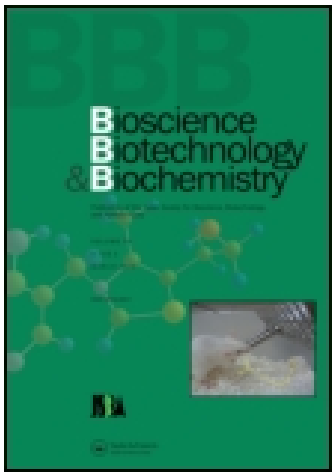

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Enzymatic Synthesis of L-Tryptophan by Enterohacter aerogenes Tryptophanase Highly Expressed in Escherichia coli, and Some Properties of the Purified Enzyme

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Enzymatic Synthesis of L-Tryptophan by *Enterobacter aerogenes* Tryptophanase Highly Expressed in *Escherichia coli*, and Some Properties of the Purified Enzyme

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We constructed two plasmids that have a strong *tac* promoter and a structural gene for tryptophanase of *Enterobacter aerogenes* SM-18 (pKT901EA) or *Escherichia coli* K-12 (pKT951EC). The tryptophanase activity of *E. coli* JM109 transformed with pKT901EA (JM109/pKT901EA) was inducible with isopropyl-β-D-thiogalactopyranoside, and 3.6 times higher than that of *E. aerogenes* SM-18. Cells of JM109/pKT901EA induced for tryptophanase synthesized L-tryptophan from indole, ammonia, and pyruvate more efficiently than *E. aerogenes* SM-18. Although JM109/pKT951EC expressed a similar level of tryptophanase activity to that of JM109/pKT901EA, the synthesis of L-tryptophan by the cells of JM109/pKT951EC did not proceed well compared with JM109/pKT901EA. Tryptophanases from *E. aerogenes* and *E. coli* K-12 were purified, and their properties were investigated. The purified *E. aerogenes* tryptophanase showed higher stability against heat inactivation than *E. coli* tryptophanase.

L-Tryptophan (L-Trp) is an essential amino acid and an economical supply is wanted. Therefore, many efforts have been made for the production of L-Trp by microorganisms. There are three methods for L-Trp production, *i.e.*, the precursor-conversion-fermentation method, direct fermentation, and enzymatic methods. As an example of the first method, genetically engineered *E. coli* strains were reported to produce more than 50 g/liter of L-Trp with continuous feeding of both glucose and anthranilate.[1] As examples of the second method, regulatory mutants of *Corynebacterium glutamicum*[2] and *Brevibacterium flavum*[3] have been reported to produce L-Trp directly from sugar materials. The productivities of these strains were 12.8 g/liter and 19 g/liter, respectively. Although direct fermentation has advantages in using cheap starting materials such as glucose or molasses, the productivities still have to be improved. Recently, improvement of the direct fermentation method by a transport mutant has been reported,[4] suggesting a new way of strain breeding in this field. The third method, enzymatic method, has merits in high yields *versus* substrates and in high purity of the products. However, demerits of this method are that the raw materials, *i.e.*, substrates of the enzymes, are expensive. Tryptophanase (Tnase) [L-tryptophan indole-lyase (deaminating), EC 4.1.99.1] is induced by L-Trp, and catalyzes degradation of L-Trp into indole, ammonia, and pyruvate. Tnase is a multifunctional enzyme that also catalyzes synthetic reactions of L-Trp from indole, ammonia, and pyruvate, or from indole and L-serine.[5,6] It has been reported that 62.5 g/liter of L-Trp was synthesized from indole, ammonia, and pyruvate by the Tnase of *Proteus rettgeri*.[7] Production from indole and L-serine by *E. coli* cells with highly expressed cloned-Tnase gene has also been reported with a yield of about one mole per liter of L-Trp.[8] From the same substrates, a similar yield of L-Trp production has been reached by *E. coli* cells with a cloned tryptophan synthase gene.[9] Although high yields of L-Trp production have been reported from indole and L-serine as described above, L-serine seems to be rather expensive as a raw material for the production. Therefore, we have tried to improve L-Trp production using Tnase from indole, ammonia, and pyruvate. In our system, one of the substrates, pyruvic acid, is supplied economically by microbial fermentation. A pyruvic acid-producing mutant of *Enterobacter aerogenes*, strain LT-94, was constructed from a high-Tnase strain, *E. aerogenes* AHU1540. After pyruvic acid was produced from glucose by the strain LT-94, L-Trp production was done enzymatically by direct addition of the substrates, *i.e.*, indole and ammonia, to the fermentation broth.[10–12] In this method, the amounts of L-Trp production depended mainly on the Tnase activity of the producer, thus it seemed necessary to increase the activity of Tnase to improve L-Trp production.[12] For this purpose, we have cloned and sequenced the Tnase gene from *E. aerogenes* and expressed it in *E. coli* by induction with L-Trp.[13]

