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Directed Evolution to Improve the Thermostability of Prolyl Endopeptidase[1]

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Prolyl endopeptidase is the only endopeptidase that specifically cleaves peptides at proline residues. Although this unique specificity is advantageous for application in protein chemistry, the stability of the enzyme is lower than those of commonly used peptidases such as subtilisin and trypsin. Therefore, we attempted to apply a directed evolution system to improve the thermostability of the enzyme. First, an efficient expression system for the enzyme in *Escherichia coli* was established using the prolyl endopeptidase gene from *Flavobacterium meningosepticum*. Then, a method for screening thermostable variants was developed by combining heat treatment with active staining on membrane filters. Random mutagenesis by error-prone PCR and screening was repeated three times, and as a result the thermostability of the enzyme was increased step by step as the amino acid substitutions accumulated. The most thermostable mutant obtained after the third cycle, PEP-407, showed a half-life of 42 min at 60°C, which was 60 times longer than that of the wild-type enzyme. The thermostable mutant was also more stable with a high concentration of glycerol, which is a necessary condition for *in vitro* amidation.

Key words: active staining, directed evolution, prolyl endopeptidase, thermostability.

Prolyl endopeptidase (PEP) [prolyl oligopeptidase, EC 3.4.21.26] specifically cleaves peptide bonds at the carboxyl side of a proline residue. It was first found in human uterus (*1*), and later shown to be a widely distributed from bacteria to mammals (*2–8*). The porcine (*9*), bovine (*10*), human (*11*), *Flavobacterium meningosepticum* (*12*), *Aeromonas hydrophila* (*13*) and *Pyrococcus furiosus* (*14*) PEP genes have all been cloned and sequenced. Amino acid sequence comparison has revealed that the PEPs constitute a new subfamily of serine proteases that is not related to the trypsin and subtilisin subfamilies (*15*, *16*).

PEP has attracted much attention in the pharmaceutical field because mammalian PEP is likely involved in the catabolism of proline-containing peptides such as substance P, neurotensin, luteinizing hormone–releasing hormone (LH-RH), thyrotropin-releasing hormone (TRH), bradkinin and angiotensin II (*17*, *18*). Furthermore, involvement of PEP in amnesia (*19*) and the generation of β-amyloid (*20*) has been suggested.

So far, PEP is the only endopeptidase known to show substrate specificity for proline, and therefore it is expected to be applicable as a catalyst for pharmaceutical production, for example the specific cleavage of precursor peptides to yield drug activity. It is also useful for *in vitro* modification of biologically active peptides, such as C-terminal amidation of LH-RH (*21*), because PEP can catalyze the coupling of peptides without side reactions. However, PEP is quite susceptible to inhibition or inactivation under conditions that are commonly applied in peptide reactions. Denaturing and/or solubility-promoting agents such as detergents and chaotropes are often added as supplements to facilitate hydrolysis of peptides. In coupling reactions, non-physiological conditions, such as high concentrations of organic solvents, and extreme pHs and/or high temperatures, are usually used to favour the coupling reaction rather than hydrolysis. The enzyme's sensitivity to such denaturative conditions greatly diminishes its utility.

In the present study, we attempted to obtain a thermostable mutant of PEP by directed evolution. As we had already cloned the PEP gene from *F. meningosepticum* (*12*), we constructed a high-level expression system for PEP in *E. coli* and established a purification procedure for this purpose. We then established a screening system in which the activity of the enzyme was monitored as the degradation of a synthetic substrate on a membrane filter. After three iterative cycles of random mutagenesis and screening, the thermostability of the enzyme increased gradually. The most improved clone, PEP-407, showed a 60-fold increase of

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Abbreviations: DMF, *N,N*-dimethylformamide; DMSO, dimethyl sulfoxide; IPTG, isopropyl-β-D(−)-thiogalactopyranoside; PEP, prolyl endopeptidase.

thermostability in terms of the half-life calculated from the first-order rate constant of inactivation at 60°C compared with the wild-type enzyme.

MATERIALS AND METHODS

Materials—Restriction enzymes and DNA-modifying enzymes were purchased from Takara Shuzo (Kyoto) and TOYOBO (Osaka). A dye terminator DNA sequencing kit was purchased from Perkin Elmer (Foster, California). Synthetic peptides for enzyme assays, *Z*-Gly-Pro-*p*-nitroanilide and *Z*-Gly-Ala-Pro-β-naphthylamide, were purchased from Seikagaku (Tokyo) and Bachem AG (Bubendorf, Switzerland), respectively. DNA purification columns were from Qiagen (Hilden, Germany). CM52 was from Whatman (Maidstone, England). Nitrocellulose membrane (Optitran BA-S 85 Reinforced NC) was from Schleicher and Schuell (Dassel, Germany). Other biochemicals were from Sigma Chemicals (St. Louis, Missouri), Wako (Osaka) and Nacalai Tesque (Kyoto).

