2. Materials and Methods

2-1. Chemicals and auxiliary enzymes

Fine chemicals such as sugars, antibiotics, acrylamide-bisacrylamide and buffer components, medium for culture of bacteria, agar were purchased from Carl Roth GmbH (Karlsruhe), substrates for FSA or TAL including sugar phosphates and inorganic salts were from Sigma-Aldrich/Fluka (München), auxiliary enzymes for photometrical analysis were from Roche Applied Science (Mannheim). Restriction enzymes for DNA cloning were from MBI fermentas (St.Leon-Rot). Anion-exchange column matrices, Q-Sepharose and MonoQ, gelfiltration matrix Superdex 200pg, hydrophobic interaction column matrix Phenyl Sepharose Fast Flow were from Amersham Biosciences/GE Healthcare (Freiburg), hydroxyapatite column matrix was from Biorad (München), Histidine-tag affinity column matrix Nickel-NTA Superflow was purchased from Qiagen (Hilden).

2-2. Bacterial strains, plasmids and oligonucleotides

Bacterial strains and plasmids in the study are listed in Table.2-1 and 2-2, respectively. Several bacterial strains or commercial plasmids were purchased from Invitrogen (Karlsruhe) or Novagen (Darmstadt), the others were inherited in our laboratory or prepared in this study.

Table.2-1. *E.coli* strains used in this work

Strain	Genotype/relevant properties	Reference
DH5α	*F^-, ϕ80dlacZΔM15 Δ(lacZYA-argF)U169, deoR, recA1 endA1, hsdR17(r_k^-, m_k^+), phoA supE44, λ^-, thi-1, gyrA96, relA*1	Hanahan, 1983
BL21(DE3) pLysS	*F^-, ompT, hsdSB(r_B^-, m_B^-), gal, dcm (DE3), pLysS(Cam^R)*	Novagen

BL21(DE3) Star pLysS	*F⁻, ompT, hsdSB(r_B^-, m_B^-), gal, dcm (DE3), pLysS(Cam^R), rne131*	Invitrogen
XL1-Blue	*recA1, endA1, gyrA96, thi hsdR17(r_B^-, m_B^+), supE44, relA1 lac[F', proAB⁺, $lacI^q$ΔZM15::Tn10(tet^r)]*	Stratagene

Table.2-2. Plasmid DNAs utilized in this study

Plasmid	Relevant properties	Reference
pUC18/19	Amp^r, *lac*POZ', *ori* colE1	Vieira&Messing 1982
pET16b	P_{T7}, Amp^r, ori_{pBR322}, *lac*I, N-term. 10xHis	Novagen
pET22b	P_{T7}, Amp^r, ori_{pBR322}, *lac*I, C-term. 6xHis	Novagen
pET28b	P_{T7}, Kan^r, ori_{pBR322}, *ori* f1, *lac*I, T7 Tag N-term. 6xHis, C-term. 6xHis	Novagen
pJF119EH	Ptac, Amp^r, *lac*I,	Fürste et al., 1986
pUC18fsa	*fsa* (mipB) gene (740bp) cloned in pUC18 (PstI/SalI)	Me. Schürmann 2001
pUC18fsaQ59E	pUC18fsa modified at *175-177 bp of *fsa* gene	This study

*: The numbers indicate orders of bases on DNA sequence of *fsa*.
(*fsa* gene: A,T,G,G,A,A, . . . = 1, 2, 3, 4, 5, 6, . . .)

pUC18fsaY131A	pUC18fsa modified at *391-393 bp of *fsa* gene	This study
pUC18fsaY131F	pUC18fsa modified at *391-393 bp of *fsa* gene	This study
pUC18fsaA129S	pUC18fsa modified at *385-387 bp of *fsa* gene	Me. Schürmann 2001
pET16fsa	*fsa* gene (717bp) cloned in pET16b (NcoI/NdeI)	Me. Schürmann 2001
pET16fsaY131A	pET16fsa modified at *391-393 bp of *fsa* gene	This study
pET16fsaY131F	pET16fsa modified at *391-393 bp of *fsa* gene	This study
pET16fsaNhis	*fsa* gene (665bp) cloned in pET16b (NdeI/XhoI)	This study
pET22fsaChis	*fsa* gene (662bp) cloned in pET16b (NdeI/XhoI)	This study
pJF119fsa	*fsa* gene (663bp) + cutting site (394bp) cloned in pJF119EH (XbaI/HindIII)	This study
pUC19ywjH	*tal* (ywjH) gene from *B. subtilis* cloned in pUC19	Me. Schürmann 2001
pET22ywjH	*tal* (ywjH) gene (641bp) cloned in pET22b (NdeI/XhoI)	This study