Recently, we have established efficient pyruvic acid production from glucose using *E. coli* W1485*lip2*, a lipoic acid auxotroph of *E. coli* K-12,[14] and its F_1-ATPase-deficient derivative.[15] Therefore, these *E. coli* strains can be used for transformation with a plasmid that can express Tnase to establish more efficient L-Trp production systems than that using *E. aerogenes* described above. To use the *E. aerogenes* Tnase gene for the above purpose, high expression of the gene seemed necessary. Also, the expression must be free from catabolite repression by glucose. Therefore, it was intended to replace the native promoter by a strong one such as the *tac* promoter.

In this study, we described construction of the high-level expression system of *E. aerogenes* Tnase in *E. coli* and its application to L-Trp synthesis from indole, pyruvic acid, and ammonia. We also described the advantage of *E. aerogenes* Tnase for this purpose and some properties of

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Abbreviations: IPTG, isopropyl-β-D-thiogalactopyranoside; PLP, pyridoxal-5′-phosphate; L-Trp, L-tryptophan; Tnase, tryptophanase.

purified *E. aerogenes* Tnase in comparison with *E. coli* Tnase.

Materials and Methods

Bacterial strains and plasmids. Bacterial strains and plasmids are listed in Table I. The construction procedures for pKT773EA, pTC1, pKT901EA, and pKT951EC are presented in Fig. 1.

Construction of plasmids. The plasmid pKT421 was digested with *Vsp*I and a 1.5-kbp fragment was isolated. This fragment contains the structural gene for *E. aerogenes* Tnase (*tnaA*) with a Shine-Dalgarno sequence, but not the promoter sequence of the Tnase operon. Their termini were filled in by the Klenow enzyme to make blunt ends, and ligated into pUC119 cut with *Sma*I. A clone that has *tnaA* with the same orientation as the *lac* promoter of the vector plasmid was selected, and designated pKT773EA. This was digested with *Xba*I, filled in by the Klenow enzyme, and digested by *Kpn*I, then the 1.5-kbp fragment containing *tnaA* was isolated. On the other hand, expression vector pTrc99A was digested with *Eco*RI, filled in by the Klenow enzyme, digested by *Kpn*I, and the 4.2-kbp fragment was isolated. These two fragments were ligated and the resulting plasmid was designated pKT901EA.

We also constructed a plasmid, pKT951EC, that is almost identical to pKT901EA except that the *tnaA* was from *E. coli* K-12. Details are in Fig. 1.

Induction study of Tnase. Strains were cultured in 10 ml of LB medium [10 g/liter Bacto tryptone (Difco), 5 g/liter Bacto yeast extract (Difco), 5 g/liter NaCl, and 50 mg/liter ampicillin sodium salt for transformants, pH 7.0] with shaking at 37°C for 3 h, and 1 ml of the culture broth was used as the seed. For *E. aerogenes* SM-18 and *E. coli* W1485, L-Trp was used as the inducer. The cells were grown for 12 h in 50 ml of LB medium with 1 g/liter L-Trp, 0.5 mM pyridoxin·HCl, and 0.1 M HEPES (pH 7.0). On the other hand, for *E. coli* JM109 transformants, IPTG was used as the inducer. The cells were grown in 50 ml of LB with 0.5 mM pyridoxin·HCl, 50 mg/liter ampicillin sodium salt, and 0.1 M HEPES (pH 7.0) for 1.5 h at 37°C with reciprocal shaking, then IPTG was added at 1.0 mM concentration and the cultures were continued for 12 h. Tnase activity was assayed by method I as described below.

