3-2. Transaldolase from *Bacillus subtilis* (TAL_{Bsu}): characterization and comparison with FSA

3-2-1. Comparison of His tagged proteins with wild type (WT) in activity and quaternary structure

Transaldolase from *B.subtilis* (TAL_{Bsu}) is a class I aldolase and has high similarity in structure to FSA (Schürmann & Sprenger, 2001, Schürmann, 2001). Indeed, they have an identity by 30% identical in amino acid sequence. Molecular masses of subunits of TAL_{Bsu} and FSA are 22.9 kDa and 23.0 kDa, respectively. Quaternary structure of TAL_{Bsu} is a homodecamer or homododecamer (Schürmann, 2001), that of FSA is a homodecamer resulting from the dimerization of two identical pentamers (Thorell et al., 2002). In spite of those similarities, FSA was shown to split Fru6P releasing DHA and GAP, but not to have transaldolase activity (Fru6P+Ery4P <-> Sed7P+GAP) (Schürmann & Sprenger, 2001, Schürmann, 2001). To investigate differences of TAL_{Bsu} from FSA in structural and biochemical characteristics, N-terminal and C-terminal His tagged TAL_{Bsu} were prepared to facilitate purification of proteins and compared with His-tagged FSA.

Both His-tagged TAL_{Bsu} were overexpressed in *E.coli* BL21(DE3)Star pLysS, and protein was induced with IPTG. Then binding of proteins to Ni-NTA superflow column was assayed. N-His tagged TAL_{Bsu} did not bind to the resin just as N-His tagged FSA did not. C-His tagged TAL_{Bsu} bound and was eluted with a buffer containing high concentration of imidazol (250mM) as in the case of C-His tagged FSA. Quaternary structures of His tagged TAL_{Bsu} were determined by gelfiltration after application into Ni-NTA superflow column. N-His tagged TAL_{Bsu} mainly came out of the column (Superdex 200pg) in 98.5~101 mL of elution volume (Fig.3-30a), whereas C-His tagged TAL_{Bsu} was seen in 85.0~110.0 mL, especially with more prominent bands in 85.0~95.0 mL of elution volume (Fig.3-30b). Compared with standard protein samples, N-His tagged TAL_{Bsu} had approximately 48000 Da of molecular mass and C-His tagged TAL_{Bsu} showed 48000~120000 Da (Fig.3-31). This indicates that N-His tagged TAL_{Bsu} is homodimer and C-His tagged is most likely a mixture of pentamer and dimer, while the WT protein appears to be homodecamer. Activities of both His-tagged proteins were assayed in crude extracts due to the difficulty of purification of N-His tagged protein by comparison of the activity with WT (Table.3-12).

