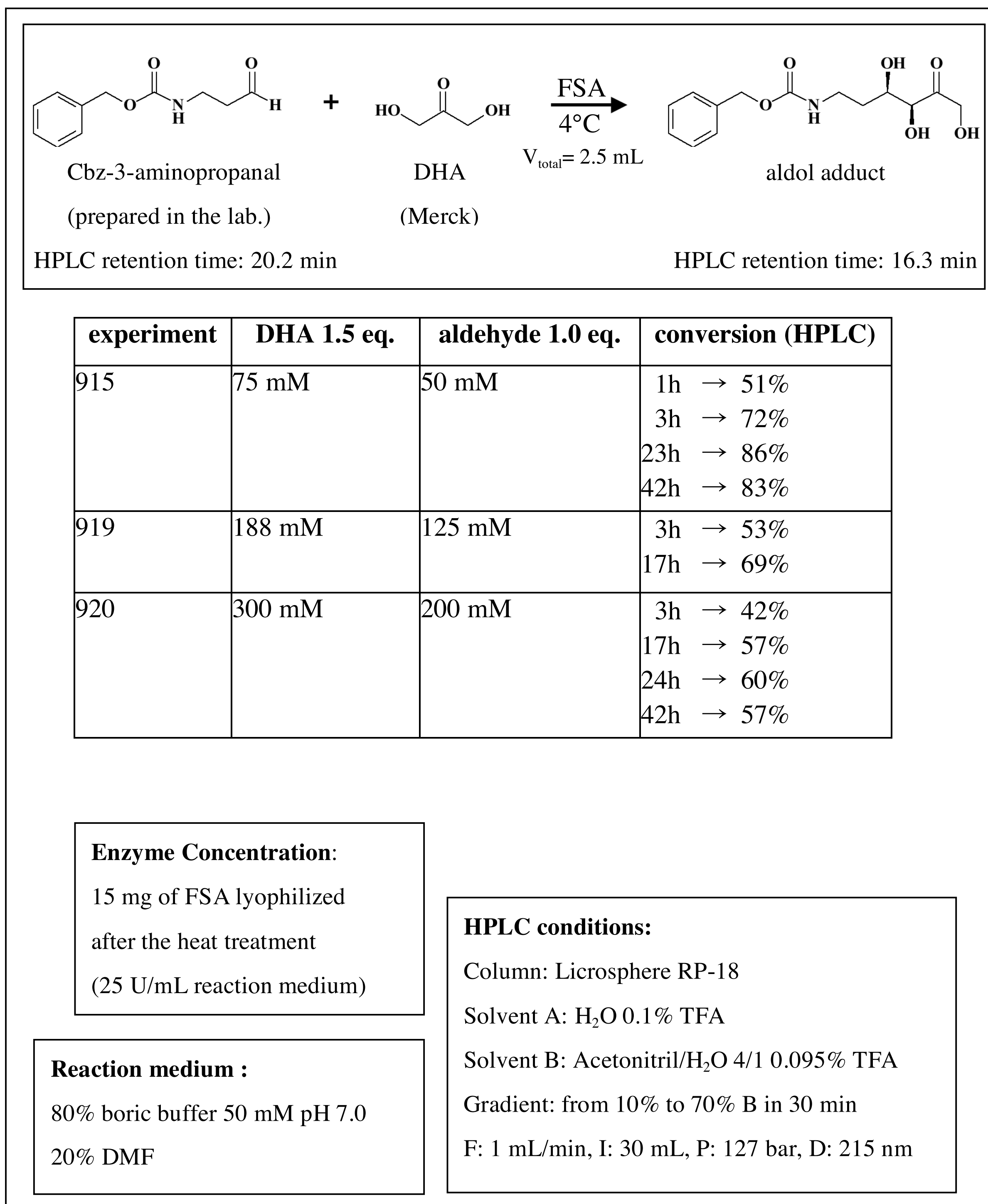
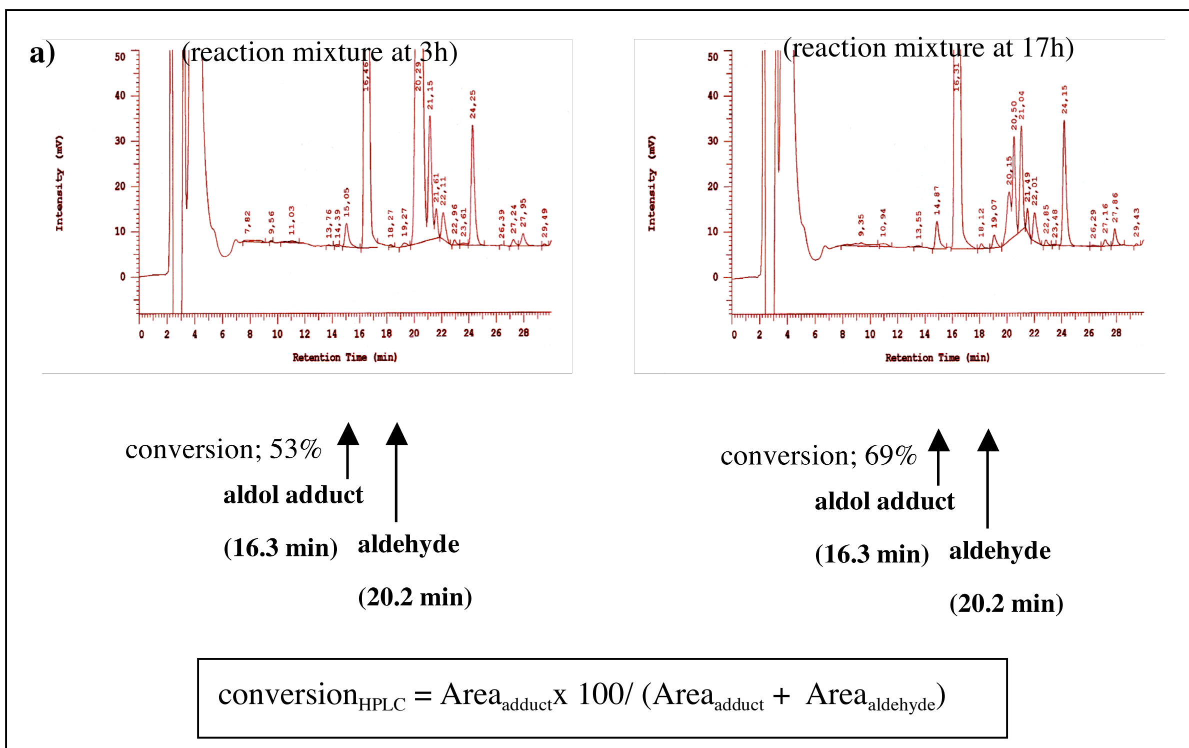
3-1-5. Application of an aminoaldehyde as substrate of FSA

