3. Results

3-1. Fructose-6-phosphate aldolase (FSA): Relationship of catalytic function and structure

3-1-1. Histidine (His)-tagged versions of FSA do not show wild-type characteristics

Up to now, wild-type FSA (WT) had been investigated in catalytic activity, structure and reaction mechanism (Schürmann & Sprenger, 2001, Schürmann Me, 2001). The next step should be further elucidation of the relationships of function and structure by preparation of mutant enzymes and purification of those. In this respect, it is important to acquire enough protein samples effectively with ease. Thus, His-tagged versions of FSA at the N-terminus (10 x His) or at the C-terminus (6 x His) were prepared (for details see M&M). These should then serve as backgrounds for mutants to get purified proteins by the application of a Ni-NTA column only. Each His-tagged FSA was overexpressed with pET system (pET16b for N-His-tagged, pET22b for C-His-tagged) in *E.coli* BL21(DE3)Star, pLysS (Invitrogen). Both His-tagged FSA versions were obtained by 20~30% of total protein in crude extracts (Fig.3-1 lane-1 & -4). However, N-terminus His-tagged FSA did not bind with Ni-NTA superflow resin (Qiagen) and came out of the column (25mL) in flow-through solution (Fig.3-1 lane-2) during the step of washing the column with a loading buffer (protein concentrations of eluted fractions were 7~45mg/L), whereas C-terminus His-tagged FSA was purified well and taken out in eluted fractions (the concentrations were 105~210mg/L) (Fig.3-1 lane-6~8). Quaternary structures of both FSAs were determined by gel filtration (Superdex 200pg). In results, both were fractionated in 75~80 mL of elution volume which corresponds to 230,000~240,000 Da molecular mass proteins (FSA wild type; 240000 Da) (Fig.3-2). This indicates that both N- and C-terminus histidine tags do not hinder the formation of quaternary structure of the enzyme. FSA activities of both His-tagged proteins were assayed in crude extracts and compared with that of FSA WT. Both proteins had lower activity than WT (Table 3-1). N-His-tagged FSA retained about 50% of WT's, C-His-tagged kept approximately only 10% of WT activity. In this respect, His-tag is unsuitable for the preparation of FSA mutants due to the difficulty of retaining the activity. In addition, N-His-tag cannot facilitate the purification owing to its lacking affinity to Ni-NTA resin. Thus, wild-type FSA was used for further investigations.

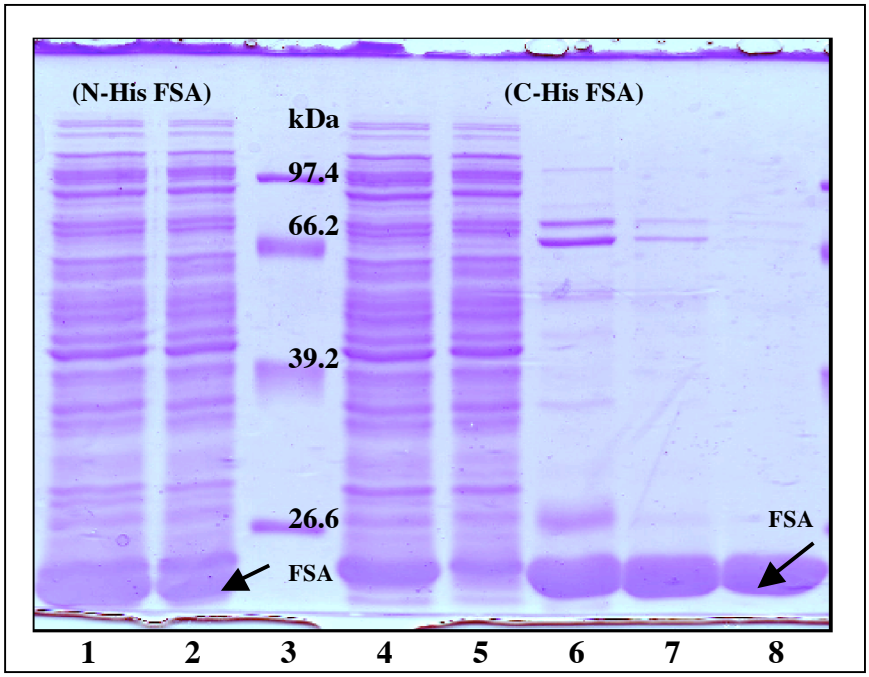
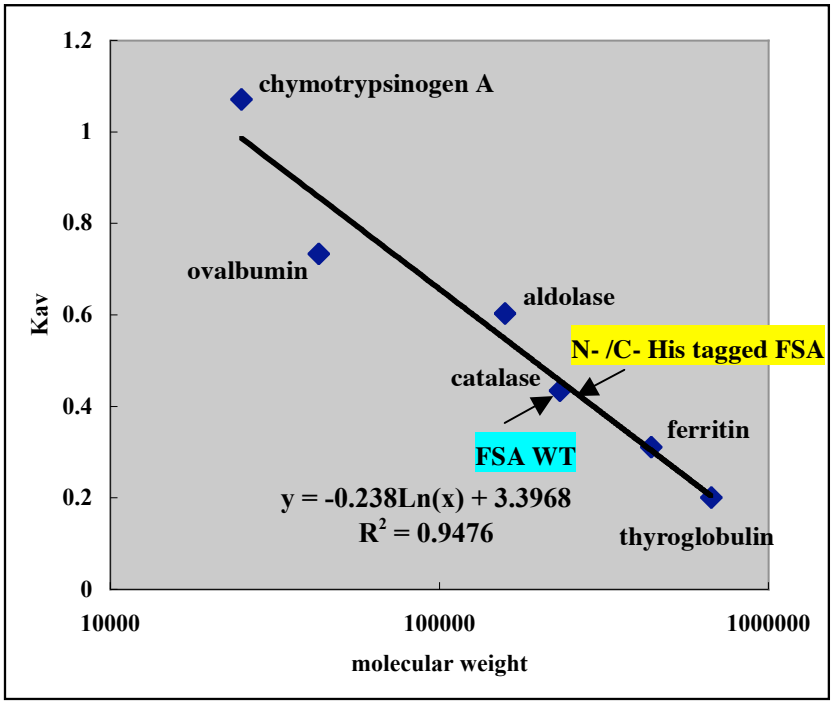


