3-1-4. Reactions using mutant FSA A129S

Another mutant enzyme, A129S, which has serine at 129th residue instead of alanine (Fig.3-11a), has high potential to catalyze the reactions using DHA as a donor substrate. Virtually, the kcat/Km value for DHA is more than 12 fold of that of WT (Schürmann, 2001). This implies that larger amounts of products can be acquired with A129S than with WT, or unfavorable reactions with WT may be accomplished with the mutant enzyme. Firstly, FSA A129S was produced in DH5α with pUC18 plasmid vector and purified with same method as WT was done (Fig.3-11b, Table 3-8).
This mutant enzyme was overexpressed well and a strong band corresponding to FSA was seen on SDS-PAGE gel (as 10~15% of total protein in crude extract). In addition, this mutant enzyme held the stability against high temperature. Thus, a heat treatment step was part of the purification protocol. However, protein solution obtained from hydroxyapatite column of the final purification step showed still a few extra protein bands on SDS-PAGE gel (Fig.3-11b). Although it contained those extra proteins, the purity appeared to be more than 90% on the gel and the specific activity was quite high enough to assay the catalytic ability of the enzyme for several substrates. Indeed, even crude extract showed higher specific activity than purified WT had (Table 3-8).

Three reactions (scheme 3-1) were probed to assay catalytic ability of both FSA WT and A129S. Dihydroxyacetone (DHA) or hydroxyacetone (HA) were used as donor substrates, and formaldehyde or glycolaldehyde as acceptor. As products, *S*-erythrulose from DHA and formaldehyde, D-xylulose from DHA and glycolaldehyde, 1-deoxy-D-xylulose from HA and glycolaldehyde were expected, respectively. Reactions were performed in 1.0 mL volumes at 37℃ for 24hours, consumptions of substrates and appearance of new peaks were investigated with HPLC. Reaction mixtures were analyzed with NMR after removal of proteins (quenching reactions with methanol -> centrifuge -> filtration by using Celite-Charcoal) by J.A. Castillo in the Institute for Chemical and Environmental Research, Barcelona, Spain.

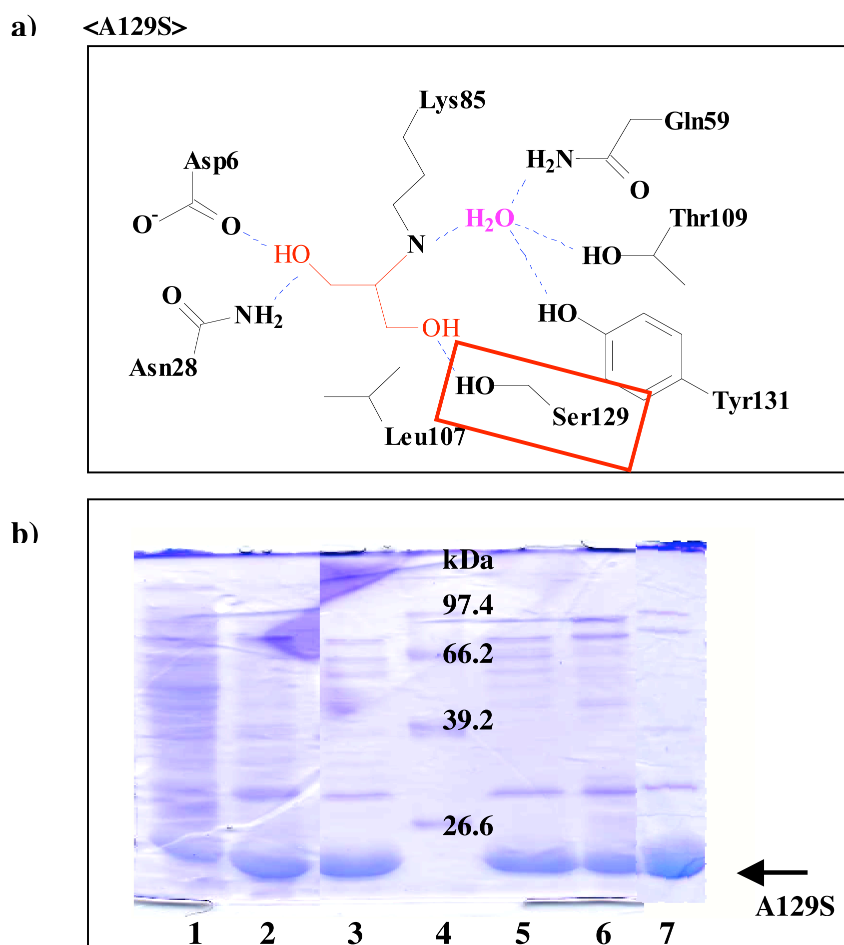
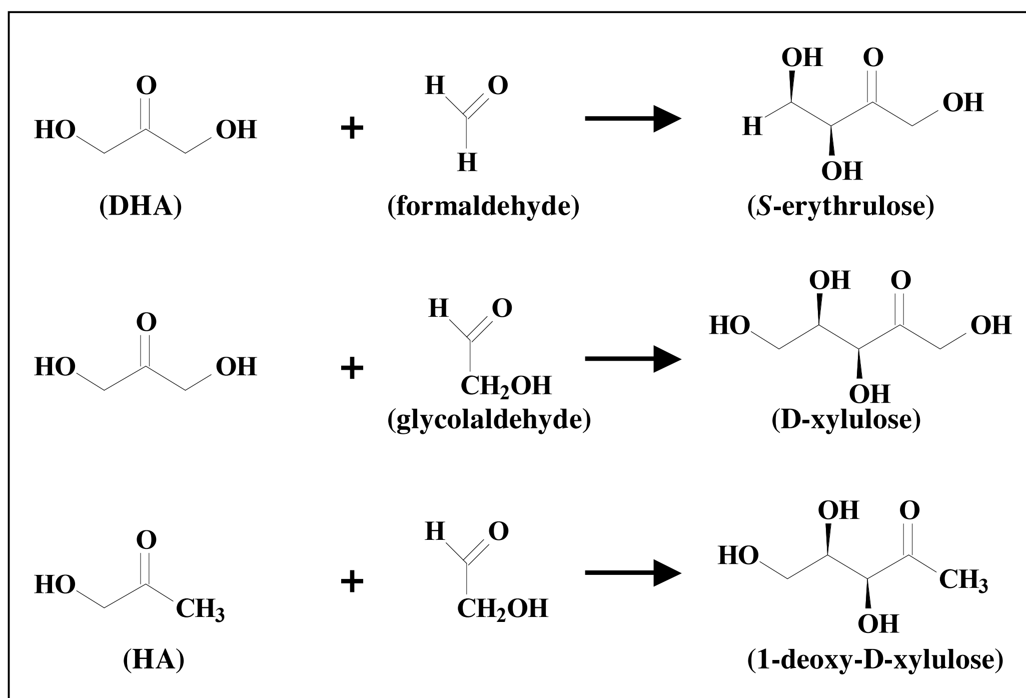


Fig.3-11 a) Two-dimensional model of the active center of FSA A129S with DHA; b) SDS-PAGE analysis of A129S on each purification step. Each lane contains 20μg of protein; lane1: crude extract, 2: protein solution after heat treatment, 4: standard protein marker, 3~6: fractions eluted from Q-Sepharose HP, 7: flowthrough from hydroxyapatite.

