the target protein was transferred into a column filled with GST Sepharose, which had been equilibrated with PBS buffer. The GST Sepharose bound with GST fusion protein was then washed with 3 volume the wash buffer, and then the GST protein was eluted with elution buffer.

GST Column Buffer: 1X PBS

Item	Concentration	weight for 500ml
NaCl	1.4 M	40.9 g
KCl	27 mM	1.006 g
Na_2HPO_4	101 mM	8.988 g
KH_2PO_4	18 mM	1.224 g

pH = 7.3

Elution buffer

Item	Concentration	weight for 50ml
Reduced Glutathione	10 mM	0.154 g
Tris-Cl (pH 8.0)	50 mM	2.5 ml 1 M Tris-Cl (pH8.0)

2.11.2 Purification of His-tagged Protein under Native Condition

Purification of His-tagged protein under native conditions is preferred in this work, because we want to wok with the naturally folded native SH3 domain to illustrate its stereo structure. However, in native conditions the potential for unrelated, nontagged proteins to interact with the Ni-NTA resin is relative high, it may be necessary to optimize the following protocol for individual target proteins.

The cell lysate has been prepared at 4 °C by the combination of treatments with lysozyme and sonication, protease inhibitors were added when necessary. The cell debris was removed from the soluble His-tagged target protein by centrifugation at 10000 g for 10 minutes; the transparent supernatant was transferred to a column filled with Ni-NTA Sepharose, which was equilibrated with the column buffer; the Ni-NTA matrix loaded with the target protein was washed with 3 volume of wash buffer. The target protein was then eluted with elution buffer.

Column buffer

Item	Concentration	weight for 100ml
NaH_2PO_4	50 mM	0.6 g
NaCl	300 mM	1.753 g
imidazole	10 mM	68.1 mg
Tween 20	0.05%	50 µl

Adjust pH to 8.0 using NaOH.

Wash buffer

Item	Concentration	weight for 50ml
NaH_2PO_4	50 mM	0.6 g
NaCl	300 mM	1.753 g
imidazole	20 mM	136.2 mg
Tween 20	0.05%	50 µl

Adjust pH to 8.0 using NaOH.

Elution buffer

Item	Concentration	weight for 50ml
NaH_2PO_4	50 mM	0.6 g

NaCl	300 mM	1.753 g
imidazole	250 mM	1.7 g
Tween 20	0.05%	50 µl

pH adjusted to 8.0 with NaOH.

2.11.3 Purification of MBP Tagged Proteins

The amylose resin (amount depending on the amount of the fusion protein to purify) in a 2.5 x 10 cm column was equilibrated by washing with 8 column volumes of column buffer. After loading with the cell lysate, the column was washed again with 8 column volumes of column buffer, the fusion protein was then eluted by elution buffer. The eluate was fractioned into 0.5 ml fractions which were analyzed by SDS page.

Column Buffer	**Elution Buffer**
20 mM Tris-HCl	20 mM Tris-HCl
200 mM NaCl	200 mM NaCl
1 mM EDTA	1 mM EDTA
Optional:	10 mM matose
1 mM azide	1 mM azide
10 mM β-ME	
or 1 mM DTT	

2.12 Purification of Proteins with Physical Methods

2.12.1 Centricon Filter Memberane

All the Centricon filter member tubes in this work are used according to the manual of the manufacturer. The appropriate volume of the sample was loaded onto the membrane and then the filter tubes were centrifuged in the SS34 rotor (Sorval) at 4000 rpm to concentrate the protein.

2.12.2 Gel Filtration Chromatography

Sephadex G-75 , with a fractionation range of 3 – 80 kDa, was used separate the SH3 domain (ca. 7 kDa) from Factor Xa (42,324 kDa, which appears on SDS page as two bands of 34 kDa and 29 kDa). PBS buffer was used as liquid phase and the flow speed of the buffer was kept constant by a motor pump; the flow-through of the column was fractioned into 1 ml fractions which were analyzed by SDS page. The procedure was carried out at 4 °C.

2.13 Protease Digestion

2.13.1 Digestion of Proteins with Factor Xa

The proteinase Factor Xa can cut any proteins containing its recognition sequence IleGluGlyArg after Arginine. The protein containing the recognition sequence for Factor Xa was solved in the cleavage buffer, and factor Xa was added in ratio of 1:100 of factor Xa to substrate. The cleavage was complete after incubation at room temperature for 16 hours.

Factor Xa cleavage buffer proposed by Novagen

Item	Concentration	weight for 100ml
Tris-HCl	50 mM	0.54 g
NaCl	100 mM	588 mg
$CaCl_2$	5 mM	1.7 mg

pH 7.5.

2.13.1.1 Preparation of ”Factor Xa Away” Resin

The slurry of “Factor Xa away” resin (Xarrest resin) was centrifuged at 1000 g for 5 min in a desktop centrifuge and the storage buffer for Xarrest resin was discarded carefully. Then the Xarrest resin was suspended and washed with 10 X resin volume (100 µl resin corresponds 200 µl slurry) of the capture buffer. After equilibration and wash the Factor Xa Away resin was ready to use.