pET16ywjHNhis	*tal* (ywjH) gene (641bp) cloned in pET16b (NdeI/XhoI)	This study
pET28ywjHChis	*tal* (ywjH) gene (644bp) cloned in pET28b (NcoI/XhoI)	This study
pETtktA	*tktA* gene from *E. coli* cloned in pET22b	Ma. Schürmann 2001
pET22chimera1	*fsa* gene (67bp), *tal* (ywjH) gene (605bp) cloned in pET22b (NdeI/XhoI)	This study
pET22chimera2	*fsa* gene (163bp), *tal* (ywjH) gene (512bp) cloned in pET22b (NdeI/XhoI)	This study
pET22chimera3	*fsa* gene (253bp), *tal* (ywjH) gene (422bp) cloned in pET22b (NdeI/XhoI)	This study
pET22chimera4	*fsa* gene (322bp), *tal* (ywjH) gene (353bp) cloned in pET22b (NdeI/XhoI)	This study
pET22chimera5	*fsa* gene (382bp), *tal* (ywjH) gene (293bp) cloned in pET22b (NdeI/XhoI)	This study
pET22chimera6	*fsa* gene (484bp), *tal* (ywjH) gene (191bp) cloned in pET22b (NdeI/XhoI)	This study
pET22chimera7	*fsa* gene (550bp), *tal* (ywjH) gene (125bp) cloned in pET22b (NdeI/XhoI)	This study
pET22chimera8	*fsa* gene (598bp), *tal* (ywjH) gene (77bp) cloned in pET22b (NdeI/XhoI)	This study

Oligonucleotides for cloning, mutagenesis and sequencing in this work are on Table.2-3.

Table.2-3. A list of oligonucleotides used in this study.

Name	Sequence (5' -> 3')	RE site
(mutagenesis of FSA)		
Q59E1	GGGGCGTCTGTTTGCCGAAGTAATGGCTACCACTGCCGAAGGG	
Q59Erev	CCCTTCGGCAGTGGTAGCCATTACTTCGGCAAACAGACGCCCC	
FSAY131A1	GCATCAATACGATTAACCGCAGGCGCAACATATTCCGCACC	
FSAY131A2	GGTGCGGAATATGTTGCGCCTGCGGTTAATCGTATTGATGC	
FSAY131F1	CCCTGAGCATCAATACGATTAACGAAAGGCGCAACATATTCCGC	
FSAY131F2	GCGGAATATGTTGCGCCTTTCGTTAATCGTATTGATGCTCAGGG	
(cloning of His-tagged FSA)		
FSAhisfor	CATTTTGAGGATGCATATGGAACTGTATCTGG	NdeI
FSAhisrev16	GCCTGCAGAACCTCGAGTTAAATCGACGTT	XhoI
FSAhisrev22	GAACGTCCTCGAGAATCGACGTTCTGCCAAAC	XhoI
(cloning of His-tagged TAL_{Bsu})		
woREfor	AGAGCGGGGGCAACCTATGTATCTCCATTCCTG	
woRErev	CAGGAATGGAGATACATAGGTTGCCCCCGCTCT	
ywjHfor2	GGCTCCATGGCCATGTTATTCTTTGTTGATACAGC	NcoI
ywjHrev2	CCCCTTCTCGAGTTTGTTCCAGTCTGCC	XhoI
ywjHfor3	CAGGGAGGCTTTTTGCATATGTTATTCTTTG	NdeI
ywjHrev3	GTTTGCCGCCCCTCGAGTTATTTGTTCC	XhoI
(error prone PCR)		
FSAstrtNcoI	GAGGATGGCCATGGAACTGTATCTGG	NcoI
(preparation of chimera enzymes)		
fsa a1	TGACAGCGCCTTCACCGCAACAACG	
fsa a2	CGCTTCATGAAGTTGCGGAAGCACAAC	

fsa a3	AATAATAGAACGCAGCTTAAGCGCGTC
fsa a4	TTCCGCTTTTAACATCTTAATAGCTGCCAG
fsa a5	CAGCGCCGACAGCAGCCCTTG
fsa a6	ATGCATTTTCAATAACTGGTGTAAGTCGG
fsa a7	CAGTAAGCAGTCCAGCGCCTGACG
fsa a8	CATCTGTTGTGCCACATCCAGTGGC
tal b2	GAATTAGGAATTCTCGCCGGTGTAACGACG
tal b3	GACGTCGTGAAAGGGTCTGTAAGCGC
tal b4	CCGAACATTACGGTGAAAATCCCAATGACG
tal b5	GGCATCAAAACAAACGTTACATTGATCTTCAATGCC
tal b6	GCGGGGGCAACCTATGTATCTCCATTC
tal b7	GGCCTTGACACGCAAATCATTGCAGCG
tal b8	AGAGGGGCTCATATCGGCACAATGCC
tal aC	ACTAAACACCCGTTAACAGACAAAGGAATCGAAC

(DNA sequencing for pET vector)

T7promotor	TAATACGACTCACTATAGGG
T7terminator	GCTAGTTATTGCTCAGCGG
pETupNde	TGTGAGCGGATAACAATTCCCCTC
pETdownBam	GTTATTGCTCAGCGGTGGCAGCAG

Red letters show target positions for alteration. “RE site” implies that the oligonucleotides had a cutting site with a restriction enzyme, indicated by blue color.