Fig.3-1. SDS-PAGE analysis of affinity of N- or C-His tagged FSA to Ni-NTA resin. Equal amounts of protein (20 μg) were applied to each lane; lane1: crude extract of N-His FSA, 2: flow-through of N-His FSA of Ni-NTA superflow column, 3: standard protein marker, 4: crude extract of C-His FSA, 5: flow-through of Ni-NTA superflow column after application of C-His FSA, 6~8: C-His FSA fractions eluted from the column. Arrows indicate His tagged FSA protein bands.

Table 3-1. Comparison of His-tagged FSAs with wild-type FSA (BL21(DE3)Star pLysS, pET16fsa) in affinity to Ni-NTA superflow, quaternary structure and specific activity.

vector	His-tag position	affinity to Ni-NTA column	quaternary structure	activity in crude extract
pET16b	N-terminal	negative	decamer	1.6±0.05 U/mg
pET22b	C-terminal	positive	decamer	0.3±0.2 U/mg
pET16b	non-tagged	-	decamer	3.2±2.0 U/mg

Specific activities of His-tagged FSAs were determined with 50mM DHA, 2.8mM GAP and proteins (approx. 20μg/mL) by taking the means of at least 3 independent measurements.



	Mr	elution volume (mL)	Kav
Blue dextran		43 = Vo	
Thyroglobulin	669000	58.5	0.201
Ferritin	440000	67	0.312
Catalase	232000	76.5	0.435
Aldolase	158000	89.5	0.604
Ovalbumin	43000	99.5	0.734
Chymotrypsinogen A	25000	125.5	1.071

Kav = (Ve-Vo)/(Vt-Vo)

Vt = total bed volume

Ve = elution volume

Fig.3-2. Determination of quaternary structures of His-tagged versions of FSA by gel filtration (Superdex 200pg - 1.6 x 60 cm). Standard samples are Thyroglobulin (MW:669000), Ferritin (MW:440000), Catalase (MW:232000), Aldolase (MW:158000), Ovalbumin (MW:43000) and Chymotrypsinogen A (MW:25000). Both N- and C-His tagged versions of FSA came out in 70.0~80.0 mL of elution volume (close to catalase) and showed peaks of proteins at 74.5 mL (theoretical MW of N-His FSA: 255kDa, C-His FSA: 241kDa).

3-1-2. Purification of FSA WT: Comparison of different methods

Purification of FSA WT from *E.coli* DH5α pUC18fsa had already succeeded by Me.Schürmann (Schürmann & Sprenger, 2001, Schürmann Me, 2001), however, pUC vector is usually not an expression vector but rather a cloning vector. Therefore, overexpression level is not high. So it was expected that other vectors as pET or pJF119 would be utilized to produce proteins. However, the conventional purification procedure (crude extract --> heat treatment --> 1st column/MonoQ) was inapplicable for samples with those vectors due to impermissible high back pressure on column possibly caused by high viscosity brought from lipoproteins in crude extracts (The column generally is not designed for the application of crude extracts). Thus, three independent samples with two different purification methods were examined (condition-1: *E.coli* BL21(DE3)Star pLysS pET16fsa with method1, condition-2: DH5α pJF119fsa with method1, condition-3: DH5α pJF119fsa with method2) (Table.3-2). In method1 an anion-exchange column, Q-Sepharose HP, (20mL; Amersham Biosciences) and a hydroxyapatite column (CHT ceramic typeI 20mL; Biorad) were adopted after heat treatment, while the hydroxyapatite column and a strong anion-exchange column, MonoQ (8mL; Amersham Biosciences), were utilized in method 2. Details of each purification method are described in Materials & Methods 2-5-4.