Table 3-8. Purification scheme for FSA A129S in method1 (DH5α pUC18fsaA129S).

sample	Amount of protein (mg)	Spec. activity (U/mg)	Amount of activity (U)	Residual FSA quantity (%)	Purification factor
crude extract	255	16.1±3.0	4104	100	1
heat treatment	83	45.8±8.0	3817	93	2.8
Q-Sepharose HP	49	65.7±15.0	3217	78	4.1
hydroxyapatite	34	91.1±10.0	3098	75	5.7

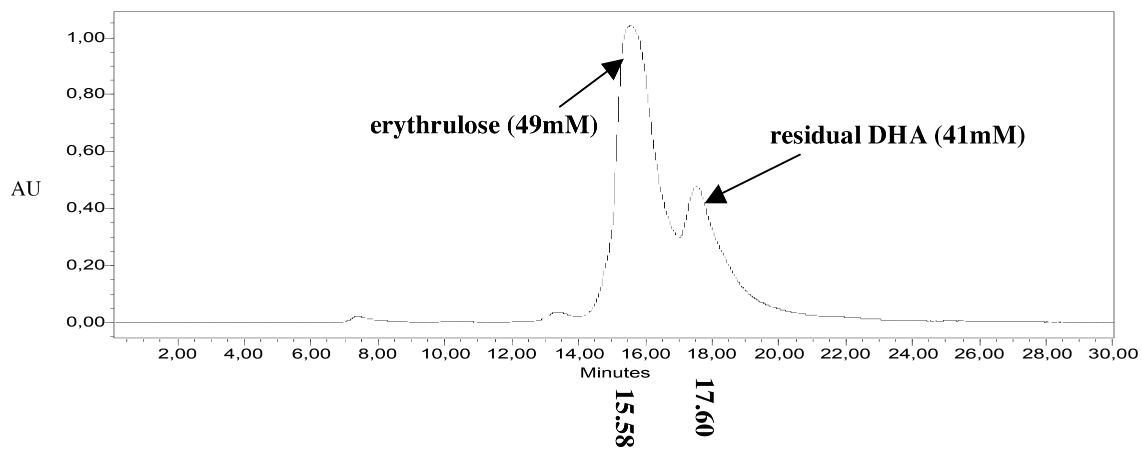
Activities were determined by measuring Fru6P production rates from DHA(50mM) + GAP(2.8mM) with 3 to 10μg proteins in a coupled enzyme assay (auxiliary enzymes: PGI and Glu6P DH cf. 2-6-1). Values were the means of at least 3 independent measurements. The amount of protein or FSA activity was in 1L culture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



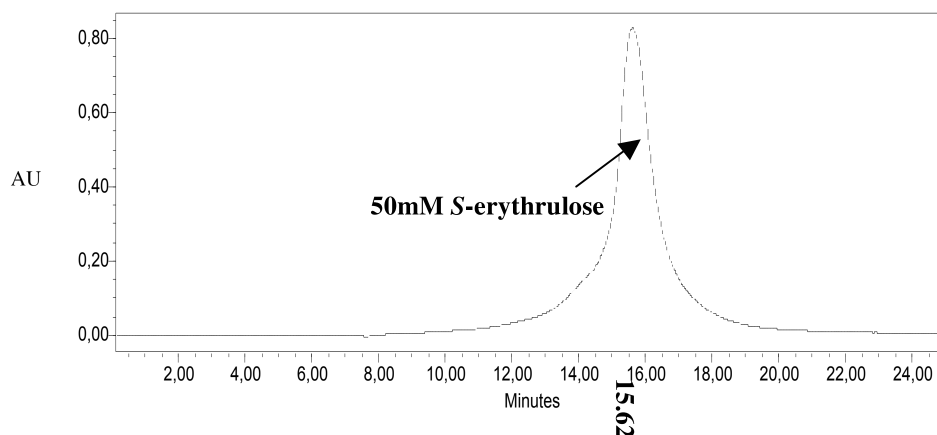
Scheme 3-1. Reactions catalyzed with FSA and expected products.

In *S*-erythrulose production from DHA (100mM initial conc.) and formaldehyde (200mM initial conc.), the decrease of DHA and a new peak corresponding to erythrulose were observed in both reactions with WT and with A129S. Wild type gave 49mM of erythrulose, the A129S mutant showed 80mM of erythrulose production, respectively. A solution without enzyme showed only a peak of DHA (formaldehyde could not be detected under the given condition) (Fig.3-12). A129S was very active for the reaction and a peak of DHA disappeared completely (Fig.3-12c), whereas a sample with WT showed residual DHA (41mM) in the reaction mixture. A sample without enzyme retained DHA at the initial concentration. Reaction mixtures were analyzed by ^{1}H-NMR (by J.A Castillo and P. Clapes, CSIC, Barcelona) to assay stereoconfiguration of products. From the result of comparison of commercial product of *S*-erythrulose (Sigma) with reaction mixtures (Fig.3-13), only *S*-enantiomer was detected in reaction mixtures. It indicates that FSA has high stereoselectivity and did not produce other stereoisomers (less than 1%). Transketolase (TKT) from *Escherichia coli* uses *S*-erythrulose as a donor substrate and transfers a dihydroxyethyl moiety from it into an acceptor substrate (Datta et al., 1960a, 1960b; Hecquet et al., 2001), thereby producing a new ketose and glycolaldehyde (scheme.3-2, Fig.3-14). In case that *S*-erythrulose exists in a reaction mixture, TKT can catalyze it and the activity can be measured.