2-3. Culture medium and culture conditions

Luria-Bertani (LB) medium was utilized for culture of *E.coli*, transformation of cells and conservation of cells on agar plates. The medium was sterilized by autoclaving at 121°C for 20 min. Several antibiotics were added under sterile condition according to need. In case of a solid medium (agar plate), 1.5% (w/v) of agar was contained.

Culture medium

LB medium (Sambrook et al. 1989)

10g	tryptone
5g	yeast extract
5g	NaCl
add 1L	H_2O

Final concentrations of antibiotics

ampicillin-sodium salt	100 mg/L
chloramphenicol	25 mg/L
kanamycin-sulfate	25 mg/L

To obtain cells for plasmid isolation, a single colony was inoculated and cultured in 4mL LB medium at 37°C overnight.

Seed-cells for storage were prepared by mixing an overnight culture with a sterile glycerol (86%) at 1 to 1 ratio, and stored in a deep freezer at -70~-80°C.

2-4. Molecular biological methods

Synthesis of oligonucleotides was ordered from biomers. net GmbH (Ulm). Shrimp alkaline phosphatase was from Boehringer Mannheim (Mannheim), other molecular biological reagents except restriction enzymes were from Roche Applied Science (e.g. Taq DNA polymerase, Pwo DNA polymerases, DNA ligases, polynucleotide kinases including several kits). DNA sequencing was ordered from GATC Biotech GmbH (Konstanz).

2-4-1. Isolation and cloning of DNA

Plasmid DNA was purified with kits, "QIAprep Spin Mini Kit"(Qiagen) or "Nucleospin Extract"(MACHEREY-NAGEL GmbH &Co.KG (Düren)) from overnight cultures. Digestion of DNA was performed by incubation with restriction enzymes in a suitable buffer and at an optimal temperature. Linear DNA fragments were separated with agarose gel electrophoresis (0.8~1.2%(w/v) in 1x TAE buffer – 40mM Tris, 20mM acetic acid, 1mM EDTA). DNA samples were mixed with 1/5 volume of a loading buffer (24%(w/v) urea, 0.2%(w/v) EDTA, 50%(w/v) sucrose, 0.1%(w/v)

bromophenolblue) and run on agarose gel including 1 μg/mL of ethidiumbromide. To isolate DNA out of agarose gels and clean it up, "QIAprep Gel Extraction Kit"(Qiagen) or "Nucleospin Extract" from MACHEREY-NAGEL was utilized. To small DNA fragments as less than 100bp, "QIAEX II"(Qiagen) and an agarose "NuSieve 3:1 Agarose" (CAMBREX (New Jersey/USA)) were successfully adopted. If necessary, linear plasmid DNA or PCR products were dephosphorylated with alkaline phosphatase from shrimp. Ligation of DNA was performed with T4 DNA ligase or "Rapid DNA Ligation Kit" (Roche Applied Science).

2-4-2. Amplification of DNA fragments

For cloning of *fsa*, *tal* or parts of those genes to prepare for chimera enzymes, DNA fragments were amplified with PCR (Polymerase Chain Reaction, Mullis & Faloona, 1987). The components of a reaction mixture are shown below, each sample volume was 50 μL. Pwo DNA polymerase was utilized in the reaction due to high fidelity.

PCR components

Template DNA (plasmid)	10~30 ng	
primer 1	10~30 pmol	
primer 2	10~30 pmol	
dNTPmix	200 μM	
polymerase buffer	1 fold	
DNA polymerase	2~5 U	in 50μL

2-4-3. Transformation of cells

50~200 ng plasmid DNA was mixed with 200 μL of ice-cold competent cells and incubated for 30 min in ice bath. Further incubation for 10 min in ice bath followed "heat shock" treatment (45°C, 90sec). After taken out of the ice bath, 500~800 μL of LB medium was added into cell suspension and it was incubated at 37°C for 30min. The cell suspension was spread on agar plates and put in the incubator (37°C).

2-4-4. Site-directed mutagenesis

To prepare for mutant enzymes at a single amino acid residue, "QuickChange

Site-directed mutagenesis Kit" was utilized. In the method, PCR was available with both a polymerase and a buffer contained in the kit. PCR products were circular plasmids and were not methylated, therefore the only parental template DNA (those were usually methylated in *E.coli* strains (dam^+)) was digested with a restriction enzyme, Dpn I (Nelson et al., 1992; McClelland & Nelson, 1992; McClelland et al., 1994) before introduction of PCR products into cells. After digestion, DNA solutions were directly utilized for transformation.