Table.3-2. Three different samples to compare in purification

sample	strain, expression vector	purification
condition-1	BL21(DE3)Star pLysS, pET16fsa	method 1
condition-2	DH5α, pJF119fsa	method 1
condition-3	DH5α, pJF119fsa	method 2

method 1: column I; Q-sepharose, II; hydroxyapatite
method 2: column I; hydroxyapatite, II; MonoQ

First, condition-1 and -2 were compared with each other (the differences were strain and plasmid vector to produce FSA) in protein quantity, specific activity and residual FSA

quantity at each step (Fig.3-3, Table 3-3 for condition-1 / Fig.3-4, Table 3-4 for condition-2). Specific activity of FSA in crude extract in condition-2 was 147% of that in condition-1, though both amounts of proteins were approximately equal. Heat treatment brought large differences between condition-1 and condition-2 in both amount of protein and residual FSA quantity (protein quantity: 37.5%(136/363mg) in condition-1, 56.1%(199/350mg) in condition-2, residual FSA quantity: 84% in condition-1, 99% in condition-2). These results indicated that the initial purity of FSA in crude extract was higher in DH5α pJF119fsa than in BL21(DE3)Star pLysS pET16fsa. In addition, more FSA was lost in condition-1 (residual FSA quantity: 84% -> 40%) than under condition-2 (99% -> 89%) in a phase of using Q-Sepharose HP as 1st column. Specific activities of elutes were 8.0U/mg (condition-1) and 11.1U/mg (condition-2), respectively. Most values in each table entry except purification factor were higher in condition-2 than in condition-1 (Table.3-3/3-4). It was apparently advantageous to use the combination of DH5α and pJF119fsa as a host cell and expression vector in FSA preparation. Then, purification under condition-3 was compared with that under condition-2. In condition-3, the same batch of protein sample as condition-2 was utilized but prepared in a phosphate buffer (10mM K_2HPO_4 + 1mM DTT pH7.0) instead of 50mM glycylglycine (pH8.0) to adopt hydroxyapatite as 1st column after heat treatment. However, specific activity in each phase under condition-3 was lower than that under condition-2 (Table.3-4/3-5), although both recovery rate of protein quantity and residual FSA activity were finally almost same (recovery rate: 28.6%(68/238mg) in condition-3, 29.7%(104/350mg) in condition-2, residual FSA activity: 89% in condition-3, 87% in condition-2). These experimental results suggested that it was a proper way to utilize DH5α pJF119fsa as a host cell and expression vector for FSA overproduction and to apply glycylglycine buffer (pH8.0) and Q-Sepharose as 1st column after heat treatment.

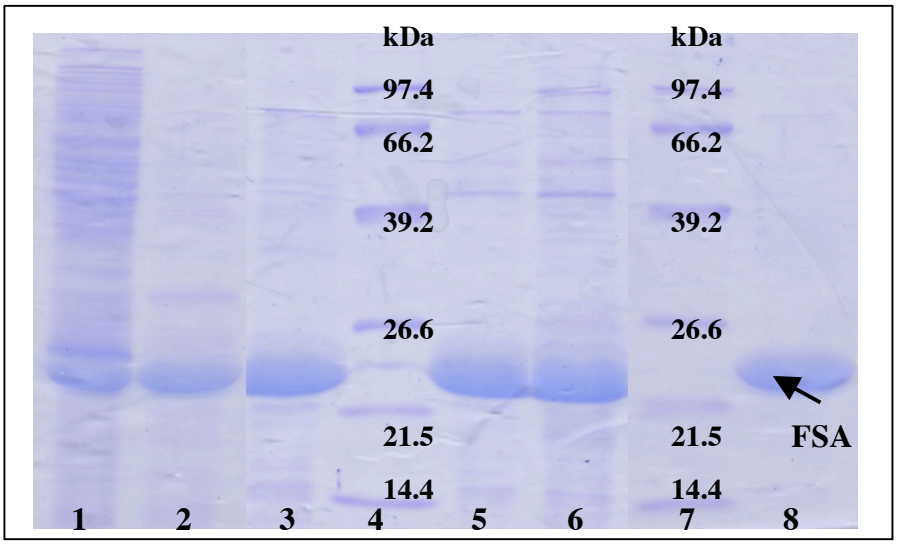


Fig.3-3. SDS-PAGE analysis of FSA (BL21(DE3)Star pLysS pET16fsa) purified with method1 under condition-1 (heat treatment -> Q-Sepharose HP -> hydroxyapatite). Equal amounts of protein (20 μg) were applied to each lane; lane1: crude extract, 2: protein solution after heat treatment, 3, 5&6: fractions eluted from Q-Sepharose HP, 4&7: standard protein marker, 8: flowthrough from hydroxyapatite. An arrow indicates the purified FSA band.

Table.3-3. Purification scheme for FSA under condition-1 (BL21(DE3)Star pLysS pET16fsa).

sample	Amount of protein (mg)	Spec. activity (U/mg)	Amount of activity (U)	Residual FSA quantity (%)	Purification factor
crude extract	363	3.2±0.5	1162	100	1
heat treatment	136	7.2±1.0	979	84	2.3
Q-Sepharose HP	58	8.0±1.8	462	40	2.5
hydroxyapatite	41	11.0±1.0	454	39	3.4

Activities were determined by measuring Fru6P production rates from DHA(50mM) + GAP(2.8mM) with 4-10 μg proteins in a coupled enzyme assay (auxiliary enzymes: PGI and Glu6P DH, Materials & Methods: 2-6-1). Values were the means of at least 3 independent measurements. The amount of protein or FSA activity was from 1L culture scale.