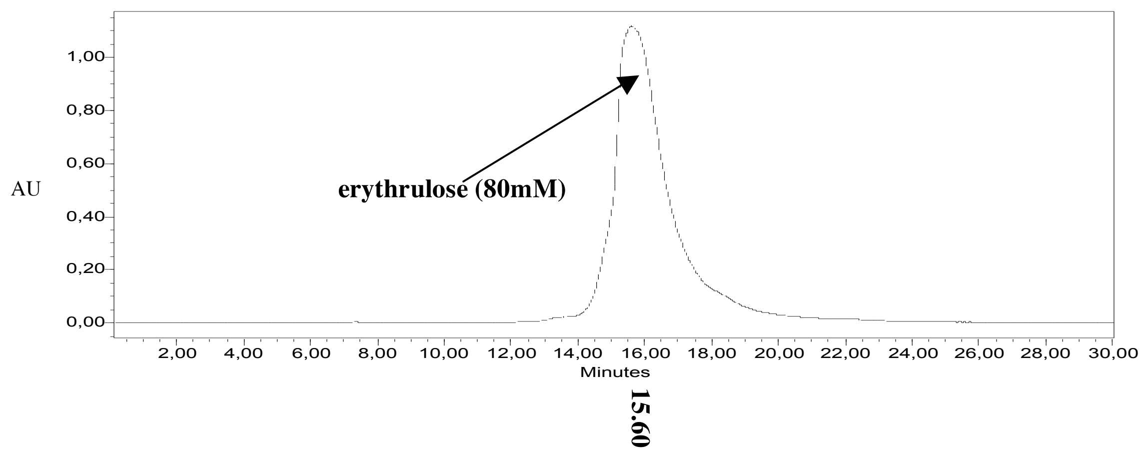
a) DHA+HCHO WT 24h



b) standard *S*-erythrulose 50mM



c) DHA+HCHO A129 24h



d) without enzyme (negative control)

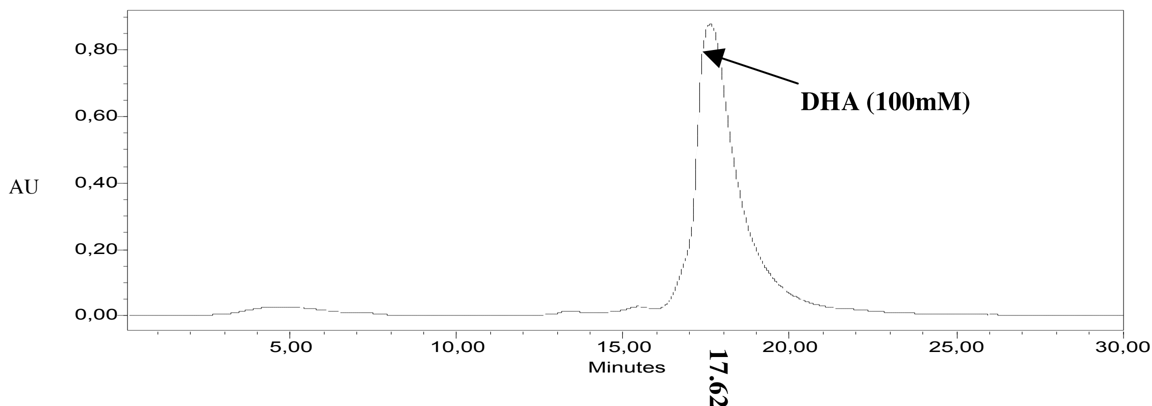


Fig.3-12. HPLC-analysis of the reaction of DHA (100mM) + formaldehyde (200mM); a) with FSA wild type (0.94 mg-protein/mL), b) 50mM standard *S*-erythrulose applied to HPLC , c) with mutant FSA A129S (0.94 mg-protein/mL), d) without enzyme, after 24h at 30°C (Each reaction volume was 1mL). Retention time: 15.6min- erythrulose, 17.5min- dihydroxyacetone detected at 192 nm. (Formaldehyde cannot be detected under the condition- column; Aminex HPX-87H, mobile phase; 5mM H_2SO_4, temp; ambient temp., flow rate; 0.5 mL/min.)

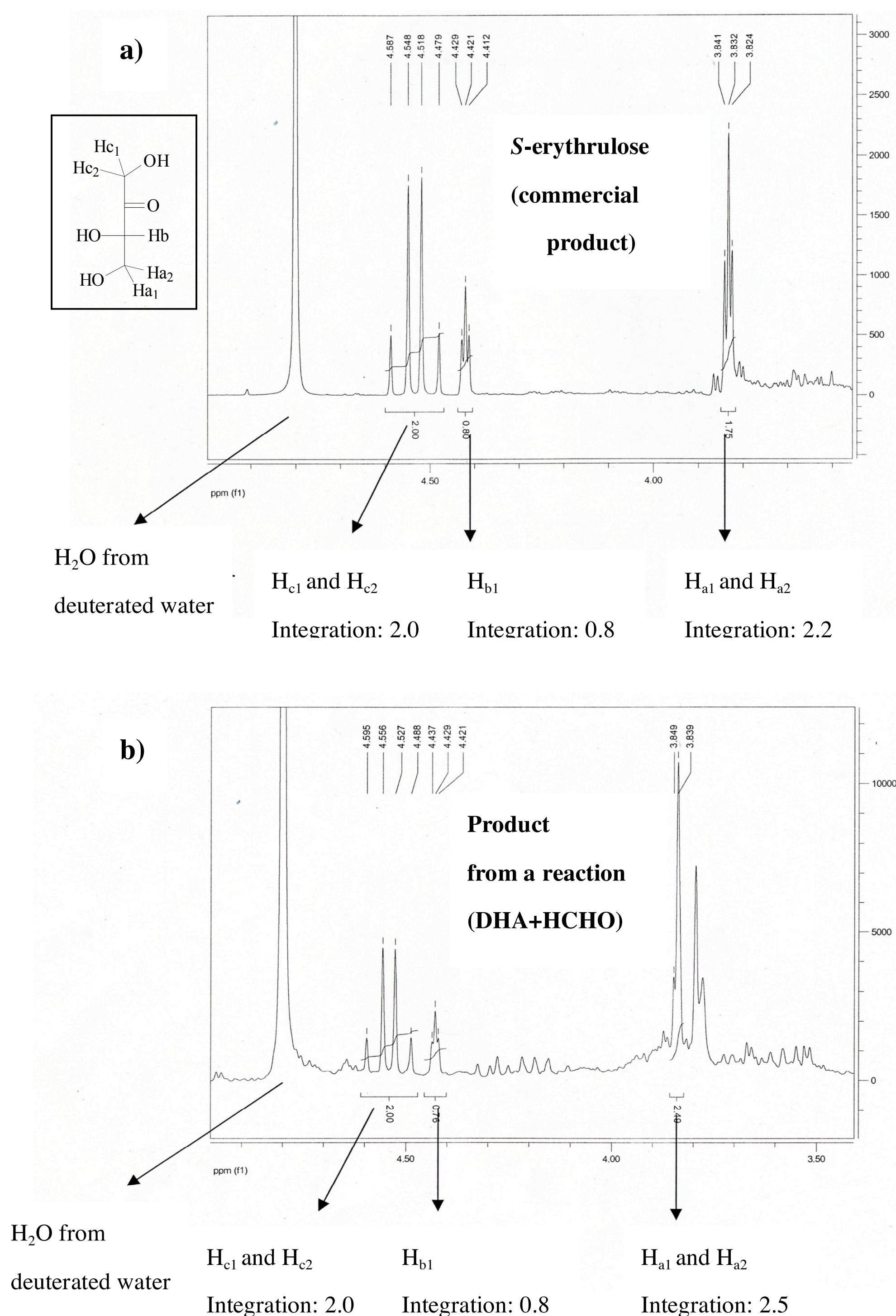
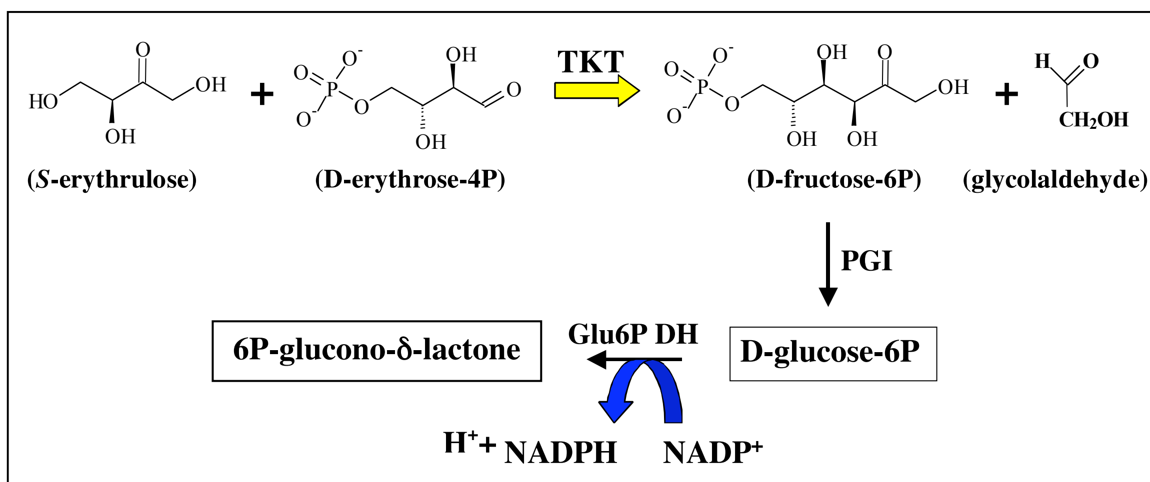