2-4-5. Mutagenesis with error prone PCR

Error prone PCR is a random mutagenesis technique to generate amino acid substitutions in proteins by introducing mutations into a gene during PCR (Cherry et al., 1999; Daugherty et al., 2000; Shafikhani et al., 1997; Turner et al., 2003; Vartanian et al., 1996; Wan et al., 1998; You et al., 1994). In this work, a commercial kit "GeneMorph II Random Mutagenesis Kit" was applied. Mutazyme II DNA polymerase in the kit was a blend of two mutant DNA polymerases that replicated at low fidelity including misinsertion or misextension (Cline & Hogrefe, 1996). The mutation frequency per gene was dependent on the amount of template DNA or cycle number of the reaction. To take low mutation frequency, 500~1000ng of template DNA as a target gene quantity was added into the reaction mixture and 20~30 of cycle number for the reaction was chosen. (If the ratio of a target gene : the whole plasmid = 1: 4, 2000~4000ng of the plasmid should be added.) Obtained PCR products were inserted into plasmid vectors with a ligase after cutting with two restriction enzymes. Ligated DNA sample was introduced into commercial competent cell (*E.coli* BL21(DE3) pLysS) in transformation (Fig.2-1).

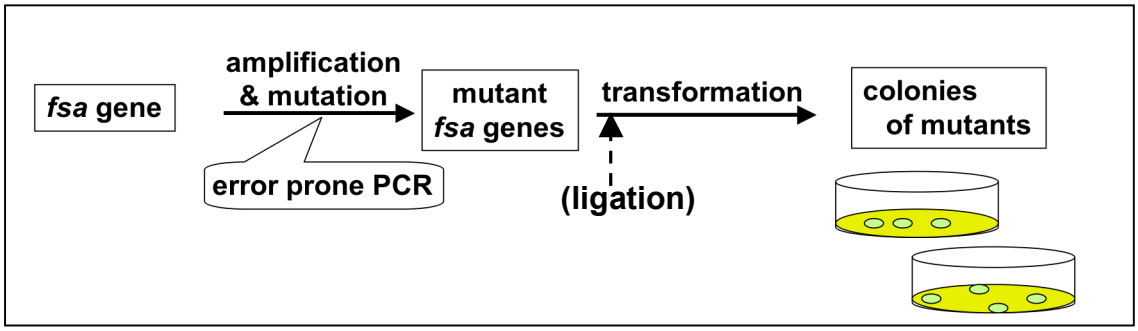


Fig.2-1. Flow of preparation of randomly mutagenized FSA in cells. PCR products were ligated to plasmid vectors and introduced into cells.

2-4-6. Cloning of DNA truncates to prepare genes encoding chimera proteins

Both truncates of *fsa* and *tal* gene were prepared to create chimera proteins by use of PCR. Each different size of truncate was amplified separately (Fig.2-2), and ligated in the combination of *fsa* with *tal* to be of a suitable size (672~675 bp). Ligated genes for full length of chimera proteins were amplified again due to the small amounts of quantities for further cloning. Amplified PCR products were cut with two restriction enzymes and inserted into plasmid vectors. Each plasmid vector containing a chimera protein gene was introduced into cells.

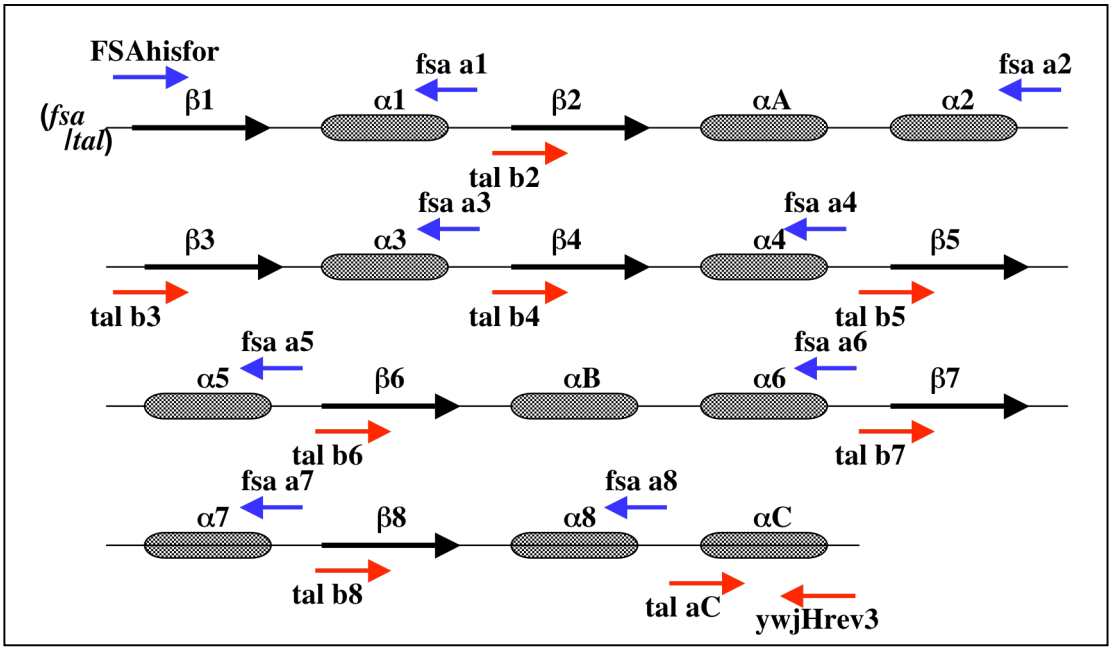


Fig.2-2. Binding positions of primers for chimera proteins on *fsa* or *tal* gene. Arrows indicate primers to prepare for *fsa* (blue) or *tal* (red) truncates, respectively. β and α are corresponding regions on genes to β strands or α helices on proteins.