In cooperation with J. A Castillo & P. Clapes (CSIC, Barcelona), an interesting aminoaldehyde (*N*-(benzyloxycarbonyl)-3-amine-propanal; *N*-Cbz-propanal) was applied to FSA (cell free extract after heat treatment: to remove a part of other proteins) and achieved a valuable reaction (Fig.3-20 & -21). The final goal of the study is to prepare for *N*-alkyliminosugars that could be antibacterial agents (Fig.3-22a~22c). The aldol reaction was tested with different quantities of substrates but same ratio of DHA to aldehyde (1.5:1). Each enzyme concentration was 25U/mL (determined by the method given on Materials & Methods 2-6-1), the amount volume of reaction mixture was 2.5mL. Virtually, 0.02 U/mg (300mM-DHA, 200mM-aldehyde at 17h) was obtained as the specific activity at 4°C, a higher value can be expected under optimal conditions. The aldol aduct was more than 99% of a mono-stereo isomer with *3S,4R* configuration, FSA showed high stereoselectivity in the reaction using an aminoaldehyde. Further reaction to iminosugar was performed chemically as depicted on Fig.3-22.



experiment	DHA 1.5 eq.	aldehyde 1.0 eq.	conversion (HPLC)
915	75 mM	50 mM	1h → 51% 3h → 72% 23h → 86% 42h → 83%
919	188 mM	125 mM	3h → 53% 17h → 69%
920	300 mM	200 mM	3h → 42% 17h → 57% 24h → 60% 42h → 57%

Fig.3-20. A novel FSA reaction using aminoaldehyde performed by J. A Castillo & Dr. P. Clapes (CSIC, Barcelona). Enzyme concentration was calculated by measuring the rate of fructose-6P formation from DHA and GAP (U = μmol/min).



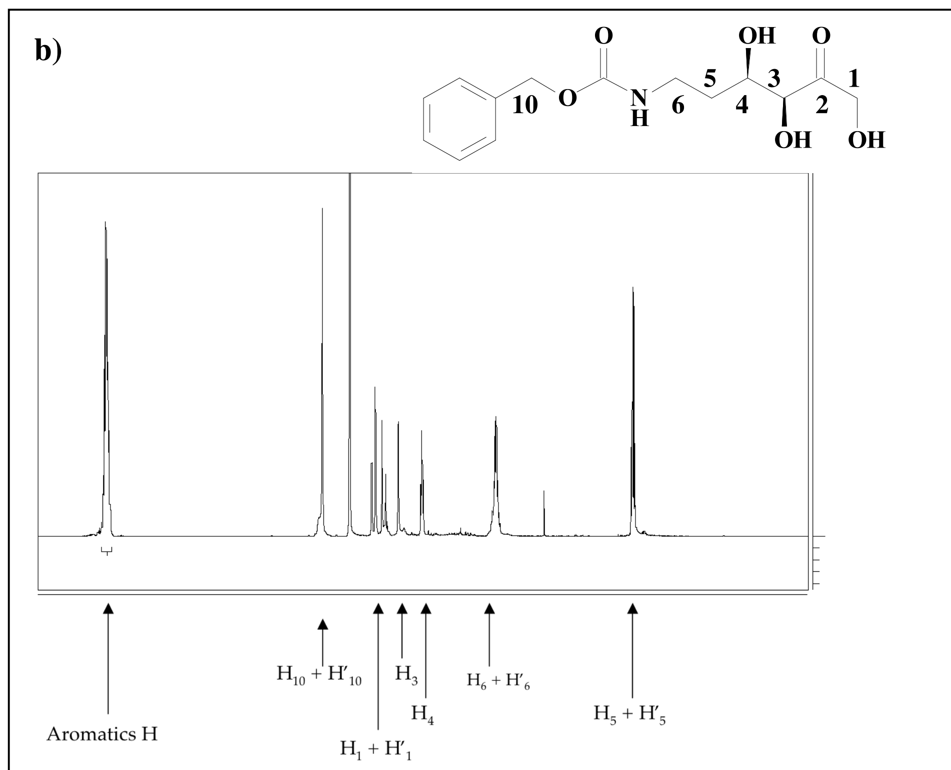


Fig.3-21. Analysis data of FSA novel reaction by J.A Castillo with a) HPLC and b) ^{1}H-NMR.