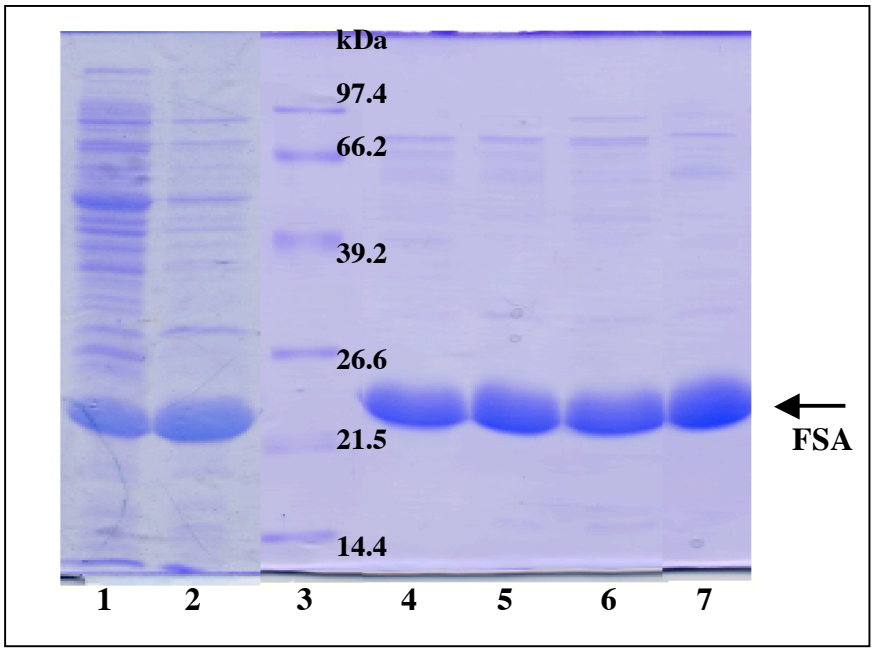


Fig.3-4. SDS-PAGE analysis of FSA (DH5α pJF119EHfsa) purified with method1 under condition-2 (heat treatment -> Q-Sepharose HP -> hydroxyapatite). Equal amounts of protein (20 μg) were applied to each lane; lane1: crude extract, 2: protein solution after heat treatment, 3: protein marker, 4~6: fractions eluted from Q-Sepharose HP, 7: flowthrough from hydroxyapatite. An arrow indicates the FSA bands.

Table 3-4. Purification scheme for FSA (DH5α pJF119EHfsa) under condition-2.

sample	Amount of protein (mg)	Spec. activity (U/mg)	Amount of activity (U)	Residual FSA quantity (%)	Purification factor
crude extract	350	4.7±1.0	1645	100	1.0
heat treatment	199	8.2±1.5	1632	99	1.7
Q-Sepharose HP	131	11.1±2.0	1459	89	2.4
hydroxyapatite	104	13.7±1.0	1429	87	2.9

Activities were determined by measuring Fru6P production rates from DHA(50mM) + GAP(2.8mM) with 4-10 μg proteins in a coupled enzyme assay (auxiliary enzymes: PGI and Glu6P DH, Materials & Methods: 2-6-1). Values were the means of at least 3 independent measurements. The amount of protein or FSA activity was from 1L culture scale.