Example of PCR components (in case of *fsa* truncates)

Template DNA (pUC18fsa)	10~30 ng		
FSAhisfor	10~30 pmol		
fsa a1 (or fsa a2~8)	10~30 pmol		
dNTPmix	200 μM		
polymerase buffer	1 fold		
Pwo DNA polymerase	2~5 U	/ 50μL	→ a *fsa* truncate

2-5. Purification and analysis of proteins

2-5-1. Overproduction of proteins and cell harvesting

E.coli cells were aerobically cultured at 37°C in Erlenmeyer flasks with shaking at 180 rpm. Liquid culture occupied one-fifth of total volume of the flask. For the expression of proteins, *E.coli* was aerobically cultured in 50~400 mL liquid culture at 25~37°C. Induction was performed by adding isopropyl-1-thio-β-D-galactoside (IPTG) at 0.5~0.6 of OD_{600nm} following a preculture starting at OD_{600} 0.1~0.2 at 37°C. The induction time depended on the protein expression system and the final IPTG concentration was 1mM.

Table.2-4. Induction time for each protein expression system

Strain	Plasmid vector	Promotor	Induction time
DH5α	pUC	*lac*	overnight (14~18 h)
DH5α	pJF	*tac*	4~5 h
BL21(DE3)	pET	*T7*	3~4 h
BL21(DE3)Star	pET	*T7*	3~4 h

Cell suspensions (400mL culture) were centrifuged (Beckman, Avanti J-25, Rotor-JA10, 6,000 rpm, 10min) and supernatants were discarded. Cells were resuspended vigorously in buffer (50mM glycylglycine, 1mM dithiothreitol (DTT) (pH8.0)). The suspension was centrifuged and supernatant was discarded again to wash cells. 10 mL of the buffer was added to the cell pellet and it was resuspended and stored at -70°C.

2-5-2. Cell disruption and preparation of crude extracts

case.1) a strain holds plasmid, pLysS (e.g. BL21(DE3) pLysS)

Frozen cells were incubated in a water bath at 30°C for 20 min to thaw them. Then, they were put in an ice bath and left for 30~40 min. Cell walls were lysed and intracellular soluble proteins were set free. The supernatant was obtained with ultracentrifuge (Beckman Coulter GmbH (Krefeld), Optima LE-80K, Rotor-TFT 70.38, 40,000 rpm, 30~40 min), and was utilized as crude extract (“cell-free extract”).

case.2) other strains

After thawing cells as above-mentioned, French Press (American Instrument Company,

French Pressure Cell Press, large cell, 1100 psig (1psi = 6,895 Pa) x3) was applied to lyse cells. Cell debris were removed with ultracentrifuge as well as mentioned in case.1, the supernatant was taken out as crude extract ("cell-free extract").

2-5-3. Heat treatment of crude extracts

Crude extract (8-10mL, 10-15 mg/mL-protein) was incubated in a falcon tube (15 mL) at 75°C for 30~40 min to denature several proteins, in case that a target protein was stable under the condition. Denatured proteins were removed with ultracentrifuge as precipitates. The supernatant was obtained as a heat-treated protein solution.

2-5-4. Purification with FPLC (Fast Protein Liquid Chromatography)

Crude extracts or heat-treated solutions were applied to a FPLC system (Amersham Biosciences/GE Healthcare, controller: LCC-501 Plus, Pump: P-500, Motor Valve: MV-7, MV-8, UV detector: control Unit UV-1, Optical Unit UV-1, Fraction collector: FRAC-100, Recorder: LKB REC102, others: Mixer, Conductivity Monitor) at 4°C as the next protein purification step (Fig.2-3).