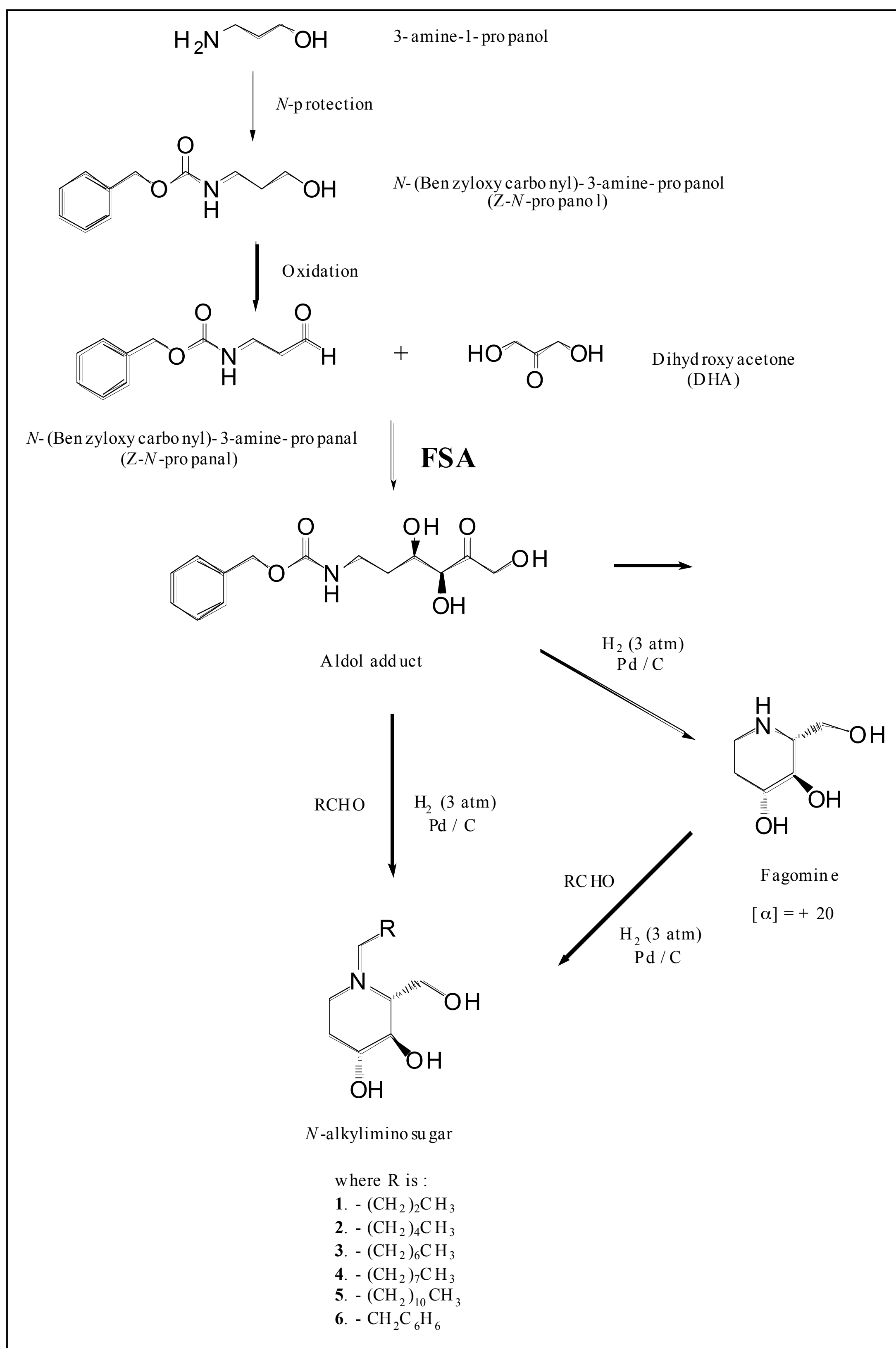


Fig.3-22a. Preparation pathway of *N*-alkyliminosugars with FSA catalyzing aldol reaction (performed by J.A Castillo and P. Clapes (CSIC, Barcelona))

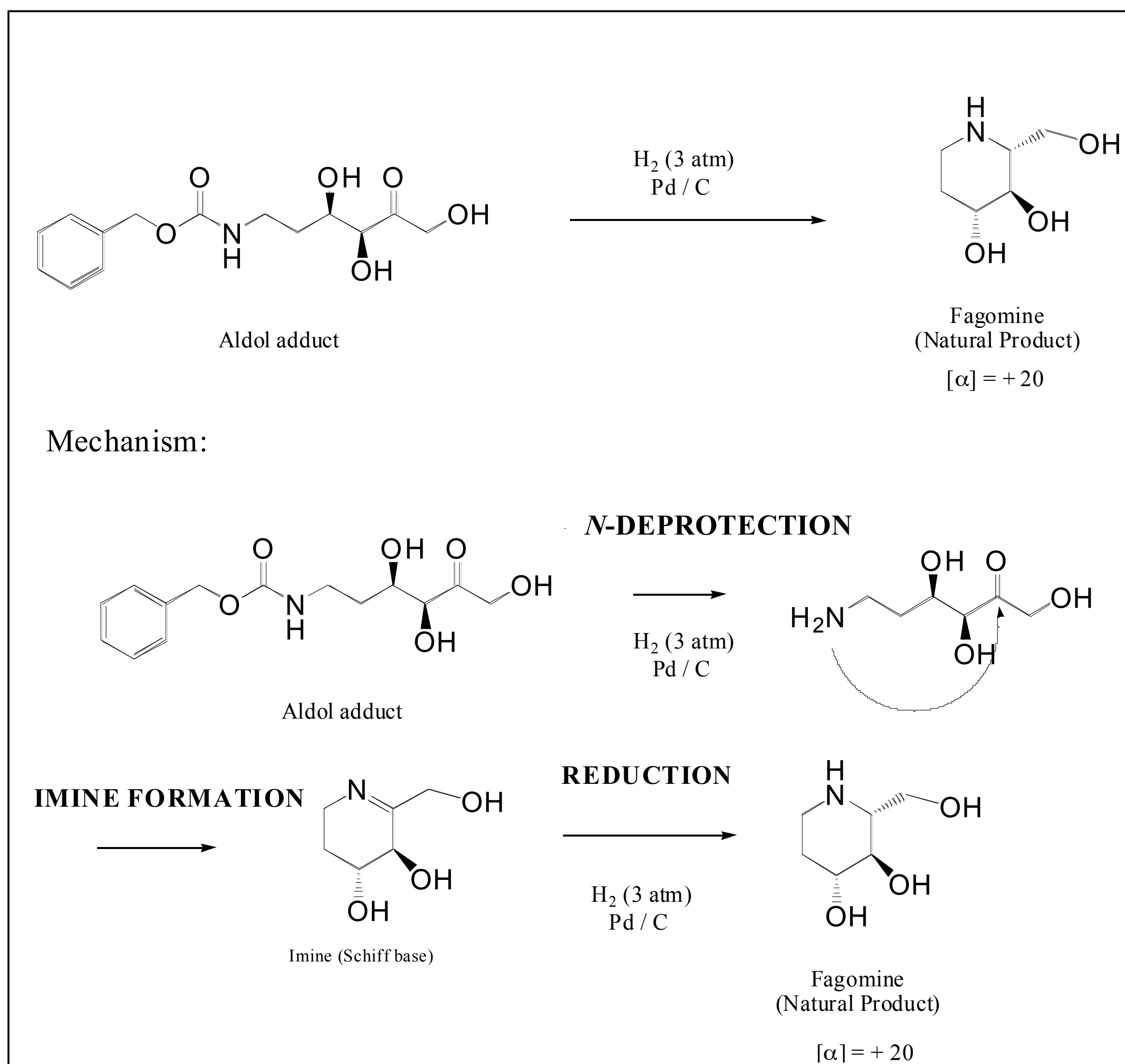


Fig.3-22b. Cyclization of an aldol aduct with a single step. (by J.A Castillo and P. Clapes (CSIC, Barcelona))