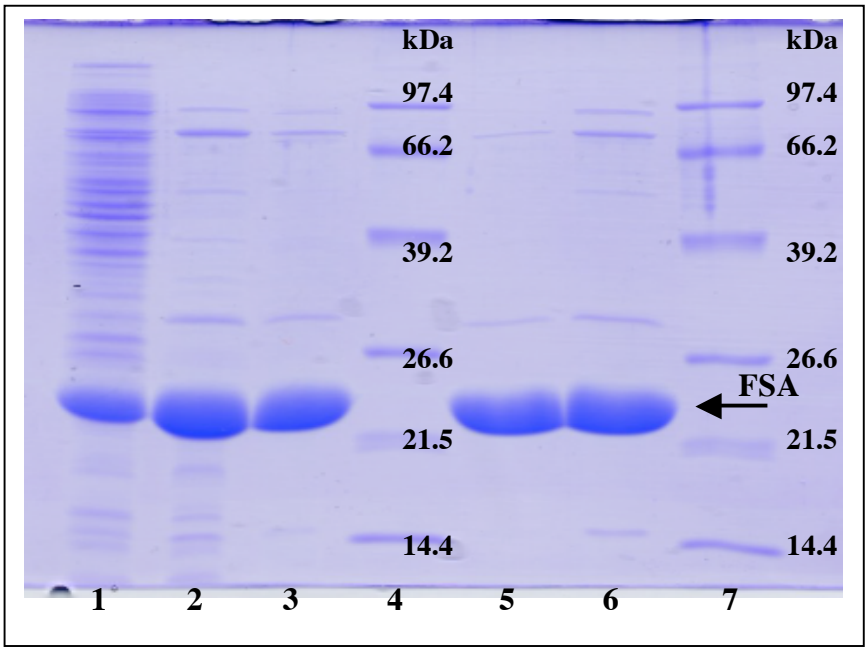


Fig.3-5. SDS-PAGE analysis of FSA (DH5α pJF119EHfsa) purified with method2 under condition-3 (heat treatment -> hydroxyapatite -> MonoQ).). Equal amounts of protein (20 μg) were applied to each lane; lane1: crude extract, 2: protein solution after heat treatment, 3: flowthrough from hydroxyapatite, 4&7: protein marker, 5&6: fractions eluted from MonoQ. An arrow indicates the FSA bands.

Table 3-5. Purification scheme for FSA (DH5α pJF119EHfsa) under condition-3.

sample	Amount of protein (mg)	Spec. activity (U/mg)	Amount of activity (U)	Residual FSA quantity (%)	Purification factor
crude extract	238	3.4±0.5	809	100	1.0
heat treatment	105	7.2±0.5	756	93	2.1
hydroxyapatite	93	8.0±0.5	744	92	2.4
MonoQ	68	10.6±0.5	721	89	3.1

Activities were determined by measuring Fru6P production rates from DHA(50mM) + GAP(2.8mM) with 4-10 μg proteins in a coupled enzyme assay (auxiliary enzymes: PGI and Glu6P DH, Materials & Methods: 2-6-1). Values were the means of at least 3 independent measurements. The amount of protein or FSA activity was in 1L culture scale.

A comparison of FSA produced under the different conditions is summarized on Table 3-6. Regarding preparation of FSA, three kinds of expression systems (P*lac* on pUC vector, P*T7* on pET vector and P*tac* on pJF vector) were tested under the control of different promoters. Induction of FSA was performed by adding IPTG (isopropyl-β-D-thiogalactopyranoside) in every system, the induction times were 14~18h for *lac*, 3~4h for T7 and 4~5h for *tac*, respectively. Although it is said that pET system is one of the strongest protein-overexpression system (Studier et al., 1990), higher specific activity and larger amount of FSA were obtained in the sample expressed with *tac* promoter. It indicates that *tac* promoter system can promote FSA production highly and cells contain larger amounts of the enzyme than the others do, which helps to purify FSA from pJF vector better than using pUC or pET vector.

Table 3-6. Comparison of FSA overexpressed from different plasmid vectors.

vector	strain	promoter	inducer (time)	specific activity
pUC18	DH5α	*lac*	IPTG (1mM) (14 – 18 h)	9.7±3.0 U/mg
pET16b	BL21(DE3) pLysS BL21(DE3)Star pLysS	*T7*	IPTG (1mM) (3 – 4 h)	11.0±2.0 U/mg
pJF119EH	DH5α	*tac*	IPTG (1mM) (4 – 5 h)	13.7±1.0 U/mg

Each vector has a different promoter, pUC18 - *lac*, pET16b - *T7* and pJF119EH – *tac*, respectively. Culture medium was LB+ampicillin (for DH5α) or LB+ampicillin +chloramphenicol (for both BL21(DE3)). Inductions were performed with IPTG (final concentration: 1.0mM) at 0.5 – 0.6 of OD_{600} for pUC18 – overnight (14~18h), for pET16b – 3~4h, and for pJF119EH – 4~5h. Specific activities were determined with 50mM DHA, 2.8mM GAP and purified enzymes (5~10μg/mL) by taking the means of at least 4 independent measurements.

3-1-3. Preparation of mutant enzymes and characterization of the active site

From the result of the 3-dimensional structure of FSA crystals and from a former report (Thorell et al., 2002, Schürmann, 2001), a two-dimensional model of the FSA active center with 8 amino acid residues had been suggested as an enzyme-substrate complex (Fig.3-6). Virtually, four of eight residues are highly conserved in the related transaldolases (Lys85, Asp6, Asn28 and Thr109; the numbering is of FSA's; (Thorell et al., 2002, Schürmann, 2001)). These residues can be considered as essential in the process of forming the Schiff-base. The reaction mechanism is that of class I aldolases. A water molecule at the active center may play an important role to transfer protons during the reaction. 3 amino acid residues, Gln59, Thr109 and Tyr131 at the active center of FSA, create hydrogen bonds with a water molecule and take part in the reaction directly or indirectly (Thorell et al., 2002). In some transaldolases, a glutamate residue is located at the corresponding position instead of Gln59 in FSA, and a phenylalanine residue is in place of Tyr131. Thr109 is conserved in both transaldolases and FSA. In this respect, Thr109 has important roles for keeping the activity and it may give negative influences on the enzymes to alter the residue into another. It was supposed that Gln59 or Tyr131 might be replaced by other amino acids to hold a water molecule and maintain the activity. To assay the hypothesis for Gln59 and Tyr131, three different mutants (Q59E, Y131A and Y131F) were prepared (Fig.3-6). Alteration of Gln59 into Glu or Tyr131 into Phe aimed for an investigation of the difference in role of the corresponding residues between FSA and transaldolases. Preparation of Y131A was to study the effect for a hydrogen bond between Tyr131 and a water molecule. Mutant *fsa* genes were prepared as described under Materials & Methods (see 2-4-4.). Expression of each mutant FSA was induced overnight in *E.coli* DH5α with pUC18 plasmid vector by adding IPTG. Crude extracts after cell breakage were analyzed with SDS-PAGE (Fig.3-7). While WT and Q59E samples showed strong bands corresponding to FSA on the gel, Y131A and Y131F appeared not to be produced well. This indicates that both Y131A and Y131F mutant enzymes may not be produced with pUC vectors or not be stable enough in cells. Thus, pET system was used to overcome the problem. In fact, two mutant samples had strong bands that were equivalent to FSA on SDS-PAGE gels after 4 hours of induction (Fig.3-8). Activities of 3 mutants and FSA WT were assayed in crude extracts.