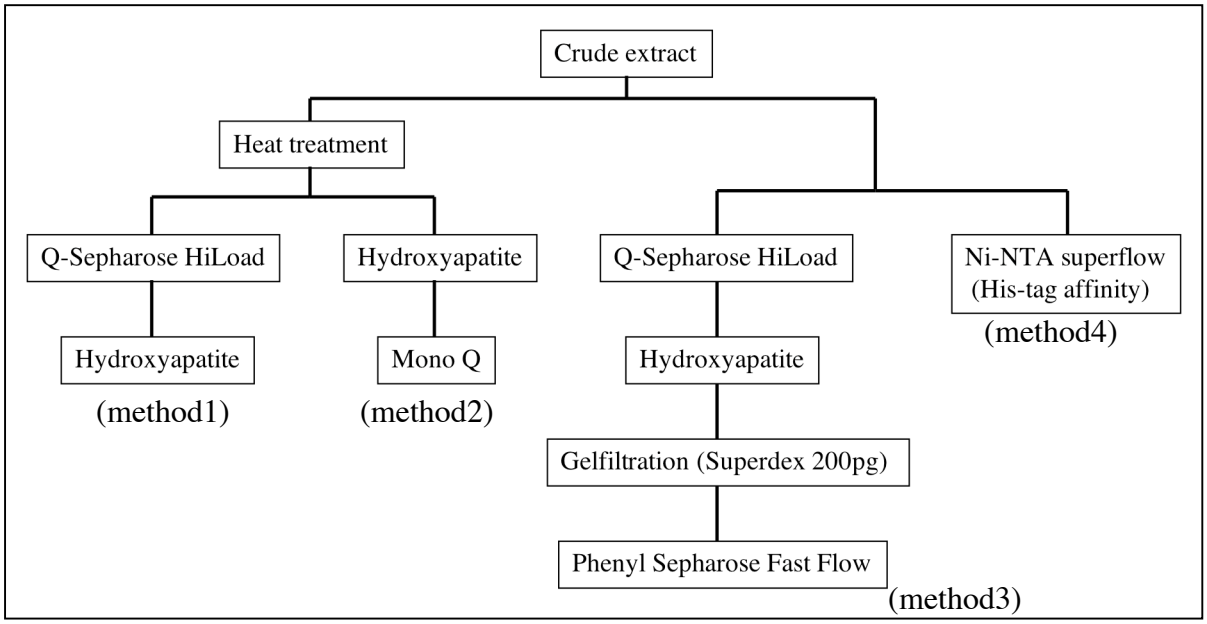


Fig.2-3. Flow of four different protein purification steps. Several mutant proteins were not subjected to heat treatment.

Anion-exchange column chromatography

Q-Sepharose HiLoad 16/10 (Amersham Bioscience, 20mL) is a strong anion-exchange column and was utilized as 1st column to remove undesirable proteins. Samples were loaded at the flow rate of 1 mL/min, 1~2 column volume of a loading buffer the gradient for protein elution was performed at 0 to 100 % for 2 column volumes. Each fraction was collected in 5~10 mL.

Loading buffer: 50mM glycylglycine, 1mM DTT / NaOH (pH8.0)

Elution buffer: 50mM glycylglycine, 1mM DTT, 0.5M NaCl / NaOH (pH8.0)

Mono Q HR10/10 (Amersham Bioscience, 8mL) has a high resolution and is the strong anion-exchange column. It was applied at the final stage of purification (usually as 2nd, 3rd or later column). Samples were loaded at the flow rate of 0.25~0.5 mL/min, the gradient (0 to 100%) was done for 2 column volumes. Each eluted sample was fractionated in 2~5 mL.

Loading buffer: 50mM glycylglycine, 1mM DTT / NaOH (pH8.0)

Elution buffer: 50mM glycylglycine, 1mM DTT, 1M NaCl / NaOH (pH8.0)

Gelfiltration

Superdex 200 prep grade (Amersham Bioscience, 150mL) is a gel permeation chromatography column which had high selectivity (Mr, globular proteins; 10^4~10^6). Samples were prepared to 1~2 % of the total gel bed volume, and loaded at the rate of 0.5 mL/min. A single buffer was utilized for sample loading and elution, the elution was performed up to 1.5~2 of the gel bed volume. Elution times (volumes) of samples were compared with standard proteins (Amersham Bioscience, Gel Filtration Calibration Kit - High Molecular Weight, Low Molecular Weight) and the molecular sizes were determined. In case that eluted protein was investigated further, each 2.5 or 5 mL sample was fractionated.

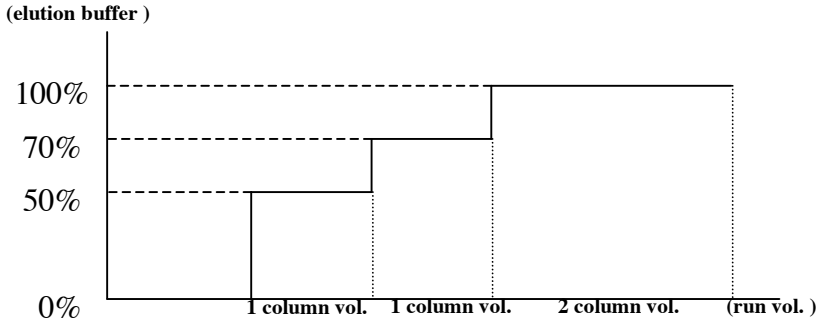
Loading&Elution buffer:

50mM glycylglycine, 1mM DTT, 0.1M NaCl / NaOH(pH8.0)

Hydrophobic interaction column (HIC) chromatography

HIC is the protein separation method based on the differences in hydrophobicity on the surface of proteins. The more hydrophobic a protein is, the higher affinity it has to HIC.