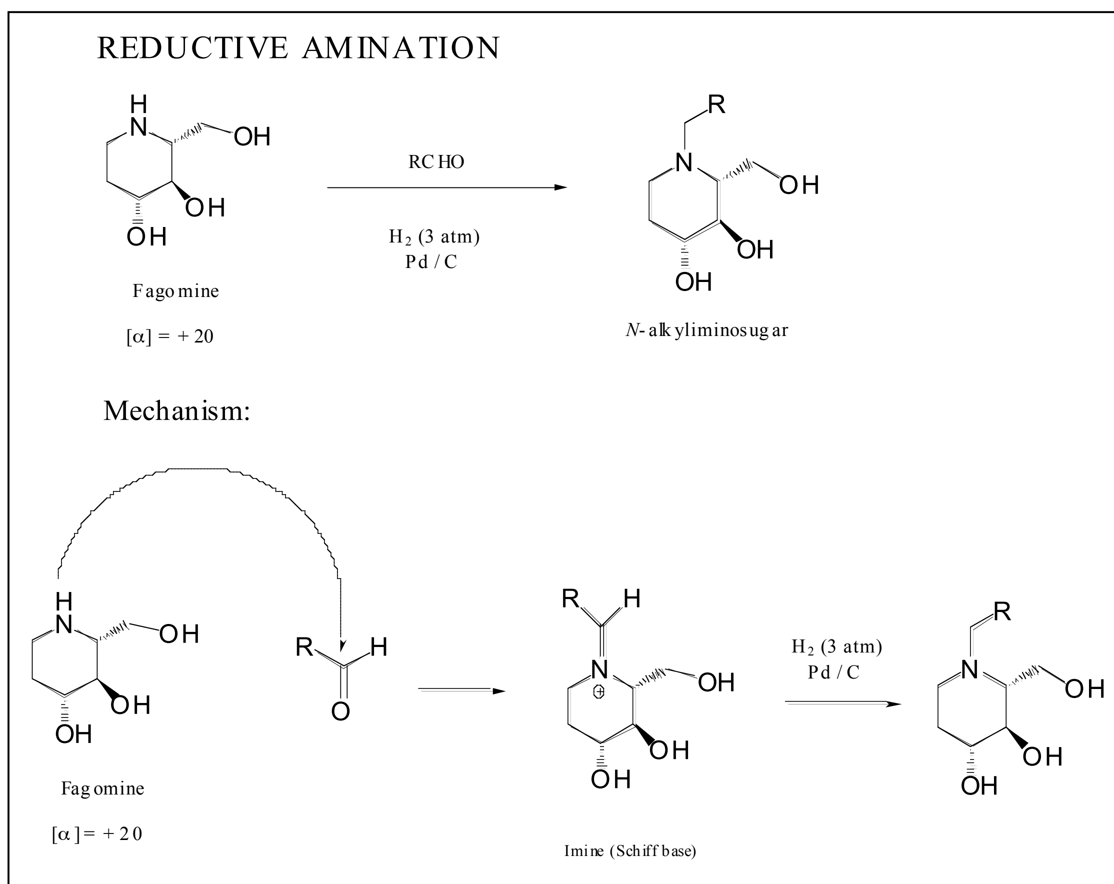
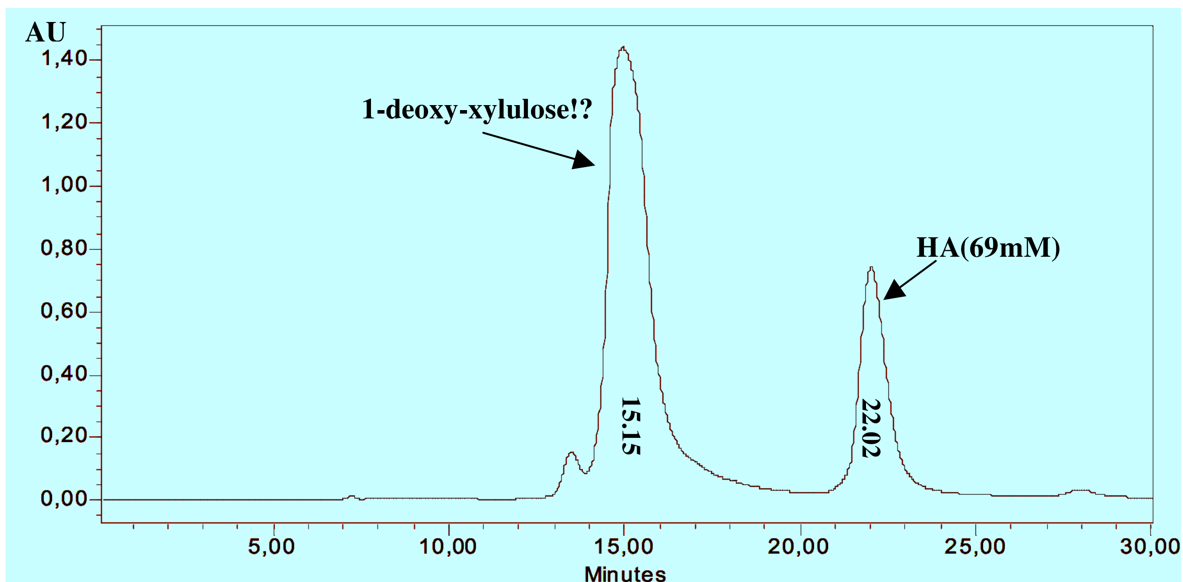


Fig.3-22c. Further reductive amination of fagomine prepared from a aldol aduct. (by J.A Castillo and P. Clapes (CSIC, Barcelona))

3-1-6. FSA reactions at low temperature

FSA activity at 4°C was investigated in two synthetic performances (a)HA+glycolaldehyde with WT, b)DHA+glycolaldehyde with A129S) as J. A Castillo (CSIC, Barcelona) adopted in his experiment. It is advantageous to stabilize unreacted aldehydes and suppress side reactions in the solutions at 4°C. As a result, a new peak expected as 1-deoxy-xylulose at 15.15 min simultaneously with the decrease of HA was observed in reaction a), a peak corresponding to xylulose, disappearance of glycolaldehyde (14.7 min) and decrease of DHA were seen in reaction b) (Fig.3-23). 68% of initial HA was consumed in reaction a) and more than 60% yield of the amount of theoretical producible xylulose in reaction b) were obtained at 24h, respectively. Those were almost equivalent to the results at 30 °C. (conversion rate: 49% in Fig.3-12a, ≥ 60% in the reaction with 200mM DHA, 200mM glycolaldehyde - data not shown)

a) HA+glycolaldehyde with FSA WT (at 4°C)



b) DHA+glycolaldehyde with FSA A129S (at 4°C)