Methods—Construction of expression plasmids: Expression plasmids for PEP were constructed from the cloned *Hin*cII–*Bam*HI fragment (2.9 kbp) harboring the PEP gene from *F. meningosepticum* (*12*). After filling the *Bam*HI site with the Klenow fragment, the fragment was subcloned at the *Bam*HI site of pUC 118 by blunt-end ligation to construct pFPEP3′. One of the three *Sca*I sites and the two *Pvu*II sites located in the coding region (at the Val583 and Thr593 positions, respectively) were deleted by cassette mutagenesis, using the synthetic oligonucleotide fragments 5′-GGGAGTAGGAGTTCTGGATATGCTTCGTTATAATA-AGTTTACTG-3′ and 5′-CAGTAAACTTATTATAACGAAG-CATATCCAGAACTCCTACTCCC-3′. A new *Eco*RI site was created immediately upstream from the initiation codon of the enzyme gene, also by cassette mutagenesis with the four oligonucleotide fragments 5′-GAATTCATGAAGTACA-ACAAACTT-3′,5′-TCTGTGGCAGTTGCAGCCTTTGCTTT-TGCAG-3′,5′-CTGCAAAAGCAAAGGCTGCAACTGC-3′, and 5′-CACAGAAAGTTTGTTGTACTTCAT-3′. The resulting fragment containing the whole open reading frame was cleaved with *Eco*RI and then subcloned into the *Eco*RI site of pKK223-3 to produce pKK-FPEP. The set comprising the promoter, the coding region and the terminator was excised with *Bbi*II from pKK-FPEP, and then blunted with the Klenow fragment, followed by ligation with the larger *Pvu*II fragment of pUC 119 to give an expression plasmid, pUK-FPEPb.

Enzyme activity assay: For evaluation of PEP activity, a solution of the enzyme or cell lysate was used as described by Yoshimoto *et al.* (*2*). For kinetic studies, Z-Gly-Pro-*p*-nitroanilide was used to monitor the initial velocity of the reaction. The substrate solution (0.05 ml of 4 mM Z-Gly-Pro-*p*-nitroanilide in 40% dioxane) was added to 0.94 ml of 0.1 M potassium phosphate buffer (pH 7.0) containing 1 mM DTT and 100 μg/ml BSA. After 3 min preincubation at 30°C, 0.01 ml of an appropriately diluted lysate or enzyme solution was added to the mixture, and then the change in absorbance was followed at 410 nm at 30°C. One unit (U) of the enzyme activity was defined as the amount of enzyme that released 1 μmol of *p*-nitroaniline per minute, corresponding to 8.87 OD/min. When stability in 50% glycerol was examined, 0.5% glycerol was present in the assay solution, which caused only a slight decrease in the activity, *i.e.* less than 3%.

Purification of prolyl endopeptidase from a periplasmic fraction prepared by the cold osmotic shock procedure: *E. coli* cells expressing PEP were harvested by centrifugation (10,000 ×*g* for 10 min) and washed with cold 0.1 M Tris-HCl buffer, pH 8.0. The washed cells were resuspended in a 0.5 M sucrose solution containing 5 mM EDTA buffered at pH 8.0 with 0.1 M Tris-HCl. Lysozyme (160 μg/ml) was added to the suspension and then the mixture was left on ice for 2 min before dilution with the same volume of ice-cold water. The diluted cell suspension was further left on ice for 30 min and then centrifuged at 10,000 ×*g* for 20 min. The supernatant was diluted again with the same volume of ice-cold water and its pH was adjusted to 7.0 with 1 *N* HCl. The crude solution obtained (periplasmic fraction) was directly applied to a CM52 column equilibrated with 20 mM sodium phosphate buffer (pH 6.8). The enzyme was eluted as a single peak with a linear gradient (0–0.25 M) of NaCl. The active fractions were combined, concentrated and dialyzed against 20 mM sodium phosphate buffer, pH 7.0. The protein concentration was estimated with an extinction coefficient E (1%/280 nm), 15.5, determined by amino acid analysis.

Error-prone PCR for random mutagenesis: The error-prone PCR conditions for random mutagenesis were optimized on the basis of the method described by Leung *et al.* (*22*). A basic reaction mixture contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% (w/v) bovine serum albumin, 0.2 mM each of four dNTPs, 10 ng/ml template plasmid, 1 μM each primer, and 25 U/ml *Taq* DNA polymerase, and the modifications made were as follows. (i) 25, 50, or 75 μM $MnCl_2$ was added with an increased concentration (5 mM) of $MgCl_2$. (ii) DMSO was added at 5 or 10%. (iii) The concentration of $MgCl_2$ was changed to 5 or 10 mM. (iv) The concentration of dGTP was increased to 0.5, 0.75, or 1.0 mM with a decrease of other dNTPs to 0.1 mM. (v) The concentration of dATP was 0.15, 0.2, or 0.25 mM with a higher concentration (1 mM) of other dNTPs in the presence of 5 mM $MgCl_2$. The PCR was carried out under all these modified conditions separately as well as the basic conditions using a Gene Amp™ 9600 PCR system (Perkin Elmer) with a program of 94°C for 1 min; 94°C for 30 s, 50°C for 30 s, and 72°C for 4 min (25 cycles); and finally 72°C for 7 min. All the PCR products were subjected to digestion with restriction enzymes and ligation with the vector fragment to give a plasmid library in order to ensure the randomness of mutations.