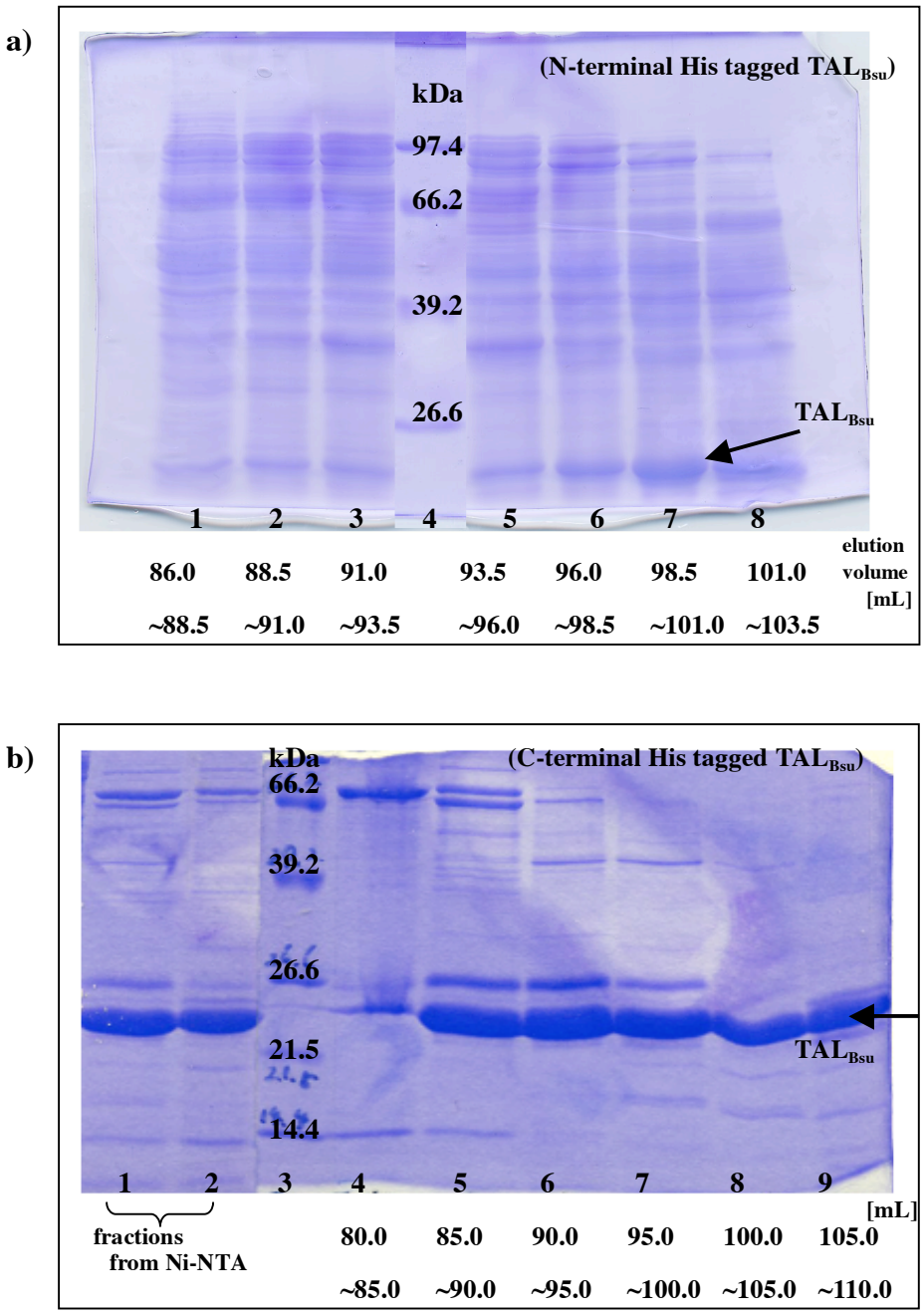
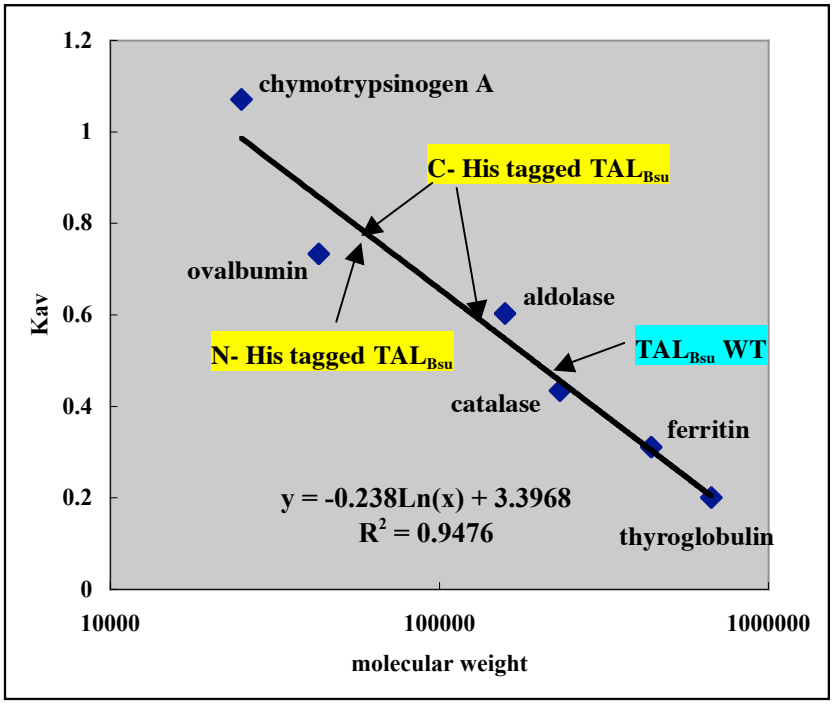


Fig.3-30. SDS-PAGE analysis of N-, or C-His tagged TAL_{Bsu} from Superdex 200pg (1.6x60cm:120mL). Equal amounts of protein (20 µg) were applied to each lane; a) N-His tagged TAL_{Bsu} (flow-through from Ni-NTA superflow) came out in 98.5~101.0 mL of elution volume, b) C-His tagged TAL_{Bsu} (eluted from Ni-NTA superflow) seen in 85~110 mL of elution volume. Correlation of molecular masses and elution volumes are the following; 240000: 75~77 mL(corresponds to decamer TAL_{Bsu}), 120000: 86~90 mL(pentamer TAL_{Bsu}), 48000: 96~107 mL(dimer TAL_{Bsu}).



	Mr	elution volume	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.3-31. Determination of quaternary structures of His-tagged TAL_{Bsu} with gelfiltration (Superdex 200pg - 1.6 x 60 cm) by comparison to standard proteins. Standard samples are Thyroglobulin(MW:669000), Ferritin(MW:440000), Catalase(MW:232000), Aldolase(MW:158000), Ovalbumin(MW:43000) and Chymotrypsinogen A(MW:25000). N-His tagged TAL_{Bsu} came out in 98.5~101.0 mL (a peak at 100mL) of elution volume (close to Ovalbumin), C-His tagged TAL_{Bsu} was seen in 85~110 mL (2 peaks for protein concentration (at 88mL and 101mL) were found near Aldolase and Ovalbumin on a chart).