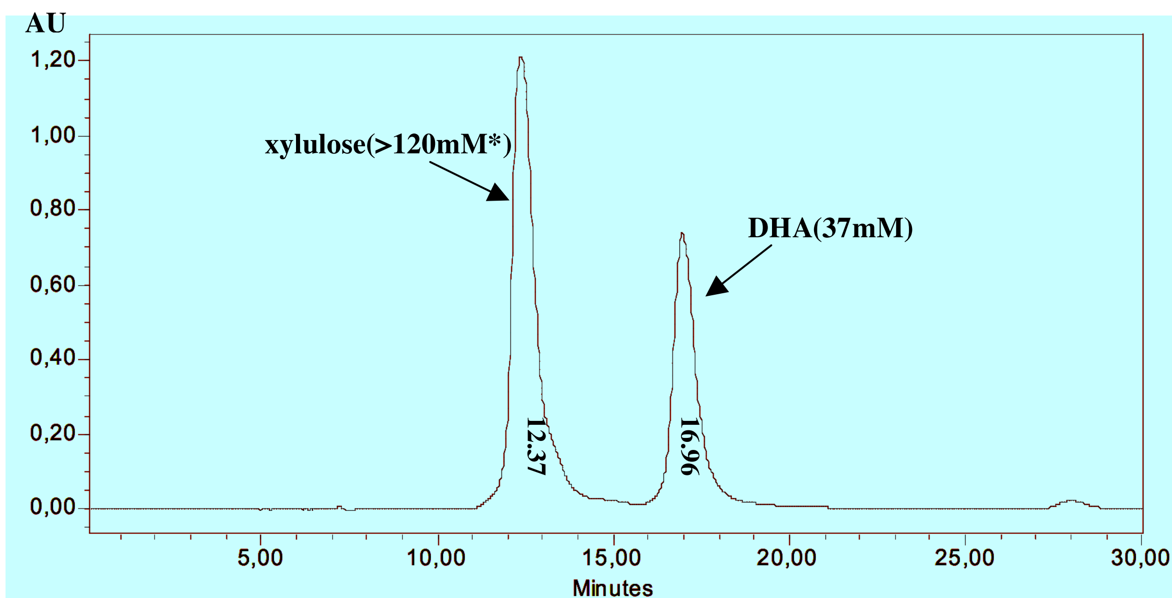


Fig.3-23. HPLC-analysis of the formation a) of 1-deoxy-xylulose from HA (200mM) + glycolaldehyde (200mM) with FSA WT (1.5mg/mL), b) of D-xylulose from DHA (200mM) + glycolaldehyde (200mM) with FSA A129S (1.5mg/mL), after 24h at 4°C. (column; Aminex HPX-87H, mobile phase; 5mM H_2SO_4, temp; ambient temp, flow rate; 0.5mL/min) *: Xylulose could not be calculated correctly because of being beyond the range of standard curve.

3-1-7. Mutagenesis of FSA using error prone PCR

So far, it had been shown that FSA activity could be improved with site-directed mutagenesis (e.g. A129S) and it might have higher activity further, but high stereo-selectivity of the enzyme was still retained. In aldolases that utilize DHAP as donor substrate, various enzymes that have different stereoselectivities are known and all four kinds of configuration at C3 and C4 positions are available (Fig.3-24).

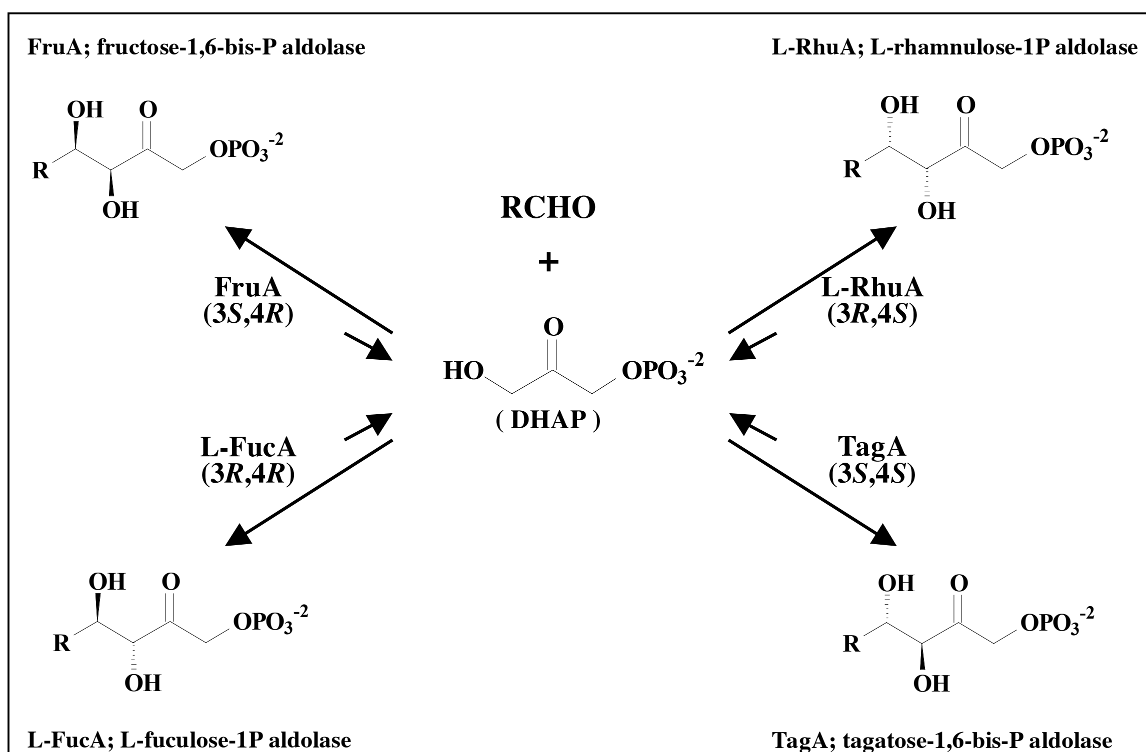


Fig.3-24. Different stereoselectivity with aldolases using dihydroxyacetone phosphate (DHAP) as a donor. An aldehyde as an acceptor substrate is available together with DHAP and new compounds are produced. (Fessner, 1992)

Those aldolases can be very valuable and useful tools in chemo-enzymatic reactions, however, some challenges remain in the high cost, chemical instability and structural inflexibility of DHAP. Therefore, it is supposed that DHA will be utilized instead of DHAP due to its low price and as it possesses two functionally equivalent nucleophilic sites (at C1 and C3 positions). But C-C bonding enzymes that catalyze DHA as donor have not been investigated except FSA up to now. FSA takes only *3S, 4R* configuration. Thus, it is expected that FSA will be altered by directed evolution to acquire new

stereoselectivities at C3 and C4 positions (Fig.3-25). Potentially possible configurations are *D-threo* (with FSA WT), *L-erythro*, *D-eythro* and *L-threo* types. For instance, when DHA and glyceraldehyde-3-phosphate are used as substrates, D-fructose-6P (*3S,4R*) with FSA WT, D-tagatose-6P (*3S,4S*), D-psicose-6P (*3R,4R*), and D-sorbose-6P (*3R,4S*) are expected products.