Assay for Tnase activity. Tnase activity was measured by indole formation from L-Trp by the enzyme reaction as follows.

Method I: This method was used to measure the activity of whole cells. The cells were collected by centrifugation and washed in ice-cold 50 mM potassium phosphate buffer (pH 7.2), and the activity of whole cells was assayed as described in our previous paper.[13] One unit (U) of Tnase was defined as the amount of the enzyme that gives rise to the formation of 1 μmol of indole per min. Specific activity was expressed in U·(mg dry cell)$^{-1}$.

Method II: The activities of purified enzyme or of enzyme solution in each purification step were assayed as described by Morino and Snell.[20] Ten microliters of enzyme solution and 1 ml of toluene were added to 0.3 ml of buffer mixture [0.12 M potassium phosphate buffer, pH 7.8; 0.2 mM reduced glutathione; 0.06 mM pyridoxal-5′-phosphate (PLP); 0.25 mg/ml bovine serum albumin], and incubated for 10 min at 37°C with gentle shaking. The reaction was started by adding 0.1 ml of 0.02 M L-Trp. After 10 min, the reaction was stopped, and the color was developed by the addition of 3.5 ml of Ehrlich reagent. After 20 min at room temperature, the color intensity was read at 570 nm. Definition of unit was identical to method I, but the specific activity was expressed as U·(mg protein)$^{-1}$. Protein concentration was measured by UV absorption at 278 nm unless otherwise stated. $A_{278\,nm}^{0.1\%}$ values for Tnases of *E. coli* and *E. aerogenes* are 0.89[21] and 0.93 (estimated from amino acid composition), respectively.

Table I. Bacterial Strains and Plasmids

Strain or plasmid	Genotype or description	Source or reference
Enterobacter aerogenes SM-18	Sm^r derived from AHU1540	10
Escherichia coli W1485	λ^- derivative of *E. coli* K-12	ATCC 12435
Escherichia coli JM109	λ^-, *recA1*, Δ(*lac-proAB*), *endA1*, *gyrA96*, *thi*, *hsdR17*, *relA1*, *supE44*, F′[*traD36*, *proAB*$^+$,*lacI*^{q}, *lacZ*ΔM15]	16
pUC119	Cloning vector, *lacP*, Ap^r	17
pTrc99A	Cloning vector, *tacP*, *lacI*^{q}, Ap^r	18
pKT421	pUC19 with *tnaA* from *E. aerogenes* SM-18	13
pMD6	pBR322 with *tnaA* from *E. coli* K-12	C. Yanofsky, 19
pKT773EA	pUC119 with *tnaA* from *E. aerogenes* SM-18	This study
pTC1	pUC119 with *tnaA* from *E. coli* K-12	This study
pKT901EA	pTrc99A with *tnaA* from *E. aerogenes* SM-18	This study
pKT951EC	pTrc99A with *tnaA* from *E. coli* K-12	This study

L-Trp Synthesis. Tnase induction was done as described in *Induction study of Tnase* except that the HEPES was not included in the induction medium. Tnase activity was assayed by method I. The cells were collected by centrifugation, washed twice with 10.8 g/liter KCl, and resuspended in 10.8 g/liter KCl to contain 4.5 mg dry weight cell per ml. One milliliter of the cell suspension was mixed with 2 ml of solution containing 28.1 mg/ml sodium pyruvate, 90 mg/ml NH_4Cl, 17.6 mg/ml indole, 0.3 mM PLP, and 2 drops of Triton X-100 (pH was adjusted to 9.0 by KOH). The resulting reaction mixture contained 0.17 M pyruvic acid, 1.12 M NH_4Cl, 0.1 M indole, and 1.5 mg dry weight cell per ml. The reaction mixture was incubated at 37°C with shaking for 6 or 24 h. The L-Trp concentration of the reaction mixture was measured as described in our previous paper.[22]