Fig.3-13. ^{1}H-NMR spectrum; a) commercial product of *S*-erythrulose, b) reaction mixture of DHA+HCHO with FSA WT (after removal of FSA). (Analysis by J.A. Castillo in Institute for Chemical and Environmental Research; Barcelona, Spain.)

A TKT reaction using D-erythrose-4-phosphate (Ery4P) as acceptor substrate was performed with utilization of 2 auxiliary enzymes (PGI, Glu6P DH) and NADP as given in scheme.3-2. Thus, presence of *S*-erythrulose can be shown in a spectrophotometric assay. An increase of NADPH was observed in both reaction mixtures of FSA WT and of A129S, indicating existence of *S*-erythrulose in the solutions (■;Fig.3-14b, ●;Fig.3-14b). In particular, A129S sample (■;Fig.3-14b) showed higher TKT activity than the sample containing 10mM of commercial *S*-erythrulose did (■;Fig.3-14a) after dilution of the sample by 25%. It implies that A129S sample contained more than 40mM *S*-erythrulose in the reaction mixture. In samples lacking Ery4P (▲;Fig.3-14b) or *S*-erythrulose (●;Fig.3-14a), the increase of Abs340 did not observed for 20 min. D-glucose-6-phosphate (Glu6P) included in commercial products of Ery4P reacted with Glu6P DH and NADP, NADPH concentration raised in 1~2 min without TKT (◆;Fig.3-14b). If both *S*-erythrulose and Ery4P exist in a reaction mixture and TKT catalyzes those, the gradual increase of NADPH concentration continues more than 5 min by the progressive production of Glu6P via Fru6P.



Scheme.3-2. Transketolase (TKT) reaction with *S*-erythrulose as donor and D-erythrose-4P as acceptor substrate. Reduction of $NADP^+$ is measured at 340nm photometrically. (cf. Ex.1 for photometrical measurement in Materials and Methods)

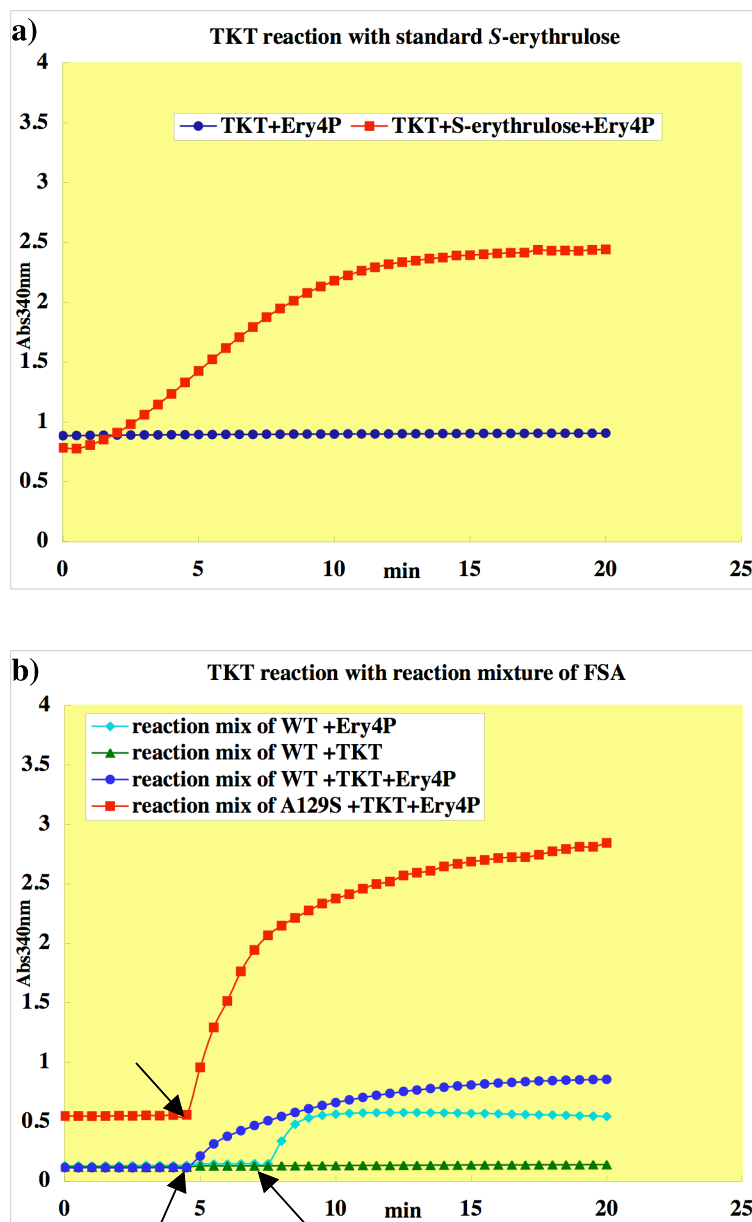
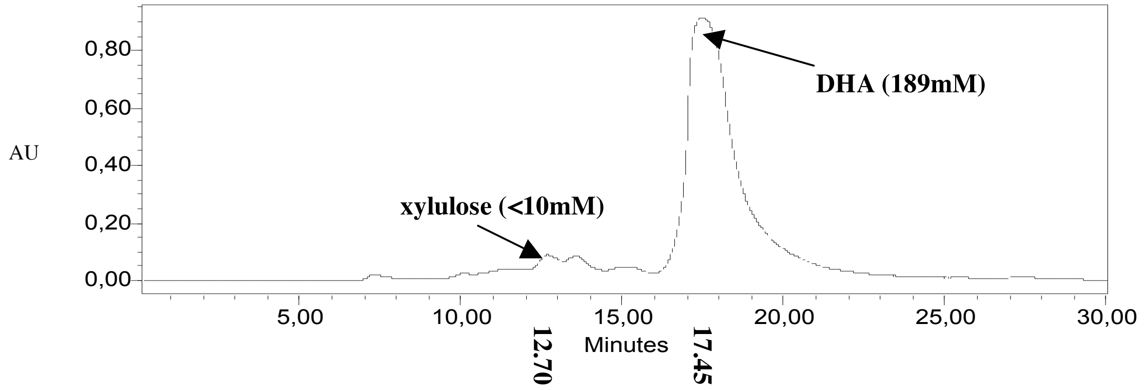