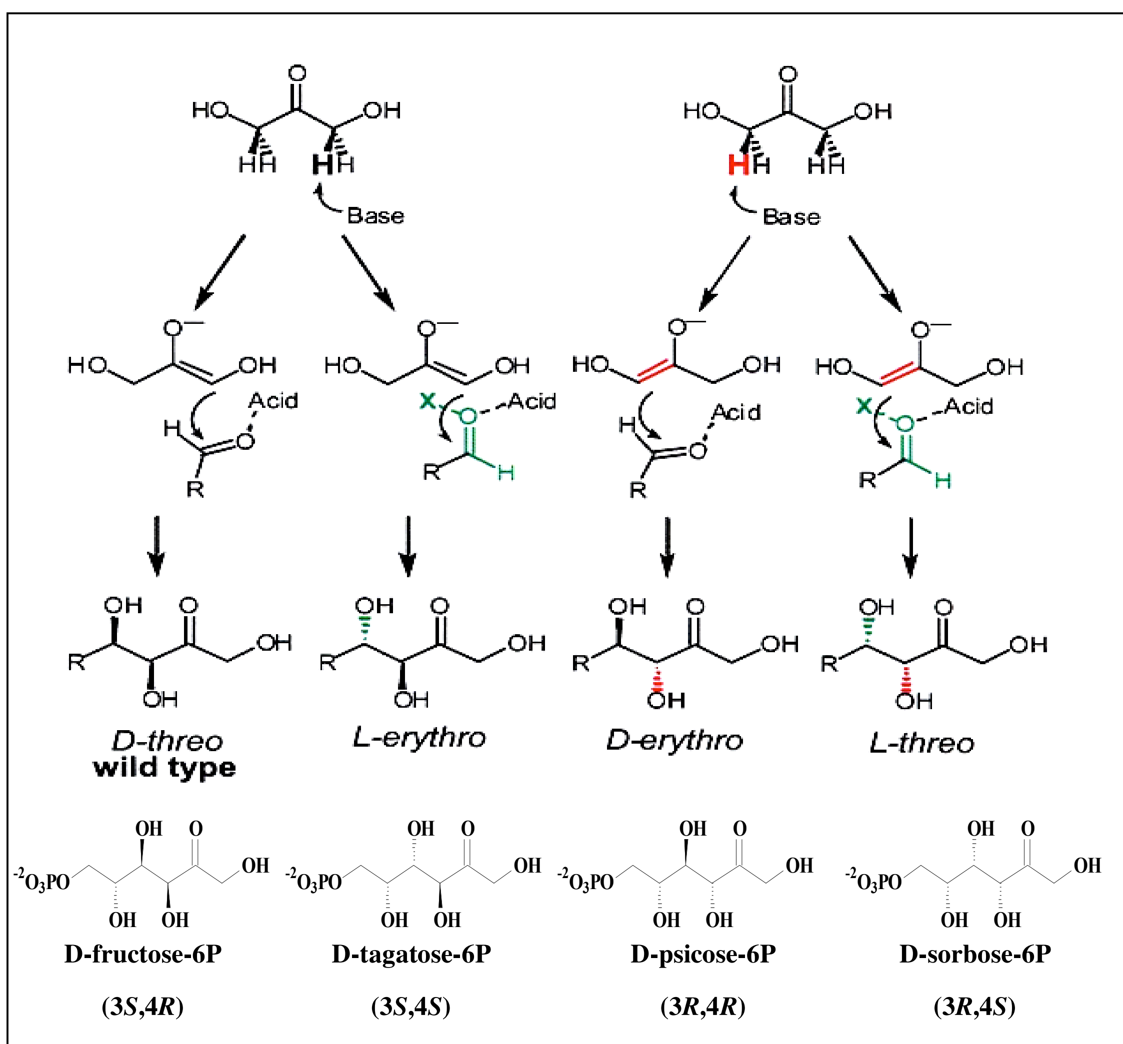


Fig.3-25. Utilization of DHA as a donor and potential reactions with FSA altered by directed evolution. FSA WT creates only products that have *D-threo* configuration (e.g. D-fructose-6P), other stereoisomers might be prepared by using FSA altered with error prone PCR. (Fessner, W.-D., Sprenger, G.A. (2004) Application for a grant of the Deutsche Forschungsgemeinschaft under SPP1170)

In an experiment, error prone PCR was executed to obtain mutant *fsa* genes (details of error prone PCR are in Materials & Methods part), PCR products were inserted into pET16b plasmid vector. The ligation mixture was introduced into *E.coli* BL21(DE3)Star, pLysS. 3,500~5,000 transformants were found on a large agar plate (ϕ145mm) of LB-medium containing ampicillin (100mg/L) and chloramphenicol (25mg/L) (Fig.3-27 :left). Several transformants were picked up and assayed for FSA activity by using a multi-well plate after enzyme induction with IPTG in liquid LB-medium.

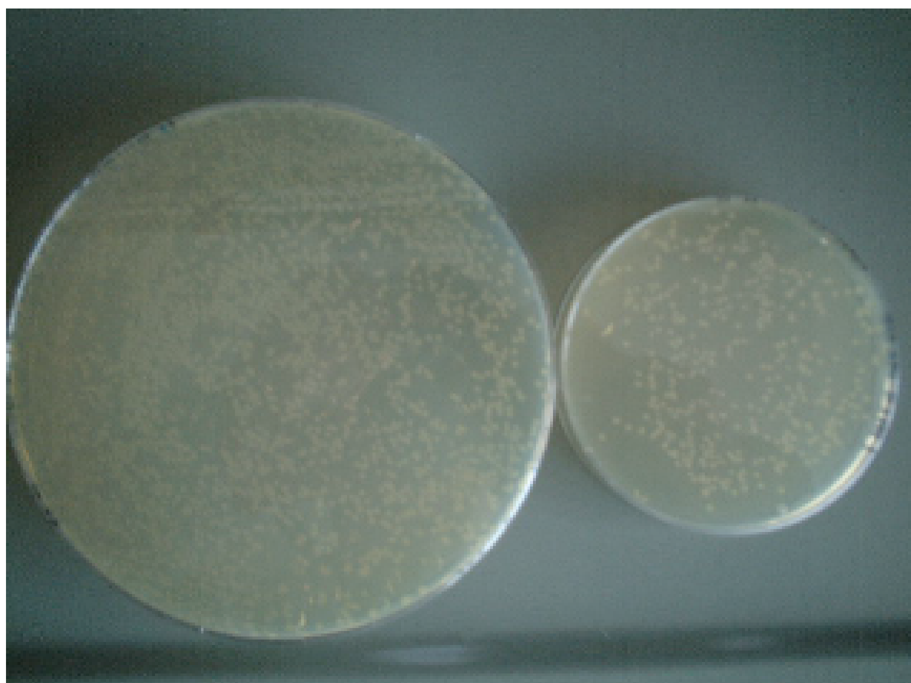


Fig.3-26. Single colonies containing FSA mutants on a large agar plate (left; ϕ:145mm) and a general size agar plate (right; ϕ:92mm). 200μl of cell suspension after transformation was spread on a large plate and 3,500~5,000 colonies were found.

As shown in Fig.3-27, FSA activity was visualized and assayed easily in a coupled assay system with the reduction of nitroblue tetrazolium (NBT) by diaphorase (Fig.3-27a). The higher activity samples have, the darker color they show (Fig.3-27b). In an experiment, eighty-four colonies were picked randomly and compared in activity with FSA WT. 16 of them showed higher activity than the average of FSA WT did, 14 samples indicated lower activity. (The others had almost same activities as FSA WT.)