As a HIC column, Phenyl Sepharose Fast Flow (Amersham Bioscience, 20mL) was applied. Buffers of protein samples were exchanged to a $(NH_4)_2SO_4$ containing buffer, then the sample was loaded with the same buffer at the flow rate of 0.5 mL/min. For elution of proteins, "step gradient" was adopted.



Each eluted sample was fractionated in 2.5~5 mL.

Loading buffer: 50mM K_2HPO_4, 1M $(NH_4)_2SO_4$ / H_3PO_4 (pH7.0)

Elution buffer: 50mM K_2HPO_4 / H_3PO_4 (pH7.0)

Hydroxyapatite column chromatography

CHT ceramic hydroxyapatite (Biorad, 20μm, TypeI, 10g (= 20mL)) was utilized as 1st or 2nd column (Biorad tech note, 2156, 2644, Kawasaki et al., 1985). After loading of a protein sample at the flow rate of 1 mL/min, the flowthrough solution was collected and concentrated with centrifuge filters, Vivaspin 20 (MWCO 100,000) (Vivascience AG (Hanover)) due to no affinity to the resin under the condition. Elution buffer was run to remove some attached proteins out of column and clean it up.

Loading buffer: 10mM K_2HPO_4, 1mM DTT / H_3PO_4 (pH7.0)

Elution buffer: 0.4M K_2HPO_4, 1mM DTT / H_3PO_4 (pH7.0)

Affinity column chromatography

His-tagged proteins were purified with Ni-NTA Superflow (Qiagen, 25mL). Crude extract samples were directly loaded to the column with a buffer containing 20mM imidazol at 1.0 mL/min. An elution buffer was run at the same flow rate without gradient. Each 5~10 mL of elute was fractionated and the exchange of buffers was done (into 50mM glycylglycine, 1mM DTT (pH8.0)).

Loading buffer:

50mM glycylglycine, 10mM imidazol, 0.3M NaCl, 1mM DTT / NaOH (pH8.0)

Elution buffer:

50mM glycylglycine, 250mM imidazol, 0.3M NaCl, 1mM DTT / HCl (pH8.0)

To purify His-tagged transketolase (TKT), buffer components were modified as below.

Loading buffer:

20mM glycylglycine, 20mM imidazol, 0.3M NaCl, 1mM DTT

1mM $MgCl_2$-$6H_2O$, 1mM thiaminediphosphate / NaOH (pH8.0)

Elution buffer:

20mM glycylglycine, 250mM imidazol, 0.3M NaCl, 1mM DTT

1mM $MgCl_2$-$6H_2O$, 1mM thiaminediphosphate / HCl (pH8.0)

2-5-5. Polyacrylamide-gel electrophoresis

Polyacrylamide-gel electrophoresis (PAGE) is advantageous for analysis of proteins size or purity (Laemmli, 1970). Proteins are segregated in accordance with the molecular size. The gel was prepared as consisting of two phases, stacking gel (4% acrylamide) or separating gel (15% acrylamide). In SDS-PAGE (Sodium dodecyl sulfate polyacrylamide-gel electrophoresis) (Laemmli, 1970), samples were denatured with SDS, linearized and negatively charged. 10~20 μg of each protein was loaded to the gel. After running, the gel was stained with CBB solution (Coomassie Brilliant Blue R-250, Biorad or Fluka).

2-5-6. Determination of protein concentration

Protein concentration in each sample was determined with Bradford method (Bradford, 1976). BSA (bovine serum albumin, Carl Roth GmbH) was used as standard protein, 25~600 mg/L of concentration of BSA solutions were prepared to calculate the correlation factor between protein concentration and absorbance of OD_{595}. That correlation coefficient was utilized to determine protein concentration for each sample.

2-6. Assay of enzyme activities

To assay enzymes in FSA or TAL activity, the following analytic methods were performed. A UV-visible spectrophotometer CARY 50 Bio (VARIAN Deutschland GmbH (Darmstadt)) was used for photometrical assay. In substrates or products

detection with HPLC, a system "Millenium^{32} Sytem" (Water GmbH, Eschborn; Waters^{TM} 717 Plus Autosampler, Waters 996 Photodiode Array Detector, Waters 510 HPLC Pump, Waters Pump Control Module) was applied.

2-6-1. Photometrical measurements

A general condition of photometrical assay in this study was the measurement of NADH oxidation or NADP reduction at 340nm (ε340 = 6.22 $cm^2/\mu mol$) with several auxiliary enzymes in 50mM glycylglycine + 1mM DTT buffer (pH 8.5) at 30°C (Schürmann Me, Dissertation 2001). (ε = molar absorption coefficient)

Ex.1 (FSA activity: Fru6P synthesis) (Schürmann Me, Dissertation 2001)

50mM DHA as donor and 2.8~3.0 mM GAP were used, Fru6P synthesis was detected by reduction of NADP with two auxiliary enzymes, phosphoglucose isomerase (PGI, Roche Applied Science) and glucose-6P dehydrogenase (Glu6P DH, Roche Applied Science).