Table 3-12. Comparison of His-tagged TAL_{Bsu} with that of wild type in affinity to Ni-NTA superflow column, specific activity and quaternary structure.

vector	His-tag position	affinity to Ni-NTA column	likely quaternary structure	activity in crude extract
pET16b	N-terminal	negative	dimer	0.28±0.17U/mg
pET28b	C-terminal	positive	pentamer & dimer mix.	0.07±0.04U/mg
pET22b	non-tagged	not tested	decamer	4.1±1.0 U/mg

Specific activities were determined in crude extracts (20~30μg protein) by measuring a TAL activity (Fru6P(10mM) + Ery4P(2mM) -> Sed7P+GAP) and taking the means of at least 3 times measurements.

Both His-tagged enzymes displayed low activities in crude extracts. Particularly C-His tagged TAL_{Bsu} held only 1.3% of what WT showed, whereas it had the affinity to Ni-NTA. N-His tagged TAL_{Bsu} didn't bind to Ni-NTA and kept 5.1% of the activity, compared with that of WT. It was similar to the case of FSA that C-His tagged protein retained lower activity than N-His tagged one. Only C-His tagged TAL_{Bsu} had the affinity to Ni-NTA column. However, His-tagged $TALs_{Bsu}$ had an altered quaternary structure, which was different from His-tagged FSAs that kept their decamer structure.

To further investigate and compare the character of TAL_{Bsu} with that of FSA, TAL_{Bsu} WT was overexpressed in BL21(DE3)Star, pLysS. In crude extract, TAL_{Bsu} was contained approximately as 15~20% of total proteins (Fig.3-32; SDS-PAGE). The enzyme was purified by using 2 different columns (Q-sepharose HP and Superdex 200pg) following heat treatment (at 75℃, for 30min) which denatures most other proteins in crude extract and can help purification of TAL_{Bsu} effectively. This step should be within 30min, otherwise, TAL_{Bsu} was precipitated or lost activity drastically. Fractions from Superdex 200pg showed only one protein band corresponding to TAL_{Bsu} at 63% of the recovery rate (residual TAL_{Bsu} quantity) (Table.3-11). This purified TAL_{Bsu} was crystallized and the structure was investigated by the group of G. Schneider in Karolinska Institute, Stockholm Sweden.

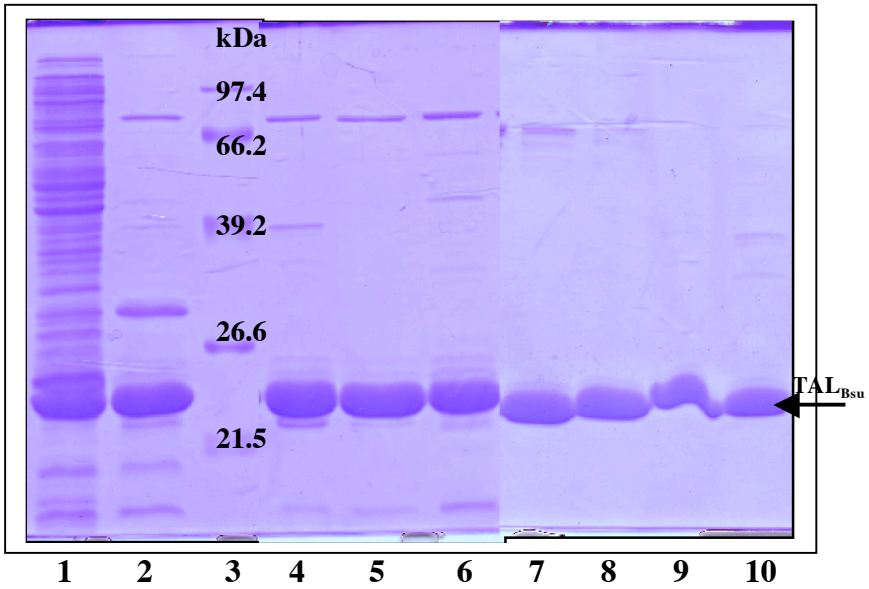


Fig.3-32. SDS-PAGE analysis of TAL_{Bsu} (pET22ywjH). Equal amounts of protein (20 μg) were applied to each lane; lane1: crude extract, 2: protein solution after heat treatment, 3: protein marker, 4~6: eluted fraction from Q-Sepharose HP, 7~10: fractions from Superdex 200pg.

Table.3-13. Purification scheme for TAL_{Bsu} (BL21(DE3) Star pLysS, pET22ywjH).

sample	Amount of protein (mg)	Spec. activity (U/mg)	Amount of activity (U)	Residual TAL quantity (%)	Purification factor
crude extract	366	4.1±1.0	1489	100	1.0
heat treatment	108	12.5±2.0	1349	91	3.0
Q-Sepharose HP	69	14.4±2.5	984	66	3.5
Superdex 200pg	42	22.4±6.0	945	63	5.5

Activities were determined with a TAL reaction (Fru6P(10mM)+Ery4P(2mM) -> Sed7P+GAP with 2-10 μg-protein at 30℃) by taking the means of at least 3 times measurements. Protein was purified by a modified method2 using Superdex 200pg instead of hydroxyapatite column. The amount of protein or TAL_{Bsu} activity was in 1L culture scale.