2.13.1.2 Removal of Factor Xa by Xarrest Agarose

Under the optimal condition (the digested protein mixture in 1X Factor Xa Cleavage/Capture Buffer), 50 ml settled resin (100 ml of the 50% slurry) can remove 4 units of enzyme factor Xa in the digestion mixture. Recovery of the cleaved target protein in the liquid phase was achieved by the supplied Spin Filters, which separated the liquid phase from the Xarrest Agarose. Two or more capture steps were used to purify the SH3 domain.

Capture/Cleavage Buffer

Item	Concentration	weight for 100ml
Tris-HCl	50 mM	540 mg
NaCl	100 mM	588 mg

$CaCl_2$	5 mM	1.7 mg

pH 8.0.

2.13.2 Protein Digestion with Thrombin

Thrombin is an endoprotease that naturally functions as a blood clotting factor to convert fibrinogen to fibrin. Human thrombin is one of the most active site-specific proteases, which is an advantage because a very low mass ratio of enzyme to target protein is needed for efficient cleavage. Although no single consensus sequence describes the thrombin cleavage site, cleavage frequently occurs after the proline-arginine residue pair when properly exposed in a three-dimensional structure.

Thrombin exhibits a very low non-specific cleavage rate under many conditions. A 1:2000 wt:wt ratio of thrombin to target protein (equivalent to one unit per milligram of target protein) is generally sufficient for cleavage in 1X Thrombin Cleavage/Capture Buffer at 20° C for 16 h. However, because each target protein presents the cleavage site somewhat differently, the digestion condition, especially thrombin concentration, temperatures and incubation times, may need to be optimized.

Thrombin Cleavage Buffer

Item	**Concentration**	**weight for 100ml**
Tris-HCl	20 mM	540 mg
NaCl	150 mM	588 mg
$CaCl_2$	2.5 mM	1.7 mg

pH 8.4.

2.13.2.1 Biotinylated Thrombin Capture

After the cleavage reaction, biotinylated thrombin was quantitatively removed with Streptavidin Agarose. One unit of enzyme biotinylated thrombin was successfully removed by 16 µl settled resin (32 µl of the 50% slurry), more than 99% of the enzymatic activity is captured by Streptavidin Agarose in 30 min incubation. The target protein was recovered by spin-filtration.

2.14 Protein Interaction

2.14.1 Two-Hybrid System

2.14.1.1 Two-Hybrid Transformation

The yeast transformation was performed essentially according to the modified Lithium acetate method by Gietz and Sugino (Gietz and Sugino, 1988). For the two-hybrid analyses, fresh yeast cell colonies of the reporter strain FH7c (or PCY2) (not older than 2 weeks) were grown on YPD medium at 30 °C till the OD_{600} of the cell culture reached 0.5; the cells were harvested by centrifugation at 1000 g for 5 minutes, washed with TE buffer, and then suspended in freshly prepared sterile 1X TE/LiAc. One µg of each of the two plasmids NDA encoding the test protein (or protein fragment), 30 µg fish DNA and 100 µl of competent yeast cells were mixed and 600 µl PEG/LiAc solution was added. This mixture was thoroughly mixed and incubated at 30 °C for 30 min. After incubation, 60 µl DMSO was added and mixed carefully, followed by a 2 minutes heat shock at 42 °C. After the heat shock, the cell mixture was cooled on ice and centrifuged at 15 000 g for 5 seconds. The cell pellet was suspended in TE buffer and plated on selective SD medium plates without tryptophan and leucine, which are the two selective markers for the two plasmids respectively. β-galactosidase activity or expression of the His3 protein was exploited to detect whether the two proteins to be tested can interact with each other. To measure the β-galactosidase activity the yeast cells containing two plasmids were lysed on a cellulose filter and incubated with X-gal as a substrate for β-galactosidase at 30 °C (Fields and Song, 1989); to monitor the expression of His3 protein the yeast host cells were grown on selective medium without histidine, where 2 - 5 mM 3-AT was used to inhibit the leaky HIS3 expression.

Solutions and Reagents

PEG/LiAc/TE	10xTE (pH=7.5)	10xLiOAc (pH=7.5)
40% PEG 1xTE, 1x LiAc	0.1M Tris-HCl 10 mM EDTA	1M LiAc *adjust pH with CH_3COOH

Amino Acid Stock Solution (10x)

L-amino acid	**mg/l**	**L-amino acid**	**mg/l**
Isoleucine	300	Methionine	200
Valine	1500	Phenylalanine	500
Adenine (Hemisulphate Salt)	200	Threonine	2000
Arginine HCl	200	Tryptophane	200
Histidine HCl Monohydrate	200	Tyrosine	300
Leucine	1000	Uracil	200
Lysine HCl	300		