Purification of Tnase. Tnase of *E. coli* JM109/pKT901EA and *E. coli* JM109/pKT951EC were induced with IPTG in cultures using a pH-controlled jar fermentor. Strains were cultured at 37°C in 60 ml LB medium containing 50 mg/liter ampicillin sodium salt with shaking for 3 h. The whole culture broth was inoculated into 3 liters of LB medium with 20 g/liter glucose, 1 mM IPTG, 0.5 mM pyridoxin·HCl, and 50 mg/liter ampicillin sodium salt in a 5-liter jar fermentor. Incubation at 37°C was continued for 9 h with aeration of 3 liters per min and agitation of 600 rpm. The pH was controlled at 7.0 with 5 N NaOH. Inclusion of glucose in the medium prevented contamination by Tnase of the host cells (*i.e.*, *E. coli* Tnase) by catabolite repression. Purification procedures of Tnase from these strains were the same as the method of Tani *et al.*[23]

Characterization of the purified enzymes.

i) Molecular mass estimation. The molecular mass markers (Boehringer Mannheim GmbH, Mannheim, Germany) and purified Tnase were mixed and put on a Sephacryl S-300 HR (Pharmacia, Uppsala, Sweden) column (26 mmϕ × 1000 mm), equilibrated with 10 mM potassium phosphate buffer (pH 7.0) containing 0.2 M KCl. Proteins were eluted by the same buffer. The protein concentration of each fraction was measured by UV absorption at 280 nm, and Tnase activity was assayed by method II.

ii) Effects of pH on the enzyme activity. Tnase activity was assayed by method II except that the 0.12 M potassium phosphate buffer (pH 7.8) of the reaction mixture was replaced by the following buffers: pH 6.0–8.5, 0.12 M potassium phosphate; and pH 8.0–10.0, 0.1 M glycine–KOH containing 0.02 M KCl.

iii) Stability studies. The buffer of the enzyme solution was replaced by 0.1 M potassium phosphate buffer (pH 7.8) containing 5 mM 2-mercaptoethanol and 0.1 mM PLP by gel filtration using a Sephadex G-25 M (Pharmacia) column. The enzyme solution was diluted to 0.2 mg/ml by the same buffer, and 30 μl each (approximately 0.12 U) was dispensed into micro centrifuge tubes. These were incubated at 37, 50, 55, 60, 65, or 70°C for 10 min, and placed on ice immediately. The remaining activities were assayed by method II, and plotted as a function of temperature. Alternatively, the enzyme was incubated at 37°C for 6, 14, 24, and 36 h, and the remaining activities were plotted as a function of time.

Other methods. Recombinant DNA manipulations and SDS–PAGE were done by standard protocols.[24] The N-terminal amino acids of purified