3-2-2. Crystallization and structural models of TAL_{Bsu} WT

TAL_{Bsu} was crystallized with the method in "Material and Methods" (by T. Sandalova, Karolinska Institute Stockholm, Sweden). 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, 2-3µl of a 12mg/ml protein solution in 50mM glycylglycine buffer (pH 8.0), 0.1M NaCl, and 1mM DTT were 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 reached 0.6 - 0.8mm (Fig.3-33). The structure of TAL_{Bsu} was determined by molecular replacement at 1.9 Å resolution and has been refined to an R-value of 21.5 % (Rfree = 24.8%) and 97% of the resides in the most favorable regions of Ramachandran plot. The asymmetric unit contains two decamers, giving a Mathews coefficient of 3.3$Å^3$/Da, which corresponds to a solvent content of 63%. The quality of the final electron density map is rather good. The main chain is well defined through the whole chain and all side chains are well defined in the density except for the loop, comprising residues 35-38 in some chains and C-terminal with rather weak electron density. In the final model, comprising two decamers, each subunit contains all 212 residues, a sulfate ion in a potential phosphate binding cleft between Arg133 and Arg181 and 3713 solvent molecules. TAL_{Bsu} monomer comprises 212 amino acids and forms a single domain with $(\beta/\alpha)_8$ -(TIM)-barrel fold similar to that of transaldolases from the thermophilic bacteria or FSA (Fig.3-34). TAL_{Bsu} is smaller than FSA, it is one of the smallest among known $(\beta/\alpha)_8$ barrel proteins. In fact, the length of the loops are shorter than in a conventional TIM barrel protein, the C-terminal helix protruding to the next subunit is shorter than that of FSA, however, it contributes to the pentamer formation as well as that in FSA (Fig.3-35a). TAL_{Bsu} forms a decamer structure, dimer of pentamers, the overall structure looks like a double doughnut shape with a thickness of 70Å and a diameter of 95Å (Fig.3-35b). The pentamer is the most remarkable substructure and forms the most extensive interactions. Upon formation of pentamers, 1500 $Å^2$ of the each subunit surface is buried from both side of the subunit, corresponding to 30% of the total solvent accessible area of the monomer. The interaction between two pentamers is less pronounced, though each subunit interacts with two subunits of the adjacent pentamer (analysis by T. Sandalova).

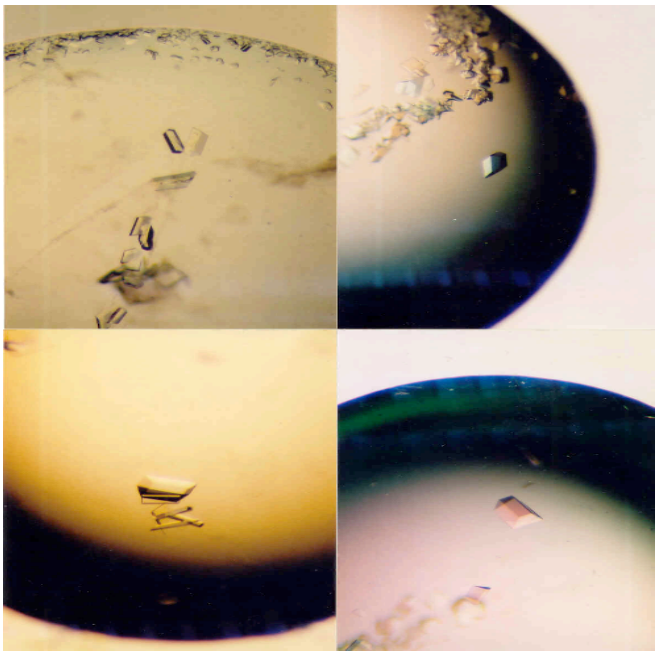


Fig.3-33. Crystals of TAL_{Bsu} (crystallized by Sandalova in Karolinska Institute, Stockholm, Sweden).