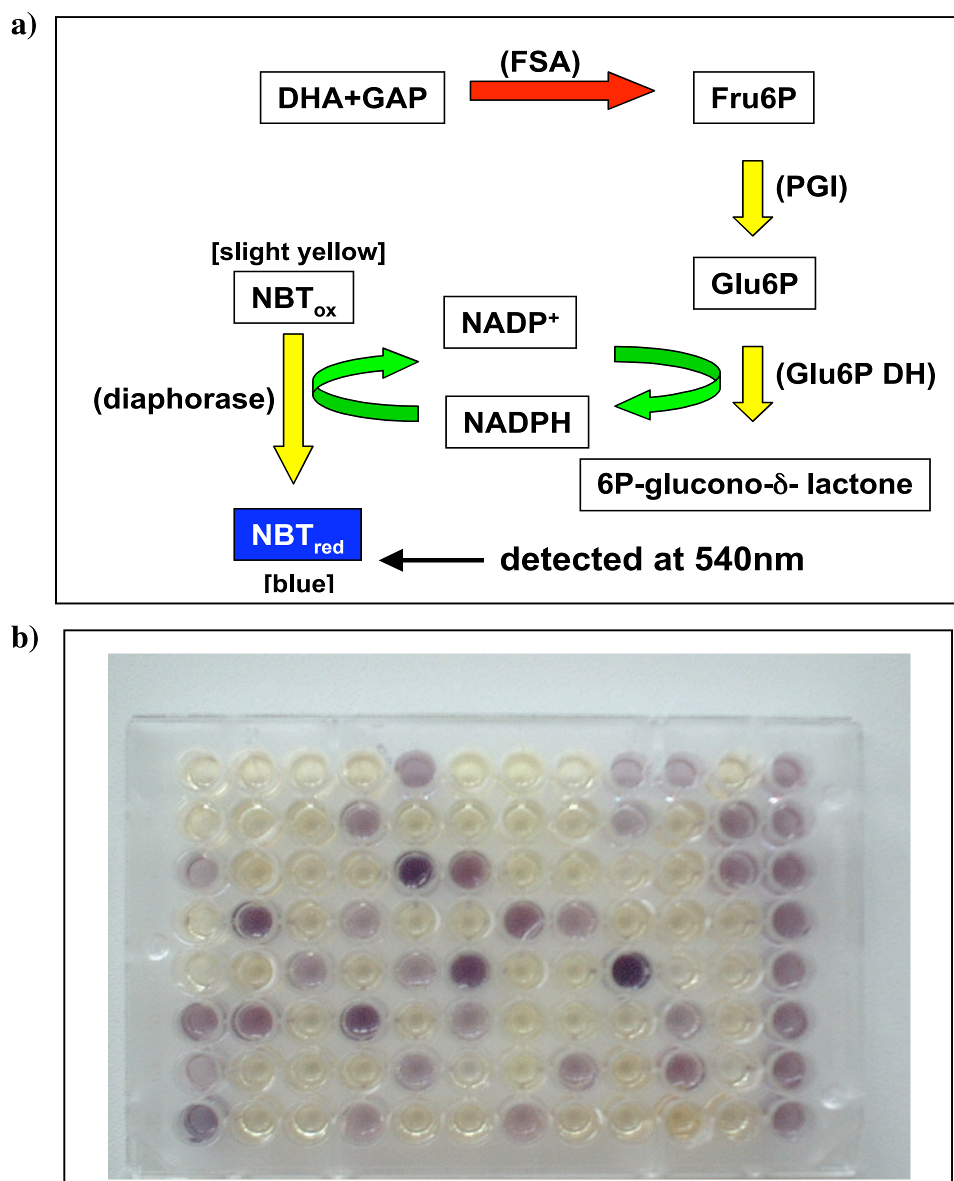


Fig.3-27. a) Reaction scheme of colorization with multi-well assay. (DHA:50mM, GAP:2.8mM, NADP:0.5mM, NBT (nitroblue tetrazolium chloride):0.5mM, PGI:1.75U/mL, Glu6PDH:0.5U/mL, diaphorase:0.3U) NADPH is oxidized by diaphorase concomitantly with NBT reduction. Reduced form of NBT shows blue color, it is detected at 540nm photometrically. b) Visualization of FSA activity by colorization with multi-well assay. The higher activity a sample has, the darker blue color it shows in 5-10 min (Details are given in Materials & Methods 2-6-3, samples on the right lane were FSA WT).

Those thirty samples were further investigated by DNA sequencing to check which nucleotides were altered and which amino acid residues were changed. Virtually, two

samples were identical to FSA WT in DNA sequence, one had a quite different sequence (no identity to known sequences) and the remaining 27 samples were mutated. Those 27 samples consisted of different variants with 1 to 6 nucleotides alteration, with 0 to 5 amino acids change or producing short proteins due to the formation of stop codon (Table.3-9).

Table 3-9. The number (N) of DNA bases and amino acids altered with error prone PCR. DNA sequences and amino acid sequences were compared with those of FSA wild type.

N of altered DNA	sample N*[1]	N of altered amino acids	sample N*[1]
1	5 samples	0	1 sample
		1	4 samples
2	5 samples	1	2 samples
		2	3 samples
3	6 samples	2	2 samples
		3	4 samples
4	6 samples	2	2 samples
		3	2 samples
		shortened*[2]	2 samples
5	3 samples	3	1 sample
		4	1 sample
		5	1 sample
6	2 samples	4	2 samples

*1; DNA sequences of 30 samples were investigated and mutations were recognized in 27 of 30 samples (by GATC Biotech GmbH).

*2; Stop codon was created on a part of *fsa* gene by alteration.

Obtained 26 mutant proteins (1 of 27 was not changed in amino acid sequence) were altered variously. 6 samples were altered at 1 residue, 7 samples at 2 residues, 7 samples at 3 residues, 3 samples at 4 residues, 1 sample at 5 residues and 2 samples were shorter ORFs formed by appearance of stop codon (Table.3-10).

Table 3-10. FSA mutants obtained with error prone PCR. Blue letters indicate mutant FSAs that showed higher activities than wild type FSA did with multi-well assay, while brown ones mean that those samples had lower activity.

The number of altered amino acids	obtained FSA mutants
1	**V88M, Y127F, H47L, A199V, G54E, K37R**
2	**A157T-T185A, P158Q-M194K, Q59H-H150Y R18C-A157G, V41F-Q193P, H150L-A214G I96V-A129T**
3	**G35C-A95T-L178P, E48V-L118P-S120T K37I-G113D-A199T, D40E-H156R-V204M A15T-E48V-S166N, Y4D-Q53H-I104V V13M-P21T-L93M**
4	**A34V-D71G-M155I-Y197H K14M-G54V-C181Y-Q212R Y112N-G124D-A129G-D210E**
5	**V10D-A58V-A95D-K154N-W211C**
appearance of stop codon*	2 samples (111 amino acids chain & 51 amino acids chain predicted)

*; Full length of FSA is 220 amino acids

For further investigation, eight samples were overexpressed in BL21(DE3)Star pLysS, proteins were induced with IPTG (1mM) in 50mL cultures. Crude extracts and protein solutions after heat treatment were examined with SDS-PAGE gel electrophoresis (Fig.3-28, Fig.3-29), FSA activity using DHA and GAP were assayed for those samples (Table.3-11).