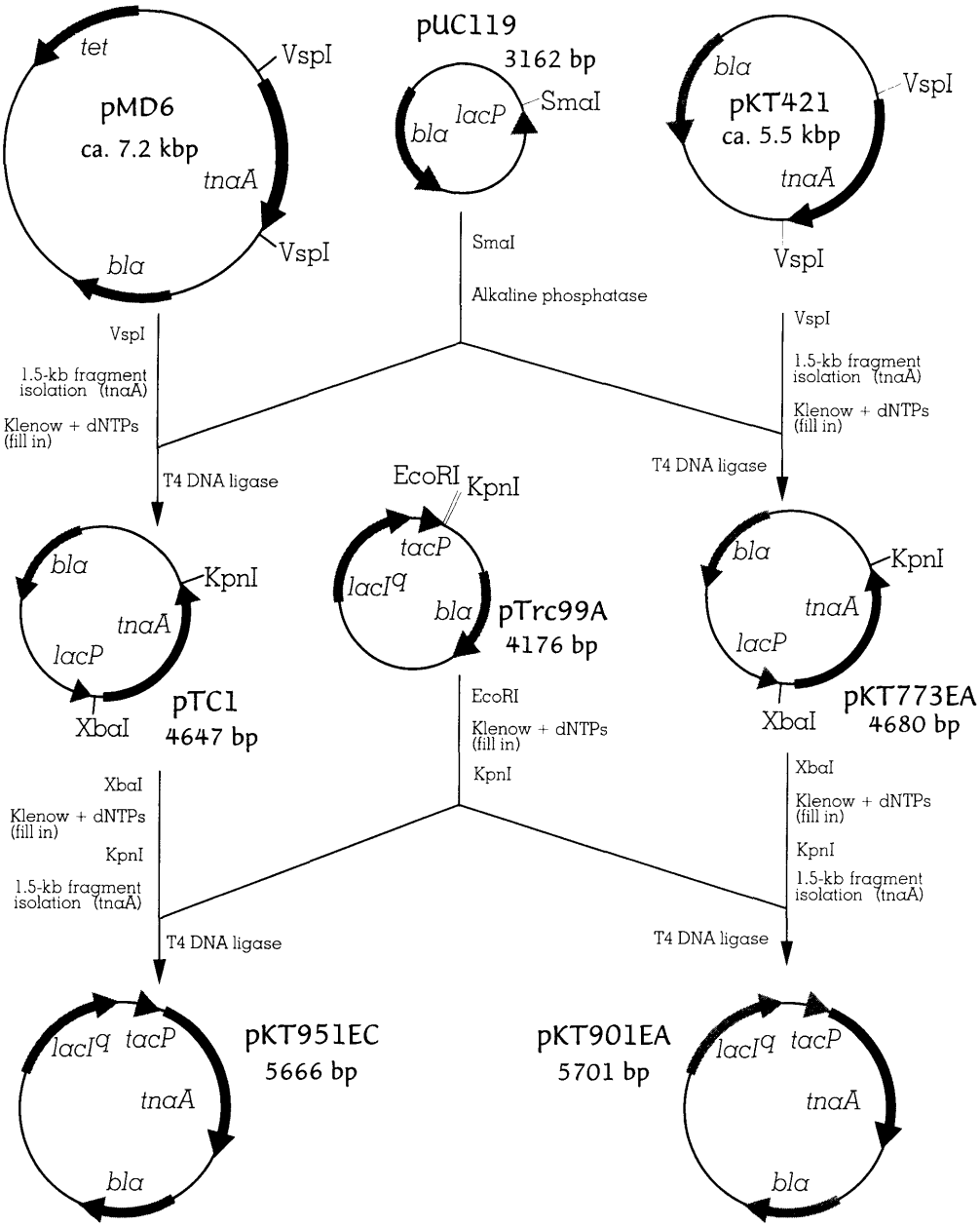

Fig. 1. Strategies of Plasmid Construction.

Abbreviations: *bla*, ampicillin resistance gene (β-lactamase gene); *tet*, tetracycline resistance gene; *lacP*, *lac* promoter; *tacP*, *tac* promoter; *lacI*^{q}, *lac* repressor gene with promoter mutation allowing elevated repressor production.

Tnase were sequenced using a Model 477A sequencer with a Model 120A PTH amino acid analyzer (Applied Biosystems). Cell growth was measured by the optical density at 590 nm.

Results

Induction study of Tnase

Table II shows the Tnase activities of *E. aerogenes* SM-18 and *E. coli* transformants under inducing or non-inducing conditions. Good growth was observed with every set of culture conditions. It seemed that Tnase coded in genomic DNA of *E. coli* JM109 or *E. aerogenes* SM-18 was induced a little, probably due to L-Trp contained in the basal LB medium. The expression in *E. coli* JM109/pKT773EA was constitutive. In contrast, the expression in *E. coli* JM109/pKT901EA was inducible. The expression level in *E. coli* JM109/pKT901EA induced with IPTG was 3.6 fold higher than that of *E. aerogenes* SM-18 induced with L-Trp.

Table II. Tnase Induction by IPTG or L-Trp

Strain	Inducer	Growth (OD_{590})	Tnase sp. act.*
E. aerogenes SM-18	—	4.85	0.17
	L-Trp	3.07	0.73
E. coli JM109/pKT773EA	—	3.76	1.02
	IPTG	4.08	2.63
E. coli JM109/pKT901EA	—	3.85	0.25
	IPTG	5.00	2.65
E. coli JM109/pTrc99A	IPTG	3.47	0.18

* Tryptophanase specific activity (U/mg dry weight cell).