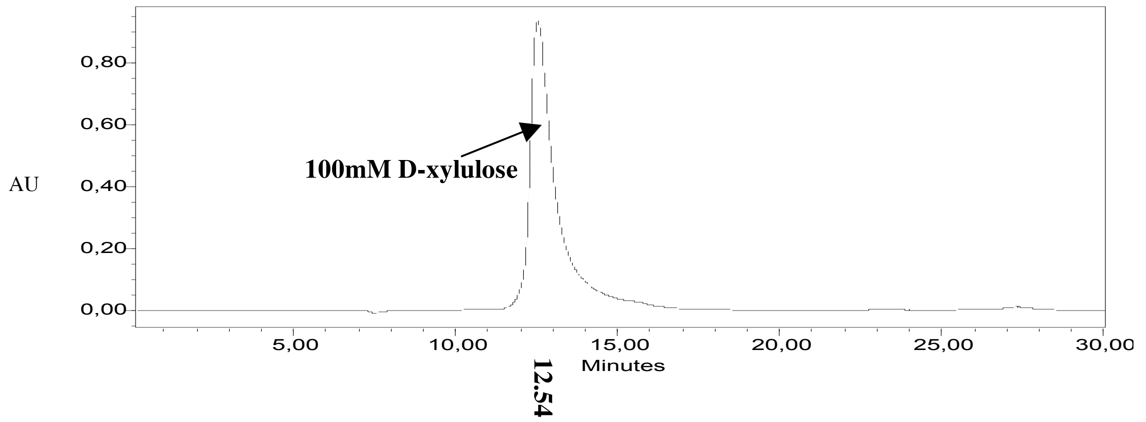
Fig.3-14. Investigation of *S*-erythrulose production with an enzymatic method by using transketolase (His-tagged TKT): a) Assay of TKT reaction with *S*-erythrulose (commercial product) and D-erythrose-4P (Ery4P), b) Assay of reaction mixtures with FSA WT and A129S (from Fig.3-12 samples). Every arrow indicates the time when Ery4P was added into each reaction mixture. Assay condition (1mL); TKT: 2.2µg, Ery4P: 2mM, $NADP^+$: 0.5mM, *S*-erythrulose: 10mM or the reaction mixture of FSA: 250µL. Glu6P in commercial products of Ery4P led to high initial values of Abs340nm in 2 samples (●;Fig.3-14a, ■;Fig.3-14a).

In the reaction of producing D-xylulose from DHA (200mM initial conc.) + glycolaldehyde (130mM initial conc.) (scheme.3-1), WT showed a low activity, little decrease of DHA (initially 200mM -> 189mM of residual) and a small peak of xylulose (less than 10mM) were found with HPLC analysis (Fig.3-15a), while A129S was more active, both DHA consumption (85mM of residual concentration) and xylulose production (equivalent to 95mM) were recognized clearly (Fig.3-15c). It indicates that the reaction was highly progressive with FSA A129S. A sample without FSA retained 98% of DHA and 95% of glycolaldehyde as initial, respectively. A small peak at the same retention time as that of xylulose (much less than 10mM) was observed (Fig.3-15d). This may suggest that chemical aldol reaction happened slightly.
NMR analysis by J. Castillo (CSIC, Barcelona) certified D-xylulose production from DHA+glycolaldehyde with FSA A129S by comparison with a commercial product of D-xylulose (Sigma) (Fig.3-16 ~ Fig.3-18). Each NMR spectrum (^{1}H-NMR or ^{13}C-NMR) had identical spectrum between a commercial product sample and a product sample with FSA despite the differences in peak size brought from the amount of product (Fig.3-16 & Fig.3-17). The close-up of peaks for both ^{1}H-NMR and ^{13}C-NMR spectrum gave the information of products in detail (Fig.3-18). Some peaks were observed in the reaction sample, but not in commercial product, it is considered that they correspond to unreacted substrates (comments by J. Castillo).

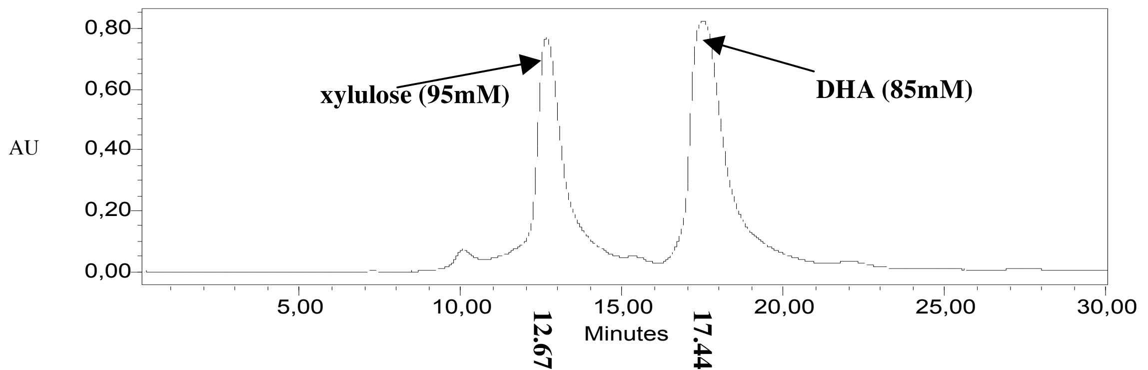
a) DHA+glycolaldehyde WT 24h



b) standard D-xylulose 100mM



c) DHA+glycolaldehyde A129S 24h



d) without enzyme (negative control)