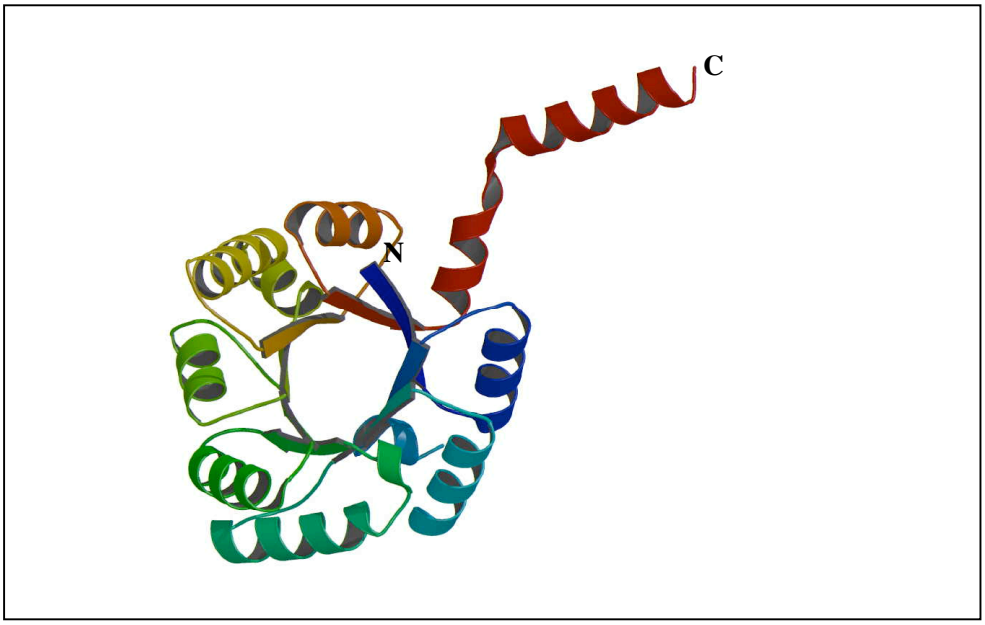


Fig.3-34. Model of a subunit of TAL_{Bsu} (by T. Sandalova in Karolinska Institute, Stockholm, Sweden). It forms a single domain with $(\beta/\alpha)_8$ -(TIM)-barrel fold similar to that of FSA. (N:N-terminal, C:C-terminal)

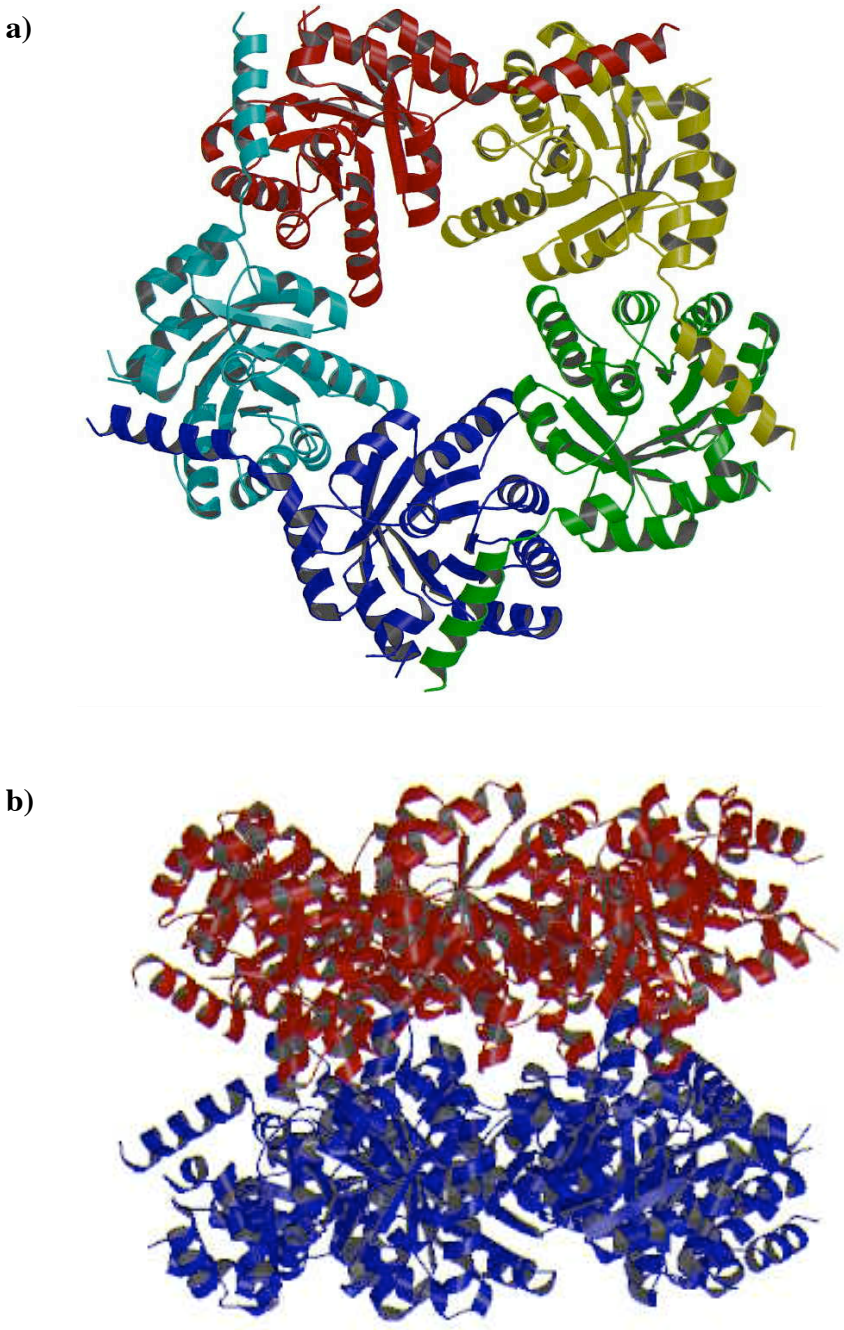


Fig.3-35. Model of quaternary structures of TAL_{Bsu}; a) a pentamer composed of five identical monomers. A C-terminal helix protrudes to the next subunit. b) overall of TAL_{Bsu} resulting from dimerization of two pentamers.