The amount of Tnase in *E. coli* JM109/pKT901EA cells was found to be over 30% of the total cell protein by densitometric analysis of SDS–PAGE (data not shown). We used JM109/pKT901EA for the following experiments.

L-*Trp Synthesis*

Figure 2 shows that L-Trp synthesis was achieved rapidly and effectively due to high Tnase activity when transformants were used. It was also indicated in Fig. 2 that L-Trp production proceeded well when *E. aerogenes* Tnase was used. The amount of L-Trp produced by JM109/pKT901EA after a 24-h reaction was 18.2 g/liter, and yields *versus* indole and pyruvic acid were 89 and 52%, respectively.

Enzyme purification

The *E. aerogenes* Tnase was purified from *E. coli* JM109/pKT901EA to give a single band in SDS–PAGE (Fig. 3). The molecular mass was estimated to be 52 kDa, and this agreed with the value calculated from DNA sequence. The purification is summarized in Table III. This preparation was applied to N-terminal amino acid sequencing. The N-terminal residue was found to be methionine and following 29 residues were readable and matched with that estimated from DNA sequence, suggesting that the preparation had good purity. We also purified *E. coli* Tnase from *E. coli* JM109/pKT951EC as shown in Table IV and Fig. 3 by the same method. From the data of Tables III and IV, it was found that the specific activity of *E. coli* Tnase was 1.4 fold higher than that of *E. aerogenes* Tnase. It was also calculated from the data that the Tnase protein amounted to 17.5% and 15.3% of the total protein of the cell extracts from JM109/pKT901EA and JM109/pKT951EC, respectively.

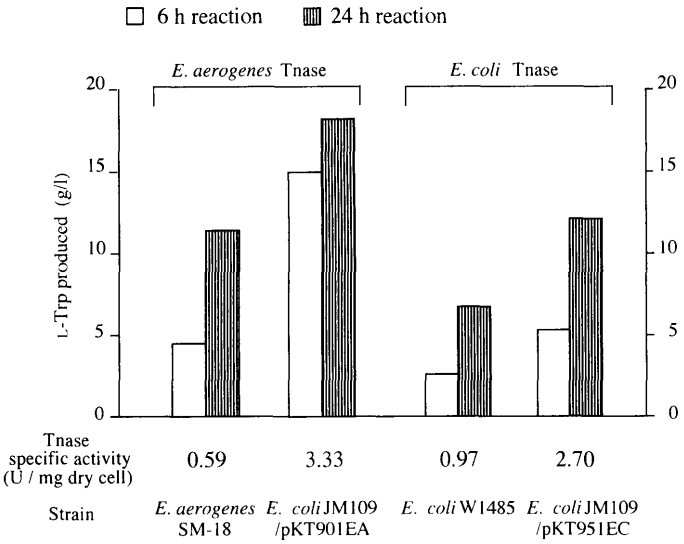

Fig. 2. L-Trp Synthesis by the Tnases from *E. coli* or *E. aerogenes*.

Specific activities of Tnase of cells used for L-Trp synthesis were measured by method I.

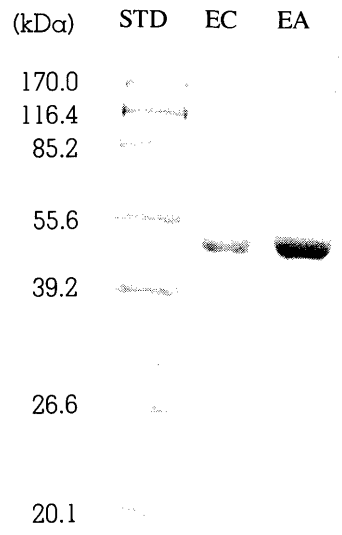

Fig. 3. SDS-PAGE of the Purified Tnases.