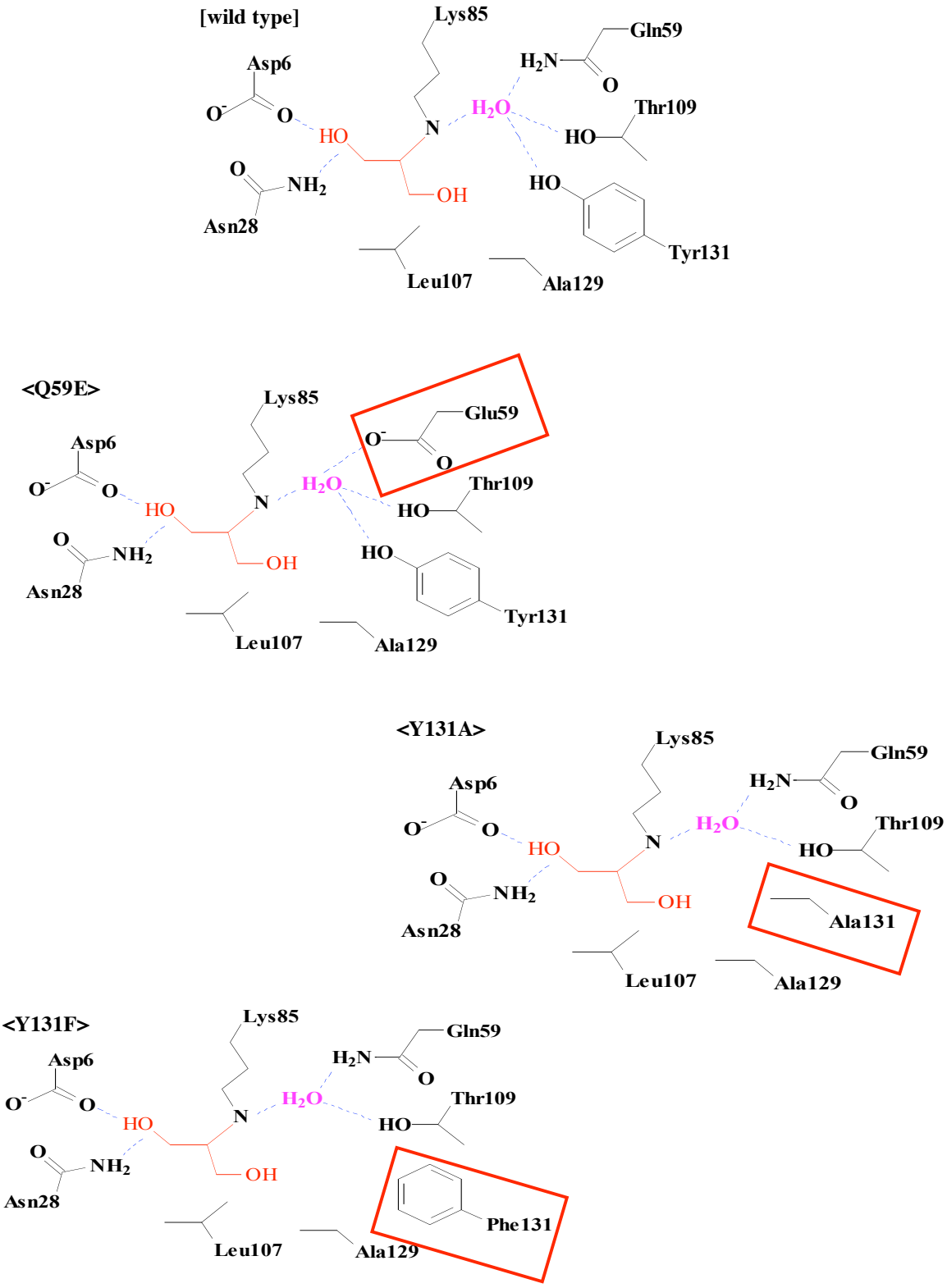


Fig.3-6. Two-dimensional models of active centers with several amino acid residues of FSA wild type and three mutants. Residues in red squares indicate altered positions.