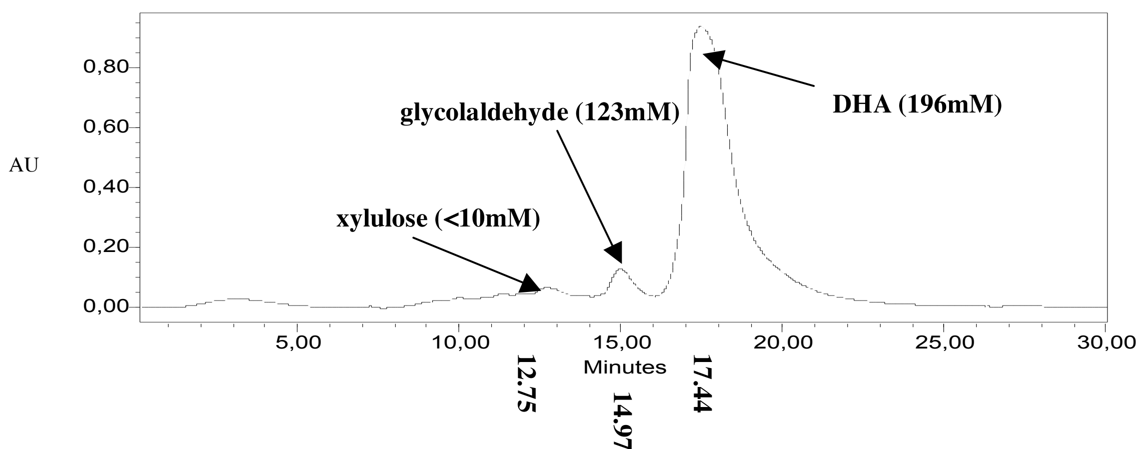


Fig.3-15. HPLC-analysis of the formation of xylulose from DHA (200mM) + glycolaldehyde (130mM); a) with FSA wild type (0.94 mg-protein/mL), b) 100mM standard D-xylulose, c) with mutant FSA A129S (0.94 mg-protein/mL), d) without enzyme, after 24h at 30°C. Retention time: 12.7min- xylulose, 15.0min- glycolaldehyde, 17.5min- dihydroxyacetone detected at 192 nm (column; Aminex HPX-87H, mobile phase; 5mM H_2SO_4, temp; ambient temp., flow rate; 0.5 mL/min).

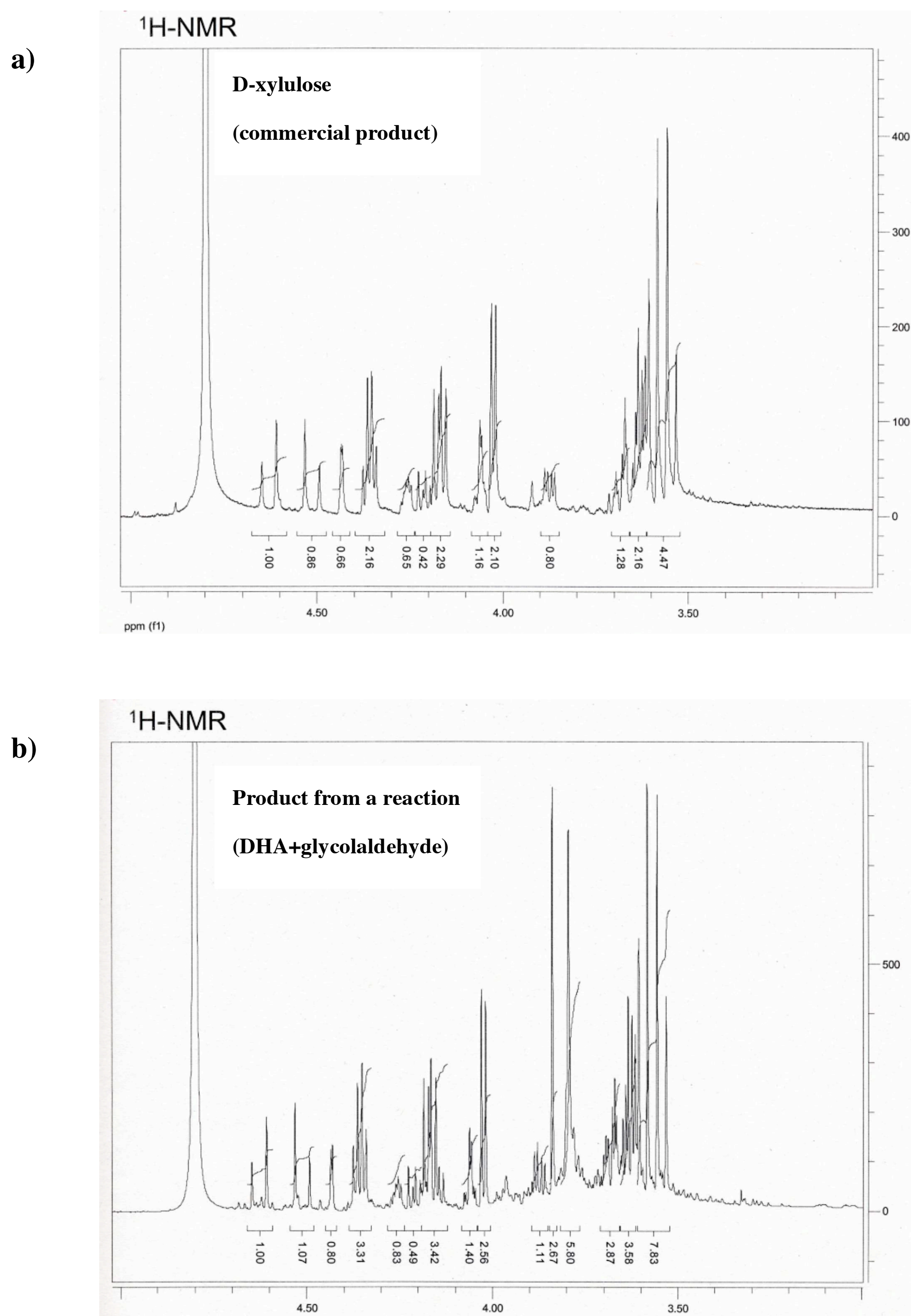


Fig.3-16. ^{1}H-NMR spectrum; a) commercial product of D-xylulose, b) reaction mixture of DHA+glycolaldehyde with FSA A129S (after removal of FSA). (Analysis by J.A. Castillo in Institute for Chemical and Environmental Research (CSIC); Barcelona, Spain.)

a)

b)