3-2-3. Substrate specificity of TAL_{Bsu}

TAL_{Bsu} was assayed with different substrates (Table.3-14) as Me Schürmann had reported on her thesis previously (Schürmann, 2001). Because *tal_{Bsu}*(ywjH) gene was newly prepared in a different vector and protein was overproduced, activities were compared with the previous report. Most activities were in the same range as reported before, however, the reaction using DHA and D,L-GAP as substrates showed a different result (< 0.02U/mg at 2.8mM of D,L-GAP) varying from reported kinetics values (Vmax: 2.8U/mg; Km: 0.24mM). 2 novel reactions, Fru6P+D-ribose and DHA+glycolaldehyde, were also investigated. D-ribose apparently was no acceptor for TAL, whereas DHA+glycolaldehyde was catalyzed at specific activity of about 10% of reaction using Fru6P as donor).

Table 3-14. Specific activities of TAL_{Bsu} for different substrates.

donor	acceptor	activity*[1] (U/mg)
Fru6P (10mM)	D-Ery4P (2mM)	22.4±6.0
	D-erythrose (0.1M)	2.5±0.3
	D-Rib5P (0.1M)	1.8±0.3
	D-ribose (0.1M)	Not detectable
	D,L-glyceraldehyde (5mM)	2.0±0.4
	glycolaldehyde (0.12M)	2.7±0.3
DHA (50mM)	D,L-glyceraldehyde-3P (2.8mM)	0.013±0.005
	glycolaldehyde (0.12M)	0.25±0.1

*1: Values are the means of at least 3 measurements.

The activities were assayed by measuring NADH oxidation concomitantly with the conversion of GAP cleaved from Fru6P into glycerol-3P or the reduction of xylulose into xylitol (cf. 2-6-1 Ex.3 or 4), otherwise, by detecting NADP reduction at 340nm (2-6-1 Ex.1) photometrically. The amounts of protein quantity were 4~20 μg/mL.

3-2-4. Chimerization of TAL_{Bsu} and FSA: assay of chimera proteins

Eight different chimera proteins consisting of both parts of TAL_{Bsu} and FSA were prepared in order to investigate the important parts to be TAL_{Bsu} or FSA, and were named chimera protein 1-8. Both FSA and TAL_{Bsu} have $(\beta\alpha)_8$–(TIM)-barrel structures, each chimera protein was designed with a gene boundary between *fsa* and tal_{Bsu} at a different (βα) barrel unit to have various proportions of TAL_{Bsu} and FSA (Fig.3-36). Practically, DNA fragments for composing chimera proteins were amplified with PCR and different *fsa* or tal_{Bsu} genes in length were obtained (Fig.3-37). Then, as shown in the model of gene combination (Fig.3-36), each *fsa* DNA truncate was ligated with a suitable tal_{Bsu} DNA fragment to have $(\beta\alpha)_8$-(TIM)-barrel structure as TAL_{Bsu} or FSA. Obtained genes for 8 chimera proteins (chimera1:672bps, chimera2~8:675bps) were inserted into pET22b plasmid vector DNA after cutting with 2 restriction enzymes (NdeI and XhoI) and cloned in *E.coli* DH5α..