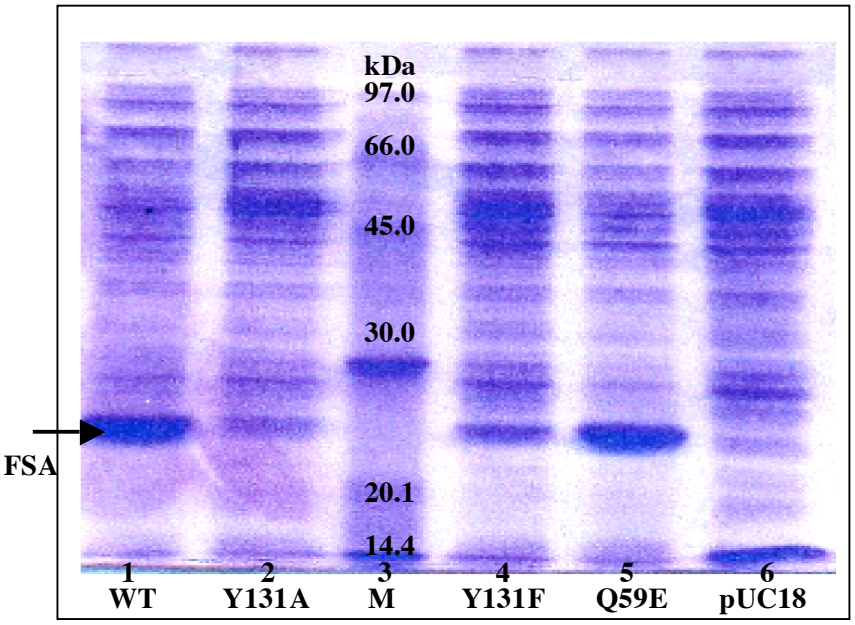


Fig.3-7 SDS-PAGE analysis of FSA wild type(WT) and three mutant proteins (Y131A, Y131F and Q59E) in crude extracts after expression with P*lac* system. A pUC18 vector containing strain was utilized as a negative control to compare *fsa* gene expressions. In each lane 20μg of protein were applied. (M: protein marker)

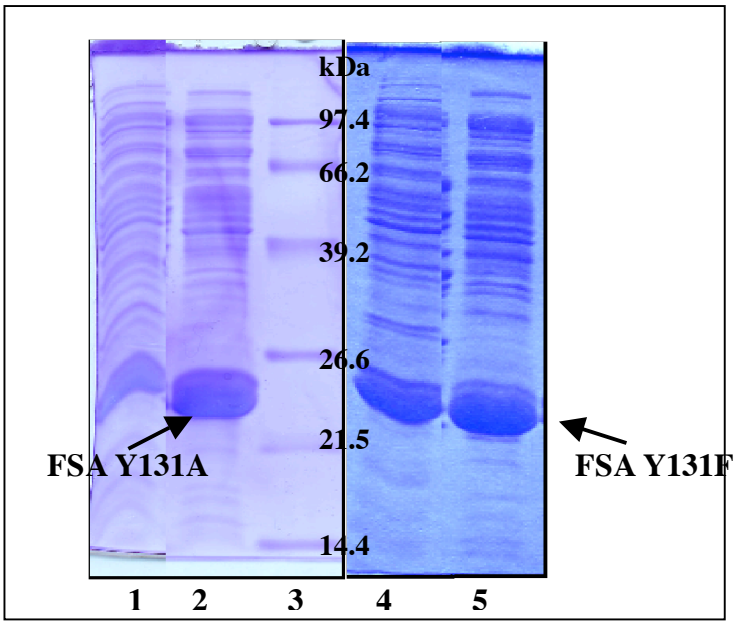


Fig.3-8. SDS-PAGE gel electrophoresis of FSA Y131A and Y131F expressed with pET system (on pET16b in *E.coli* BL21(DE3)Star pLysS): lane1- crude extract of FSA Y131A, 2- a fraction of Y131A eluted from Q-Sepharose, 3- standard protein marker, 4- crude extract of FSA Y131F, 5- a fraction of Y131F eluted from Q-Sepharose. Each sample contained 20μg of protein.

Table 3-7. Activities of FSA mutants in comparison with wild type.

mutant name	vector	activity in crude extract	with purified enzyme
wild type	pUC18	3.2± 0.3 U/mg (100%)*[1]	9.7± 3.0 U/mg (100%)*[2]
Q59E	pUC18	3.1± 0.4 U/mg (97%)*[2]	6.4± 0.5 U/mg (66%)*[2]
Y131A	pET16	not detectable*[1]	-
Y131F	pET16	not detectable*[1]	-

Activities were determined by measuring Fru6P production rates from 50mM DHA + 2.8mM GAP with 17~30 μg/mL proteins.

*1: values are the mean of two measurements

*2: values are the mean of three or more measurements

As a result (Table 3-7), in crude extract, Q59E retained more than 95% of the activity of the wild type in crude extract, whereas Y131A and Y131F showed no detectable activity. In this respect, the alteration of Gln59 into Glu59 did not lead to drastic change in function, while Y131 residue was essential for activity of FSA.