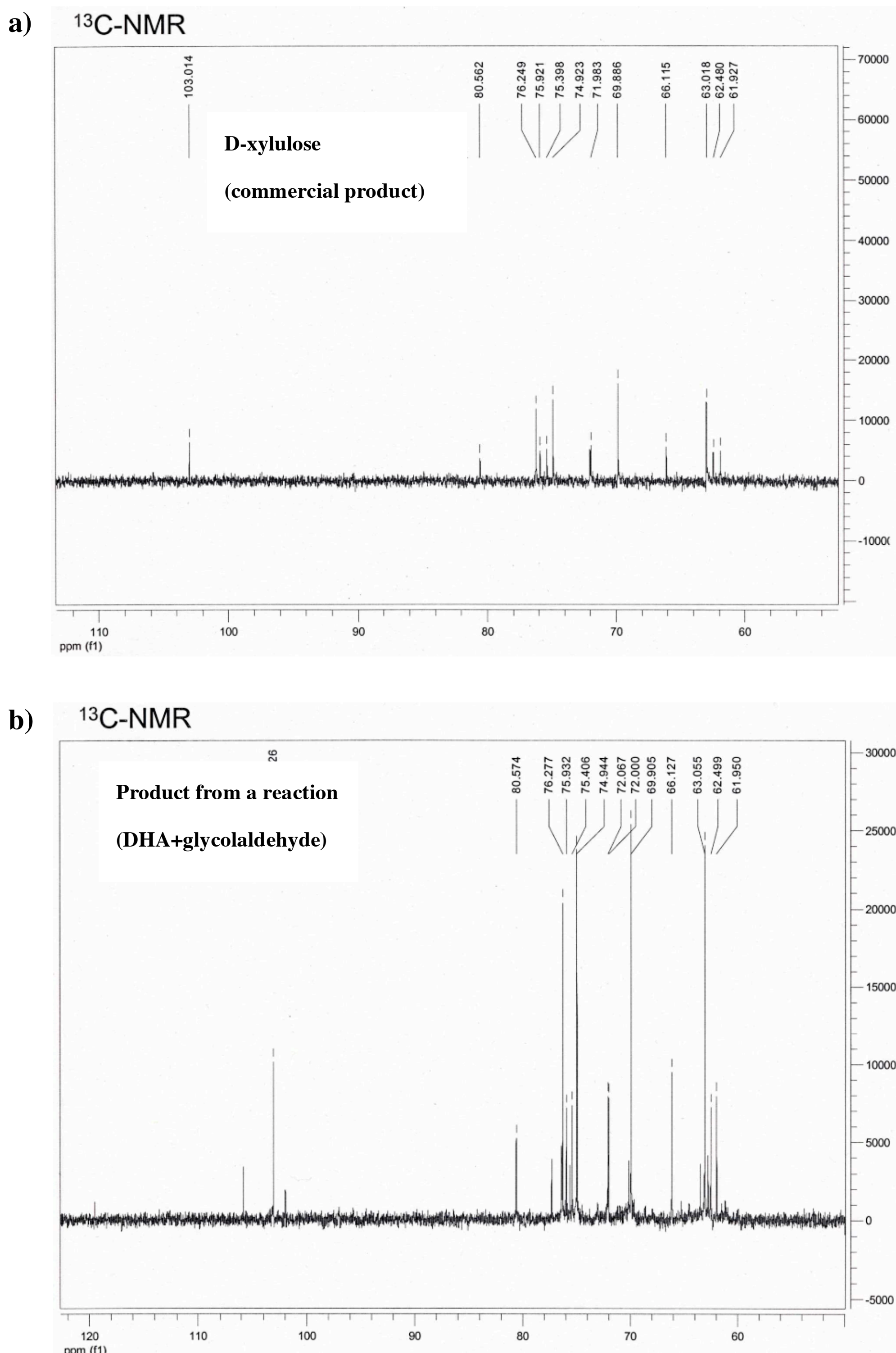


Fig.3-17. ^{13}C-NMR spectrum; a) commercial product of D-xylulose, b) reaction mixture of DHA+glycolaldehyde with FSA A129S (after removal of FSA). (Analysis by J.A. Castillo in Institute for Chemical and Environmental Research(CSIC): Barcelona.)

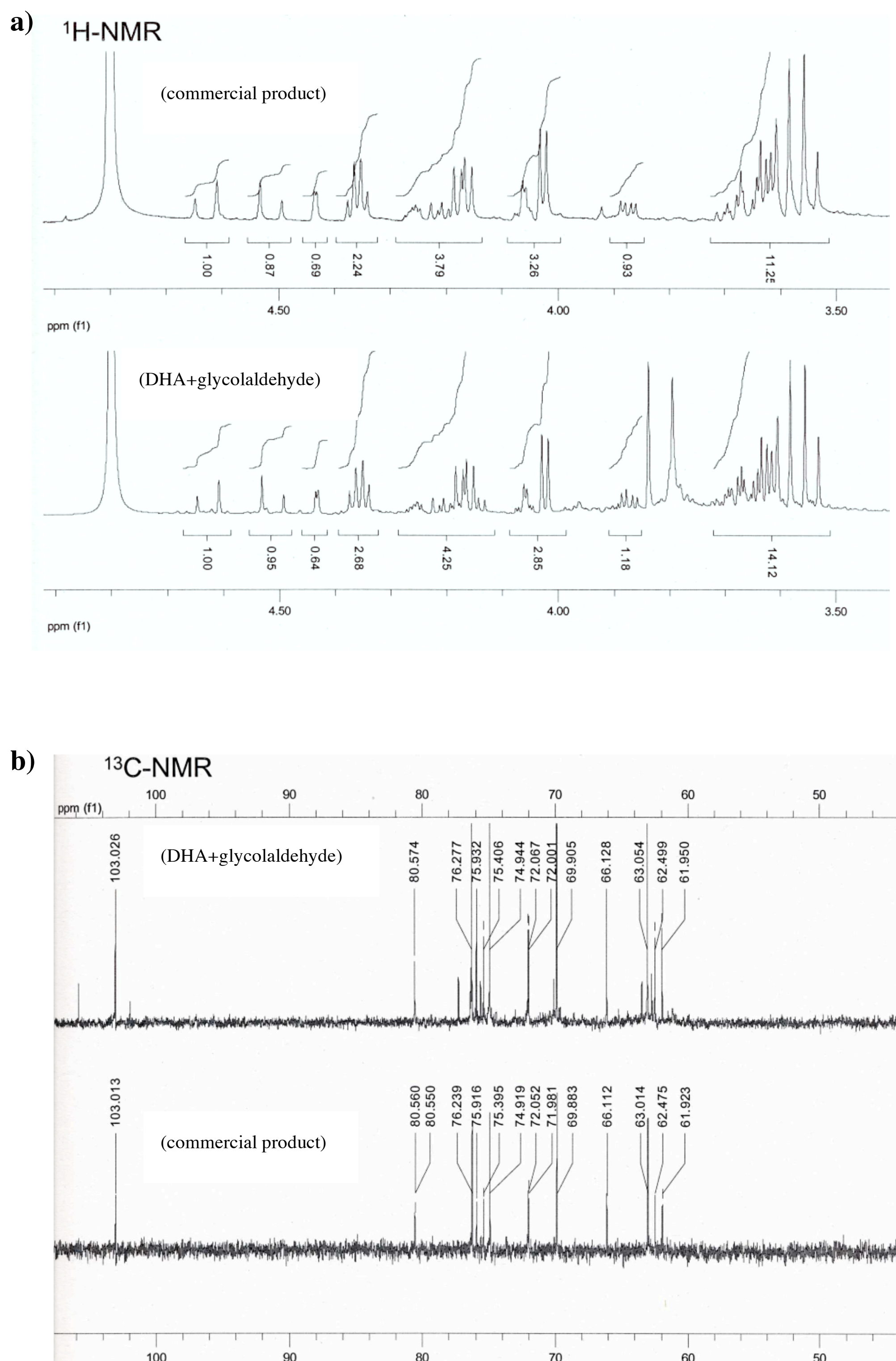
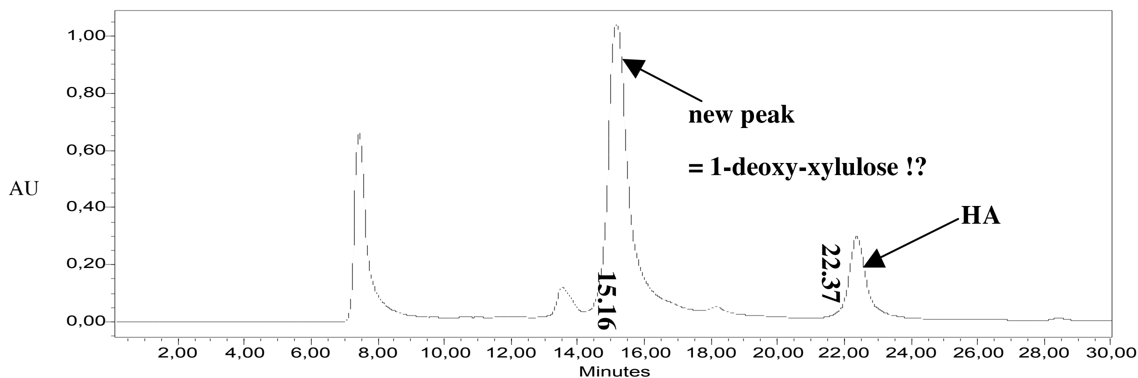