Lanes: STD, molecular mass standards; EC, *E. coli* Tnase; EA, *E. aerogenes* Tnase. Protein bands were stained with Coomassie Brilliant Blue R-250.

Table III. Summary of Purification of *E. aerogenes* Tnase from *E. coli* JM109/pKT901EA

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)
Sonic disruption	1510*	4440	2.9	100
Protamine sulfate	1220*	4390	3.6	99
Ammonium sulfate fractionation	960	4400	4.6	99
Heat treatment	620	3970	6.4	89
Ammonium sulfate precipitation	311	3820	12.3	86
Q-Sepharose chromatography	196	3280	16.7	74

* Protein concentration was measured by the Bradford method with bovine serum albumin as the standard.[25]

Table IV. Summary of Purification of *E. coli* Tnase from *E. coli* JM109/pKT951EC

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)
Sonic disruption	1560*	5630	3.6	100
Protamine sulfate	1390*	6730	4.8	120
Ammonium sulfate fractionation	414	4260	10.3	76
Heat treatment	244	3970	16.3	71
Ammonium sulfate precipitation	199	3980	20.0	71
Q-Sepharose chromatography	155	3650	23.5	65

* The same as in Table III.

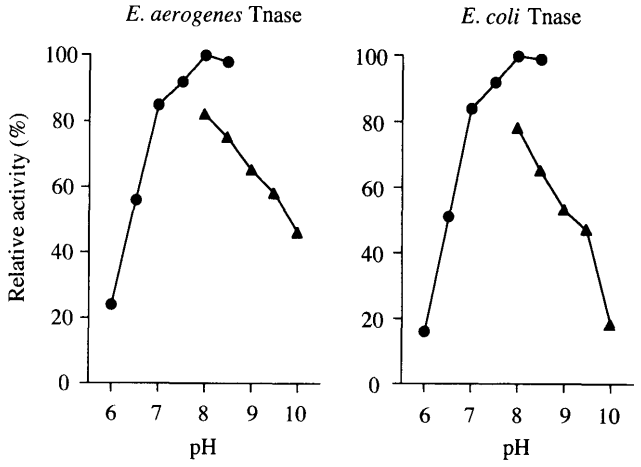

Fig. 4. Effects of pH on Activities of Tnases from *E. aerogenes* and *E. coli*.

Activities were assayed by method II. The buffer systems were 0.12 M potassium phosphate buffer (—●—), or 0.10 M glycine-KOH buffer with 0.02 M KCl (—▲—).

Properties of purified enzyme

Gel filtration analysis of *E. aerogenes* Tnase showed that the molecular mass of the native enzyme was 210 kDa. Therefore, it seemed that the enzyme is a tetramer with identical subunits like other Tnases from some bacteria (reviewed by Snell[26]). The pH profiles of enzyme activities are presented in Fig. 4. Optimum pH values for L-Trp degradation reaction for Tnases from *E. aerogenes* and *E. coli* were found to be 8.0. The results of heat inactivation analysis are shown in Fig. 5. The thermal stability after 10 min of incubation of *E. aerogenes* Tnase was 5°C higher than that of *E. coli* Tnase. We also examined the effects of long-term incubation at 37°C on the enzyme activity. As shown in Fig. 6, the activity of *E. coli* Tnase reduced to less than 1% after incubation for 24 h, while *E. aerogenes* Tnase retained 54% of the original activity. The SDS-PAGE analysis of each Tnase revealed that the protein band of the treated sample was not different from that of the non-treated one. Thus, it seemed that the inactivation by long-term incubation was not due to degradation by contaminating protease(s) (data not shown).

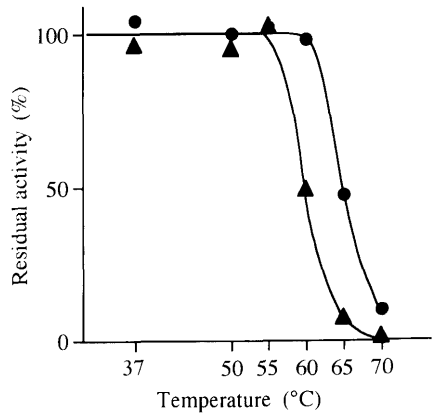

Fig. 5. Heat Inactivation Curve of Tnases from *E. aerogenes* and *E. coli*.