All three mutant proteins were denatured by heat treatment at 75℃ for 40 min and precipitated through centrifugation at 14,000 rpm for 30 min. This suggests that structural balance of each mutant enzyme might be damaged and that even a single amino acid alteration affects stability.

Q59E was successfully purified by use of 4 different columns (Q-Sepharose HP, hydroxyapatite, Superdex 200pg and PhenylSepharose F.F.) without heat treatment (SDS-PAGE: Fig.3-9 & 3-10) (see method3 in “Materials & Methods”). The activity of purified Q59E (> 95%, Fig.3-10; lane: 6~9) maintained approximately only 66% of that with purified WT (Table 3-7). The mutant enzyme was crystallized by the group of G. Schneider at the Karolinska Institute, Stockholm Sweden. From the analysis of crystals, the conformation of amino acid residues at the active center and whole enzyme structure appeared to be similar to those of WT from the results of analysis of crystals.

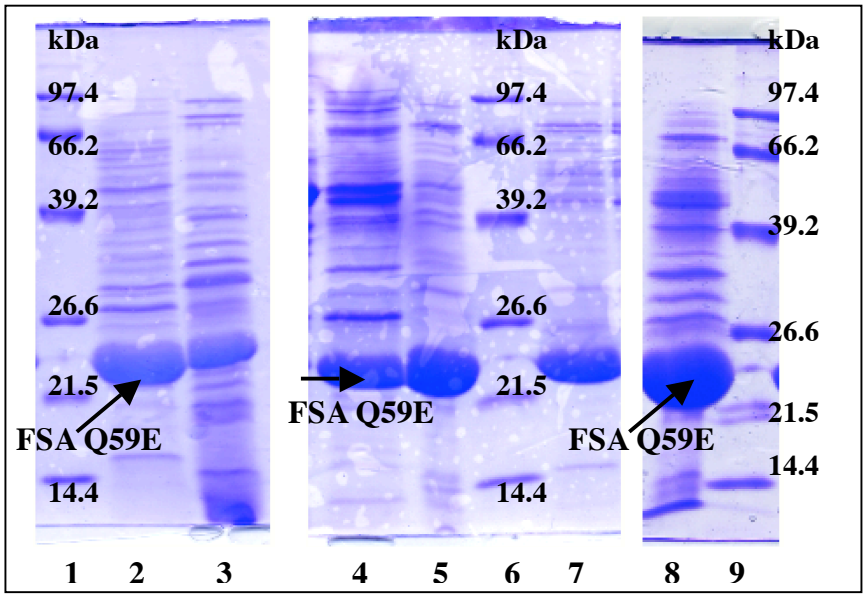


Fig.3-9. SDS-PAGE analysis of FSA Q59E in each purification step. Equal amounts of protein (20 μg) were applied to each lane; lane1, 6&9: standard protein marker, 2: crude extract, 3: protein solution after heat treatment (activity was lost), 4~5&7: fractions eluted from Q-Sepharose HP, 8: flowthrough from hydroxyapatite. Protein solution after heat treatment was not used for further purification.

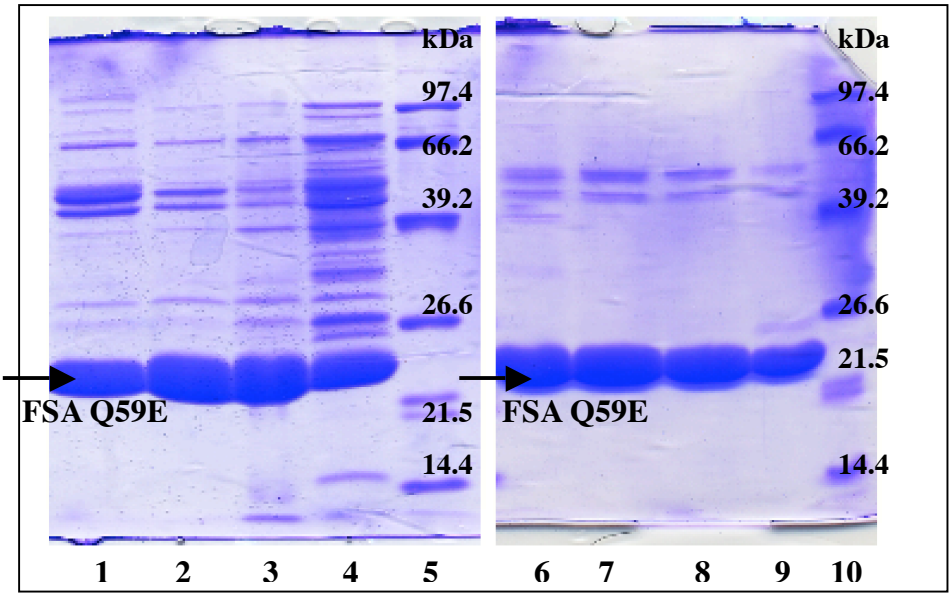


Fig.3-10 SDS-PAGE analysis of FSA Q59E in each purification step. Equal amounts of protein (20 μg) were applied to each lane; lane1~4: fractions from Superdex200pg, 5&10: standard protein marker, 6~9: fractions from PhenylSepharose F.F.