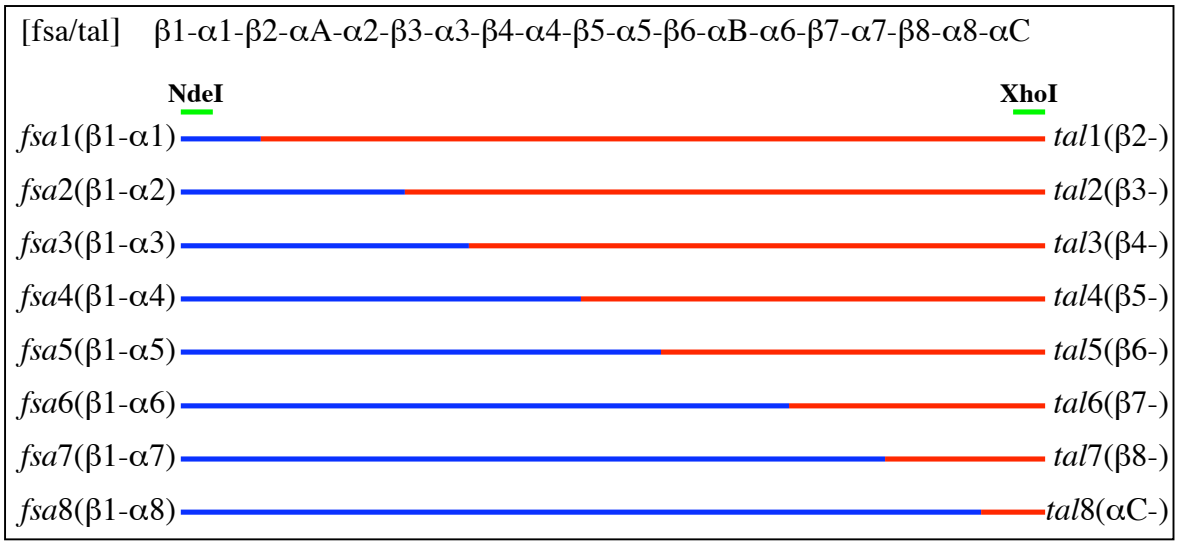


Fig.3-36. Model of gene combinations with *fsa*(blue) and tal_{Bsu}(red) for 8 chimera proteins. Length of whole chimera genes and *fsa* or tal_{Bsu} fragment are the following: *chimera*1-672bps; *fsa*1(67bps)-*tal*1(605bps), *chimera*2-675bps; *fsa*2(163bps)-*tal*2(512bps), *chimera*3-675bps; *fsa*3(253bps)-*tal*3(422bps), *chimera*4-675bps; *fsa*4(322bps)-*tal*4(353bps), *chimera*5-675bps; *fsa*5(382bps)-*tal*5(293bps), *chimera*6-675bps; *fsa*6(484bps)-*tal*6(191bps), *chimera*7-675bps; *fsa*7(550bps)-*tal*7(125bps), *chimera*8-675bps; *fsa*8(598bps)-*tal*8(77bps). Cutting sites with restriction enzymes exist at both edges of gene (NdeI; *fsa* side, XhoI; tal_{Bsu} side).

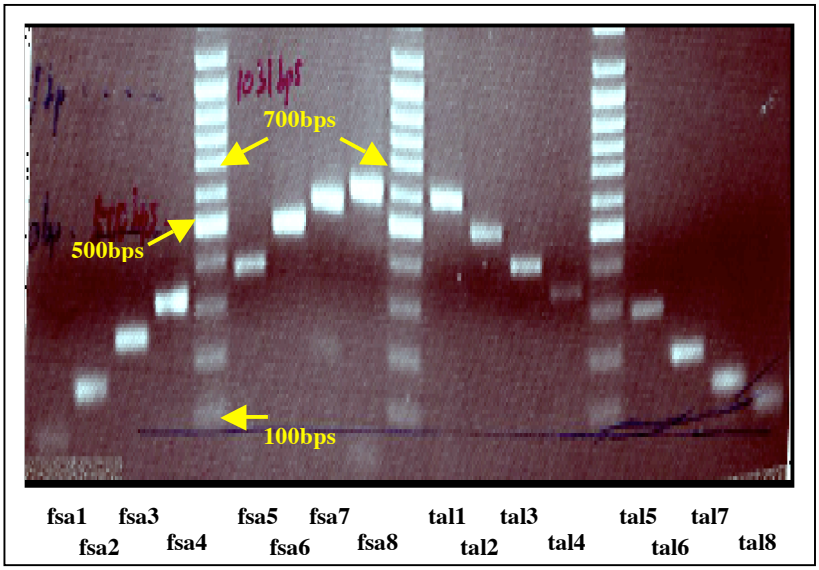


Fig.3-37. PCR products of *fsa* or tal_{Bsu} truncates: *fsa*1-67bps, *fsa*2-163bps, *fsa*3-253bps, *fsa*4-322bps, *fsa*5-382bps, *fsa*6-484bps, *fsa*7-550bps, *fsa*8-598bps, *tal*1-605bps, *tal*2-512bps, *tal*3-422bps, *tal*4-353bps, *tal*5-293bps, *tal*6-191bps, *tal*7-125bps, *tal*8-77bps.

After the confirmation of DNA sequences of the genes, plasmid DNAs containing chimera genes, pET22chimera1~8, were introduced into *E.coli* BL21(DE3)Star pLysS. Production of chimera proteins was examined with SDS-PAGE for whole cell samples (cell suspensions were loaded to SDS-PAGE) (Fig.3-38) or for cell free extracts after cell lysis (Fig.3-39). In whole cell samples, chimera4, chimera6 and chimera8 showed corresponding bands to chimera proteins on a gel, whereas the others could not be detected. Actually no difference was found between pET22b and chimera 1, 2, 3, 5 and 7 in protein bands on the gel (Fig.3-38). In crude extract samples, chimera6 protein would not be detected. It might be precipitated as cell pellet, while chimera4 and chimera8 indicated corresponding protein bands (Fig.3-39). Chimera proteins are probably very unstable and they might be degraded in cells during cultivation.

Although only small amount of FSA/TAL protein were visible in some samples, all 8 crude extracts were assayed for TAL and FSA activity. Neither TAL activity nor FSA activity was observed in any sample (data not shown). This implies that these chimera proteins were relatively unstable and in addition they are neither encoding TAL nor FSA activity.