YPD (pH=6.)	**SD-Medium (pH=6.0)**
2%Select Peptone 1% yeast extract 2% glucose	0.17% yeast nitrogen base 0.5% Ammonium sulphate 100 ml of 10x amino acid sol.
*pH was adjusted with KOH *YPD and SD plates contained additional 2% agar * PJ69-4a cells require 0.003 % adenine which is added to the medium	

2.14.1.2 β-galactosidase Filter Assay

The yeast strain PCY2 was simultaneously transformed with two plasmids and the cells were grown on SD-trp-leu plates. Three clones were streaked on SD-trp-leu plates and were grown for 2-3 days before they were tested for β-galactosidase activity by the filter assay (Fields and Song, 1989). The cells were transferred to the sterile filters by stamping, lysed by freezing in liquid nitrogen and thawed again at room temperature. After the filters were thawn, cell lysates were incubated in the Z-buffer/x-gal solution, protected from light, for 0.5-8 hours or until the blue color of the positive control developed. The blue color indicates that the fusion proteins expressed from the two plasmids physically interact and activate the transcription of a lacZ fusion gene in which β-galactosidase is under the control of a GAL4 operator.

Z-Buffer (pH = 7.0)	**X-gal Stock Solution**	**Z-Buffer/X-gal Solution**

16.1 g/l Na_2HPO_4 x 7 H_2O 5.5 g/l Na_2HPO_4 x H_2O 0.75 g/l KCl 0.246 g/l $MgSO_4$ x 7 H_2O	20mg X-gal in DMF (store in dark)	100 ml Z-Buffer 0.27 µl β- mercaptoethanol 1.67 ml X-gal solution

2.14.1.3 Construct of ScPex5p, ScPex13p and ScPex14p used in the TH Analyses

The complete ScPex5 ORF was amplified out of the *Saccharomyces cerevisiae* genomic DNA by the Polymerase Chain Reaction with the primers RE248 and RE249. The amplified DNA fragment was then cloned into pSK+ plasmid servicing as the DNA template for the cloning of all the following ScPex5 fragments. For two-hybrid analysis the following fragments of ScPex5p: ScPex5pF1 (1-312aa, PCR primers: RE191, RE194), ScPex5pF2 (1-180aa, PCR primers: RE191, RE192), ScPex5pF3 (181-312aa, PCR primers: RE193, RE194), ScPex5pF4 (181-240aa, PCR primers: RE193, RE320), ScPex5pF5 (241-312aa, PCR primers: RE321, RE194), ScPex5pF6 (201-226aa, PCR primers: RE323, RE324), were cloned into pPC86 (GAL4 activation domain fusion vector) by EcoRI and SpeI, which were introduced into these ScPex5p fragments by the corresponding primers; while the ScPex13p fragments ScPex13pSH3 (309-370aa) and ScPex13pSH3F (285-386aa) were amplified by the Polymerase Chain Reaction with the primers RE162, RE163 and RE160, RE161 respectively, then were restricted with BamHI, SalI, which were introduced into the PCR products with the corresponding primers. These PCR fragments digested with the restriction enzymes were then cloned into pPC97 (DNA binding domain fusion vector) between the restriction sites of BglII and SalI.

2.14.2 Biacore Measure

The experiments to detect interactions between proteins and to measure the binding constants of the interaction of the proteins by biosensor method were carried out by using the BIAcore JM45 equipment.

2.14.3 Spot Blot Scanning

2.14.3.1 Synthesis of Peptides on Membrane

Cellulose membranes were used as supporting base for spots containing certain peptides. The hydroxyl-groups of the cellulose were activated by chemical treatment. Subsequently, the activated hydroxyl-groups on the certain position of the membrane were linked with a β-alanine by the dehydration reaction. These β-alanines were linked to another β-alanine as a start point for the peptide synthesis, guided by certain peptide sequences.

2.14.3.2 Ligandblot Analysis

The spot membrane was washed 3 times with 10 ml TBS buffer and the unspecific binding sites on the membrane were blocked by incubation with 10 ml blocking buffer for 10 hours. Then the membrane was washed again with 10 ml TBS for 10 min. After this treatment, the membrane was incubated with targeting protein, fused to GST, in 10 ml TBS buffer with a concentration of ca. 30 µg/ml for 10 hours, then was washed 3 times with 10 ml TBS buffer. Subsequently, the membrane was incubated with mono-colonial antibody against GST in 10 ml TBS buffer for 3 hours, washed 3 times with TBS buffer, following an incubation with the second antibody in 10 ml TBS for 1 hours. Finally, the membrane was photo-chemically activated with ECL solution and then the digital image of the luminescence spot on the membrane was captured.

TBS (Tris-Buffered-Saline)	**T-TBS**	**Blocking Buffer**
50 mM Tris	50 mM Tris	10 % Blocking Reagent in T-TBS
137 mM NaCl	137 mM NaCl	10 % (w/v) Saccharose
2.7 mM KCl	2.7 mM KCl	
pH 8.0 mit HCl	0.05 % Tween 20	
	pH 8.0 mit HCl	