Ex.2 (FSA activity: cleavage of Fru6P) (Schürmann & Sprenger, 2001)

For the Fru6P cleavage assay, 10~50 mM of Fru6P as a single substrate was added, GAP resulting from the cleavage was detected by use of an auxiliary enzymes mixture, triose-phosphate isomerase + glycerol-3P dehydrogenase (TIM/Gly3P DH, Roche Applied Science) concomitantly with oxidation of NADH.

Ex.3 (Classical TAL activity) (Tsolas &Horecker, 1972)

10mM Fru6P as donor and 2mM Ery4P as acceptor were used catalyzed into Sed7P and GAP were produced. GAP was detected as well as in Ex.2.

Ex.4 (xylulose production)

50mM DHA or 10mM Fru6P as donor, glycolaldehyde (120mM) as acceptor were substrates for xylulose production. GAP released from Fru6P was detected. Otherwise, xylulose was reduced into xylitol with sorbitol dehydrogenase (SDH, Roche Applied Science) and detected by simultaneous NADH oxidation (pH 7.5~8.0) (Kersters et al., 1964; Lindstad et al., 1998; Ng et al., 1992).

Ex.5 (utilization of various acceptors) (Tsolas &Horecker, 1972)

10mM Fru6P as donor and various acceptor substrates were utilized in reactions. Activities were assayed by detection of GAP from Fru6P by use of NADH and TIM/Gly3P DH.

2-6-2. Analysis with HPLC (High-Performance Liquid Chromatography)

Detection column for substrates and reaction products was Aminex HPX-87H (Biorad, 300mm x 7.8mm), 5mM H_2SO_4 was applied as a mobile phase. The flow rate for measurement was 0.5 mL/min, column temperature was ambient and the wavelength for detection was 192 nm. Samples were loaded after removing proteins by adding methanol, centrifuge (Beckman Coulter GmbH, Microfuge 22R Centrifuge, 14,000 rpm, 4°C, 20~30min) and a passage through Celite(545)-charcoal (Fluka). Reaction products were compared with standard substances in retention time.

2-6-3. Colour assay of FSA activity

Cells (BL21(DE3) pLysS + pET16mutant fsa) were incubated with shaking at 165rpm in 1mL of LB medium containing 1mM IPTG and antibiotics (ampicillin and chloramphenicol) at 37°C overnight by use of a deep-well plate (deep-well incubation). Cells were collected with a centrifuge (SIGMA Laborzentrifugen GmbH (Osterode am Harz), SIGMA 3K-1, 4,000 rpm, 4°C, 10min). Buffer (50mM glycylglycine + 1mM DTT (pH 8.5)) and DNase (final conc. 2mg/mL) were added after discarding supernatant. Samples were frozen at -20~-70°C for 1h and incubated at 30°C for 20min. Crude extracts were obtained by centrifuge. 20~30μg protein was added into the reaction mixture.

Components of coloring test in FSA activity

crude extract	20~30 μg protein
PGI (1mg/mL)	1μL
Glu6P DH (0.1 U/μL)	1μL
NADP (50mM)	2μL
DHA (2.5M dimer)	2μL
NBT (nitroblue tetrazolium chloride, Roche Applied Bioscience)	
(50mM in 70% DMF)	2μL
diaphorase (Sigma-Aldrich, 0.03 U/μL)	2μL
50mM glycylglycine + 1mM DTT (pH8.5)	add up to 200μL
GAP	2μL

The reaction was started by adding GAP at ambient temperatures. Time course was

measured at OD_{540} with a multi-well microplate and Microplate Scanning Spectrophotometer (BIO-TEC Instrument (Vermont USA), Power $Wave_x$).

2-7. Crystallization of proteins (by T. Sandalova at Karolinska Institute)

Crystals of TAL_{Bsu} are obtained in hanging drops by vapor diffusion method at 20ºC. Crystal screens (Hampton Research) were used to establish initial crystallization conditions, which were then refined in a finer grid. The crystals were obtained under several different conditions, all of them contain PEG and different salts. The best diffracting crystals were obtained in 20% PEG 4K, 0.2M of Li_2SO_4, 20% of glycerol, 100 mM Tris HCl pH 9.0 at room temperature. Typically, 2-3ml of a 12mg/ml protein solution in 50mM glycylglycine buffer (pH 8.0), 0.1M NaCl and 1mM DTT was mixed in a 1:1 ratio with the crystallization reservoir solution and equilibrated against 1ml of reservoir solution. Diamond-shaped crystals grew several weeks until they reach 0.6 - 0.8mm.