Enzymes were incubated for 10 min at various temperatures. Residual activities were assayed by method II, and expressed in percentages of the activity of non-treated enzyme. Symbols: ●, *E. aerogenes* Tnase; ▲, *E. coli* Tnase.

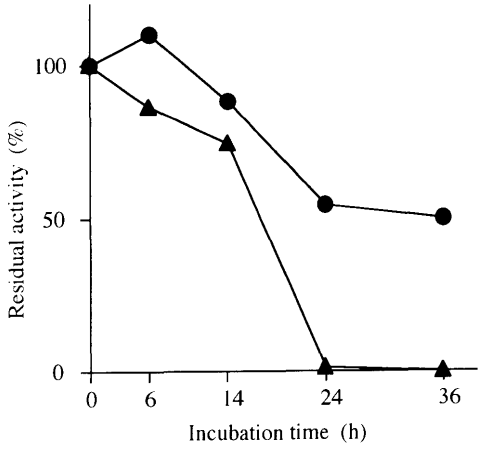

Fig. 6. Inactivation of Tnases from *E. aerogenes* and *E. coli* by Long-term Incubation at 37°C.

Residual activities were assayed by method II, and expressed in percentages of the activity of non-treated enzyme. Symbols: ●, *E. aerogenes* Tnase; ▲, *E. coli* Tnase.

Discussion

We have subcloned the *E. aerogenes* Tnase gene into expression vector pTrc99A, and the Tnase was expressed at high levels by induction with IPTG. The activity of *E. coli* JM109/pKT901EA was 3.6 times higher than that of the wild type strain *E. aerogenes* SM-18. *E. aerogenes* SM-18 is originally a high producer of Tnase, and Tnase was accumulated to 15% of total protein when L-Trp was used as the inducer.[13] Therefore, we considered that the expression level of 30% measured by densitometry in *E. coli* JM109/pKT901EA is at the maximum level. The Tnase expression of *E. coli* JM109/pKT773EA was constitutive, and not inducible (Table II). This may be explained by the conditions that the single *lacI* gene on the bacterial genome could not produce sufficient repressor to titrate the *lac* operator on the high copy number plasmid. In contrast, the Tnase of *E. coli* JM109/pKT901EA was inducible due to the $lacI^q$ gene on the plasmid. We are now producing both pyruvic acid and Tnase in the same batch culture for more convenient L-Trp production using *E. coli* W1485*lip2*,[14] which harbors pKT901EA. In this process, we found that the timing of initiation of induction is critical for efficient production of both pyruvic acid and Tnase (unpublished data). Therefore, the inducible feature seemed to be favorable, though the use of expensive IPTG must be avoided by using some other cheap inducer such as lactose.

We have demonstrated that the elevated expression of Tnase by recombinant DNA techniques is effective for L-Trp production from indole, ammonia, and pyruvate (Fig. 2). It was considered that *E. aerogenes* Tnase was superior to *E. coli* Tnase qualitatively in L-Trp production, because the L-Trp yield by the cells of JM109/pKT901EA after 6 h of reaction was about three times higher than that by the cells of JM109/pKT951EC, although the specific activity of Tnase in each transformant showed a small difference (Fig. 2). At present, we have no data concerning kinetic parameters for synthetic reaction of *E. aerogenes* Tnase. However, it may be assumed that better stability of *E. aerogenes* Tnase against heat treatment and long-term incubation (Figs. 5, 6) is one of the reasons why L-Trp production proceeded well by *E. aerogenes* Tnase.

The amount of L-Trp obtained, 18.2 g/liter, is rather low compared with other results using Tnase or tryptophan synthase.[7-9] This was simply because the concentration of pyruvic acid in the reaction mixture, about 15 g/liter, was chosen to be similar to that produced by *E. coli* W1485*lip2*.[14] It seems possible to increase the L-Trp yields by raising the concentrations of the substrates, though it was not the aim of this report.

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