In crude extracts, 5 of 8 mutants showed a strong band on the SDS-PAGE gel corresponding to FSA (220 amino acids; 23kDa) as well as WT did, whereas 3 samples did not (Fig.3-28). 4 mutant samples (K37R, H47L, V88M and I96V+A129T) retained FSA activity (72~148% of that WT had), the others (Q59H+H150Y, Y112N+G124D +A129G+D210E, 111 amino acids ORF and 52 amino acids ORF) did not have activity (Table3-11). Molecular masses of 2 short proteins would be 12.3 kDa and 5.8 kDa, respectively, those 2 short proteins and a mutant FSA Y112N+G124D +A129G+D210E might be unstable and degraded easily, thus they couldn't be seen on the gel.

4 mutant samples showing FSA activity in crude extracts and FSA WT as a positive control were incubated at 75℃ for 40min (heat treatment), supernatants after ultracentrifugation (18,000 x g) were applied to SDS-PAGE gel electrophoresis (Fig.3-29), and FSA activity for each sample was measured. In result, 4 samples except V88M held FSA in the solution after heat treatment and showed activity (53~110% of that with WT), while V88M lost both the corresponding band on the gel and the activity (Fig.3-29 & Table.3-11). The alteration of Val88 into Met apparently made protein structure unstable, the mutant FSA was denatured by heat treatment and precipitated through centrifugation (cell-free extract after heat treatment did not contain the corresponding protein on SDS-PAGE). Interestingly, activities in crude extracts did not always fit in those with a simple assay using multi-well plates. Indeed, four of eight samples indicated higher activity than WT in multi-well assay method, though those retained lower or almost no activity in usual assay method. Thus, the standard assay is required to evaluate exact activities of mutant samples after multi-well plates assay.

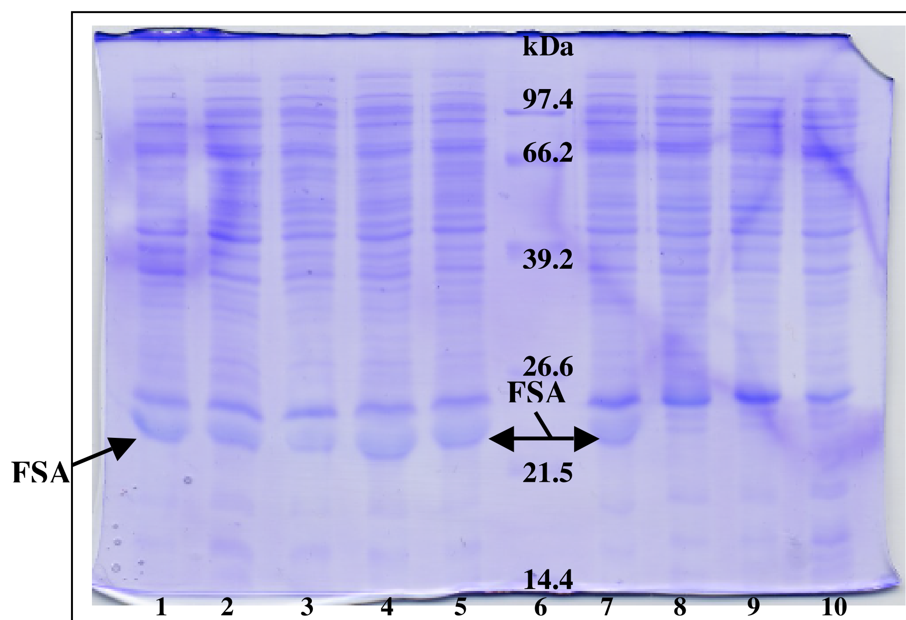


Fig.3-28. SDS-PAGE analysis of mutant FSAs in crude extracts expressed with pET system (on pET16b in *E.coli* BL21(DE3)Star pLysS). Equal amounts of protein (20 µg) were applied to each lane; lane1: WT, 2: V88M, 3: Q59H+H150Y, 4: H47L, 5: K37R, 6: standard marker, 7: I96V+A129T, 8: short protein (111 amino acids), 9: short protein (52 amino acids), 10: Y112N+G124D+A129G+D210E.

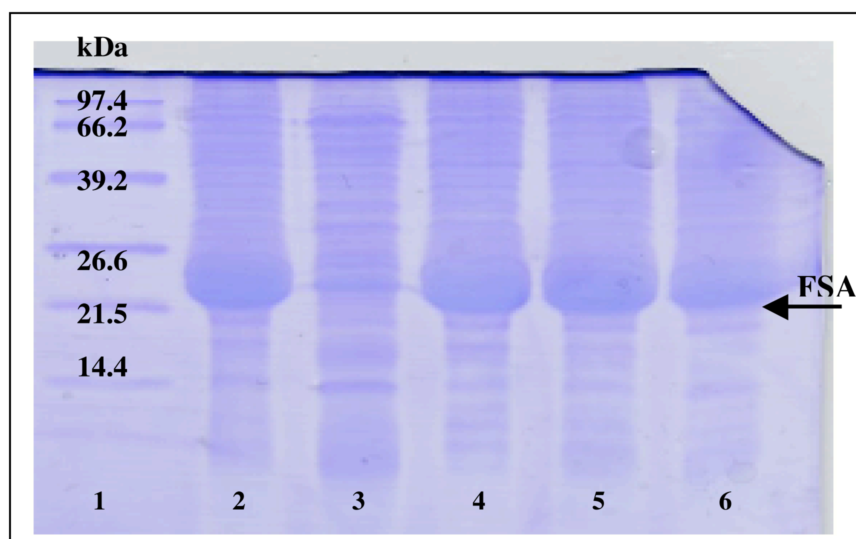


Fig.3-29. SDS-PAGE analysis of mutant FSAs after heat treatment. Equal amounts of protein (20 µg) were applied to each lane; lane1: standard marker, 2: WT, 3: V88M, 4: H47L, 5: K37R, 6: I96V+A129T.

Table 3-11. Assay of mutant FSAs obtained by error prone PCR.

sample	multi-well assay (comparison with WT)	Spec. activity(U/mg) (in crude extact)	activity(U/mg) (after heat treatment)
WT	-	2.9(100%)	5.8(100%)
K37R	**higher**	4.3(148%)	6.4(110%)
H47L	**higher**	4.3(148%)	5.8(100%)
V88M	**lower**	2.2(76%)	denatured
Q59H-H150Y	**higher**	0.1(3%)	not measured
I96V-A129T	**higher**	2.1(72%)	3.1(53%)
Y112N-G124D -A129G-D210E	**higher**	0.0(0%)*	not measured
short protein 1 (111 amino acids)	**higher**	0.0(0%)*	not measured
short protein 2 (52 amino acids)	**lower**	0.0(0%)*	not measured

*: Expected proteins were not recognized in crude extracts with SDS-PAGE.

Specific activities were determined by measuring Fru6P production rates from DHA(50mM) + GAP(2.8mM) with 10-25 μg proteins. Both multi-well and usual photometric assays were done one time, respectively. FSA samples were prepared by culture of *E.coli* BL21(DE3)Star pLysS + pET16b for each mutant FSA in 50mL LB medium including antibiotics and by induction with IPTG for 4h.