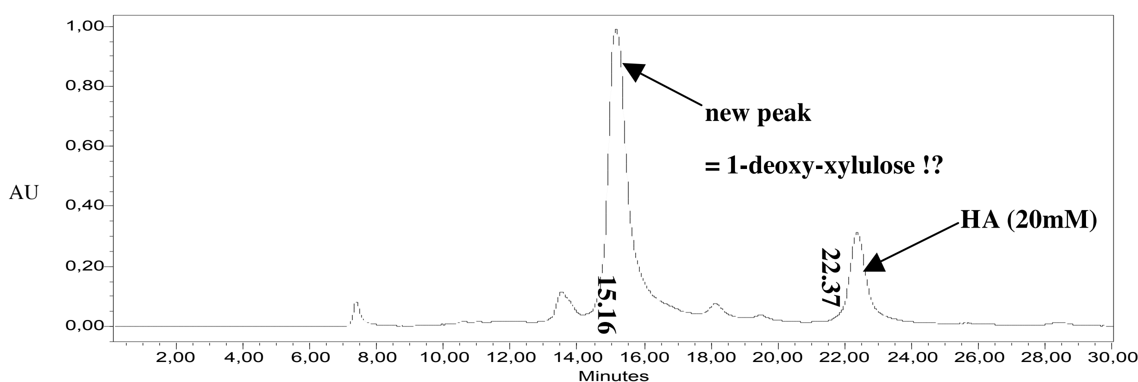
Fig.3-18. Comparison of D-xylulose (commercial product) with product from DHA+glycolaldehyde with FSA A129S (after removal of FSA); a) in closeup ^{1}H-NMR spectrum b) in closeup ^{13}C-NMR spectrum; (Analysis by J.A. Castillo in Institute for Chemical and Environmental Research; Barcelona, Spain.)

In the third reaction on scheme.3-1, when hydroxyacetone (HA) was utilized as a donor instead of DHA, decrease of HA (50mM -> 20mM; peak at 22.4 min) and appearance of a large peak (at 15.16 min) overlapping with glycolaldehyde (14.91 min) were observed for both samples of WT and A129S samples (Fig.3-19). Peaks at 15.16 min had different spectra of absorbance between 200nm and 400nm, compared with that of glycolaldehyde (14.91 min). Therefore it is supposed that this peak can correspond to 1-deoxy-D-xylulose. In both samples residual HA was approximately 20mM (initial concentration was 50mM), same size of a new peak at approximately 15.2 min was found, which implies that the same amount of product was contained in each reaction mixture and the catalytic abilities of both FSA WT and a mutant A129S for HA were not different. A peak at approximately 7.5min was seen in both samples, but it was unidentified. That peak was detected only in samples containing enzymes, but it didn’t appear for a sample without enzyme solution.

a) HA+glycolaldehyde WT 22h



b) HA+glycolaldehyde A129S 22h



c) without enzyme (negative control)

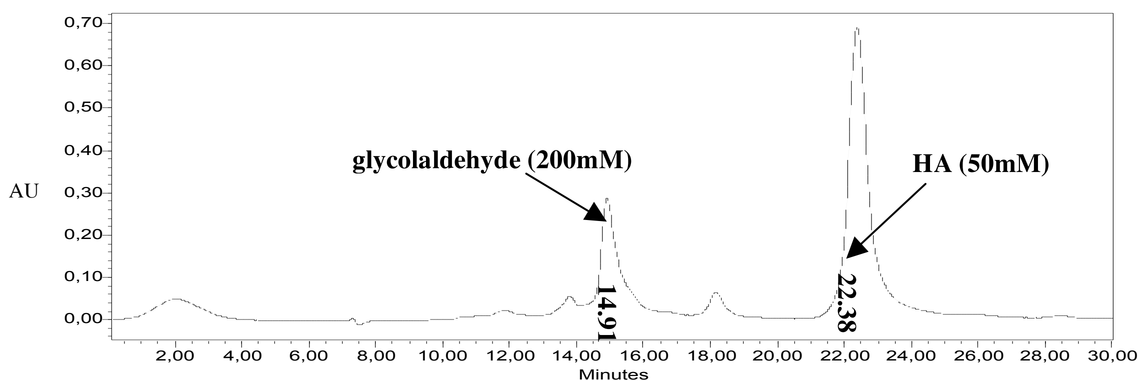


Fig.3-19. HPLC-analysis of the formation of 1-deoxy-xylulose from HA (50mM) + glycolaldehyde (200mM); a) with FSA wild type (0.41 mg-protein/mL), b) with mutant FSA A129S (0.48 mg-protein/mL), c) without enzyme, after 22h at 30°C. Retention time: 14.9min- glycolaldehyde, 15.2min- new peak (= 1-deoxy-xylulose?), 22.4min- hydroxyacetone detected at 192 nm (column; Aminex HPX-87H, mobile phase; 5mM H_2SO_4, temp; ambient temp., flow rate; 0.5 mL/min).