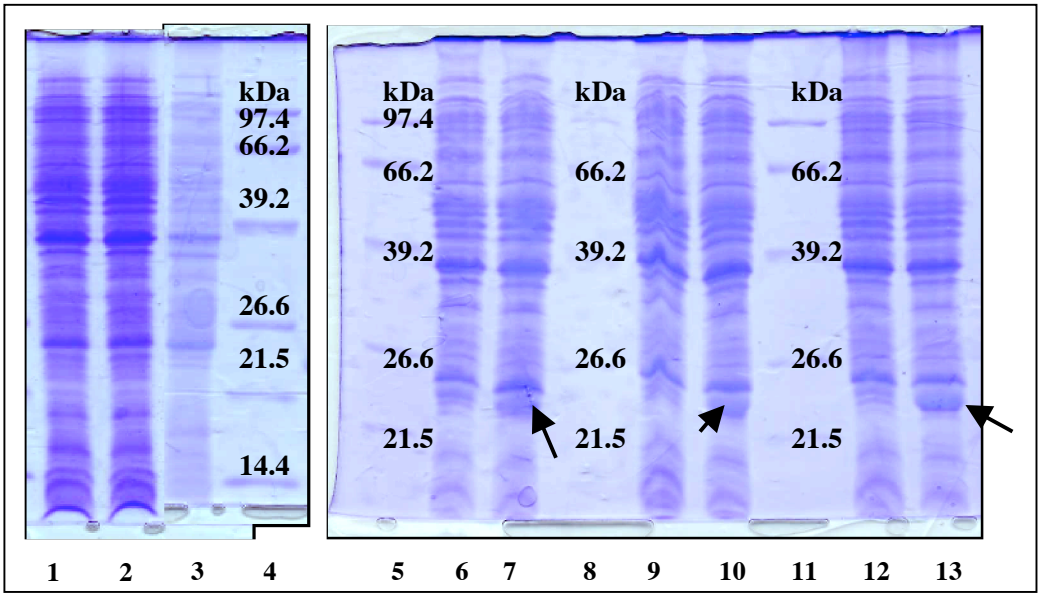


Fig.3-38. Expression of chimera proteins - whole cells (cell suspensions were loaded) on SDS-PAGE; lane1: pET22b, 2: chimera1, 3: chimera2, 4,5,8&11: protein marker, 6: chimera3, 7: chimera4, 9: chimera5, 10: chimera6, 12: chimera7, 13: chimera8. Arrows indicate chimera proteins.

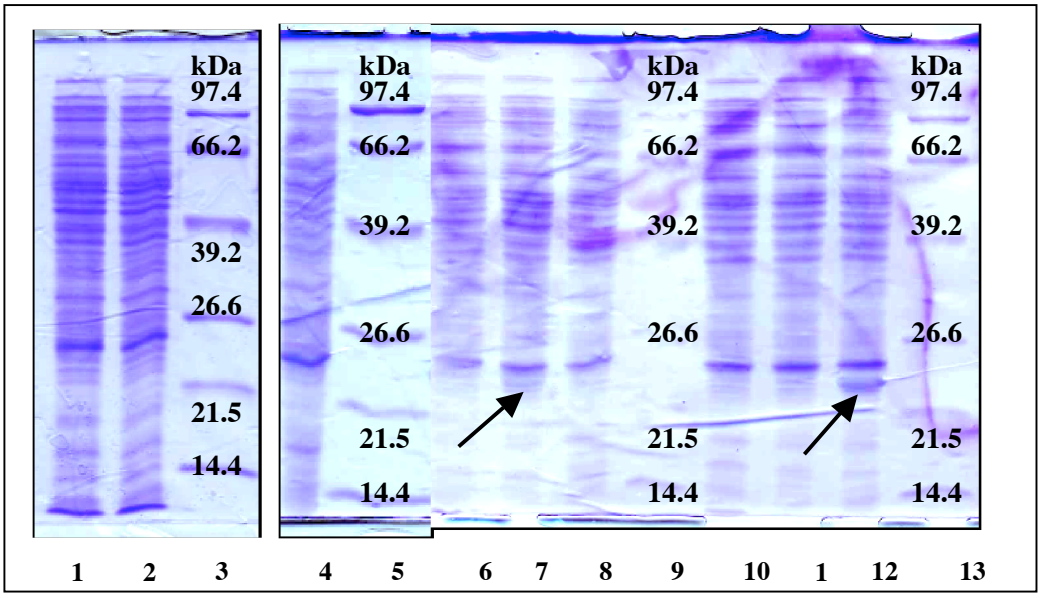


Fig.3-39. Expression of chimera proteins - crude extracts on SDS-PAGE; lane1: pET22b, 2: chimera1, 3,5,9&13: protein marker, 4: chimera2, 6: chimera3, 7: chimera4, 8: chimera5, 10: chimera6, 11: chimera7, 12: chimera8. Arrows indicate chimera proteins obtained in crude extracts.