Screening for thermostable mutant clones: *E. coli* JM109 was transformed with the ligation mixture for the mutant library and then plated on nitrocellulose filters placed on TY agar plates (1% bacto-tryptone, 0.1% glucose, 0.8% NaCl, 1.5% agar, pH 7.0) containing 100 μg/ml ampicillin. The plates were incubated at 37°C and replicas of each filter were prepared when the colonies appeared on the filters. Both the original and replica filters were incubated on the TY agar plates at 37°C for a few hours and overnight, respectively. The originals were then stored at 4°C and the replicas were subjected to active staining. Lysis of the cells was performed by soaking the filters at room temperature in lysis buffers: 50 mM Tris-HCl (pH 8.0), 1 mg/ml lysozyme, and 1% Triton X-100 for 30 min. The filters were washed in 20 mM sodium phosphate buffer (pH 7.0) to remove bacterial debris and then blocked with 5% skim

milk in 20 mM sodium phosphate (pH 7.0) to prevent nonspecific adsorption of the substrate peptide. After incubation in 20 mM sodium phosphate buffer (pH 7.0) for 15 min at an appropriate temperature (50–61°C) to select several colonies, the filters were stained with 0.2 mM Z-Gly-Ala-Pro-β-naphthylamide and 0.01% Fast Garnet GBC sulfate salt. Selected clones were isolated and subjected to secondary screening to confirm their thermostability and to choose the best clone. They were inoculated on to new filters on TY agar plates again, and then active staining was performed at a temperature 1–2 degrees higher than that used for the first screening.

RESULTS

Expression of Prolyl Endopepidase in E. coli—In our cloning study on the PEP gene from *F. meningosepticum* (*12*), the *Hin*cII-*Bam*HI fragment of the gene was subcloned into pUC19 to construct the plasmid pFPEP3. The *E. coli* JM109 strain transformed with the plasmid clearly exhibited enzyme activity in the cell suspension assay described under "MATERIALS AND METHODS." Optimization of the expression level was carried out, and a plasmid with the *tac* promoter and rrnB ribosomal RNA transcription terminator, pUK-FPEPb, was constructed as described under "MATERIALS AND METHODS," and used for further studies. *E. coli* JM109 was transformed with pUK-FPEPb, and transformants were selected on LB plates containing 50 μg/ml ampicillin and grown in LB medium with ampicillin overnight. For expression of PEP, 100 ml of a high-density growth medium, CIRCLEGROW (Bio101 inc, Carlsbad, CA), containing 100 μg/ml ampicillin was inoculated with 2 ml of the overnight culture and shaken at 37°C. The transformants were found to express PEP in a soluble and active form, which was easily obtained in the cell lysate, at sufficiently high levels (8,060 U/liter culture) even without IPTG induction. For purification, *E. coli* cells were harvested 24 h after inoculation with preculture.

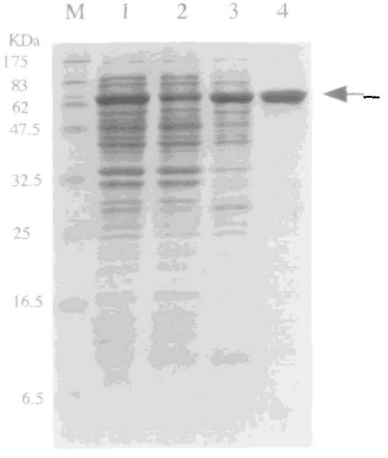

Fig. 1. **Expression of PEP in *E. coli* JM109.** After osmotic shock, the precipitate was separated from the supernatant (periplasmic fraction) by centrifugation and sonicated to prepare the spheroplast sample for gel electrophoresis. The whole cell extract (lane 1), and the spheroplast (lane 2) and periplasmic (lane 3) fractions from 25 μl of culture were run on a 12.5% SDS–polyacrylamide gel together with 2.0 μg of purified PEP (lane 4) and marker proteins (lane M). The band corresponding to PEP is indicated by the arrow.

Purification of Recombinant Prolyl Endopeptidase from the Periplasmic Fraction—The results of DNA sequencing suggested that PEP from *F. meningosepticum* possessed an N-terminal signal sequence. Therefore we subjected *E. coli* cells expressing PEP to the cold osmotic shock procedure, and measured the enzyme activities released into the supernatant (periplasmic fraction) and entrapped in the spheroplast fraction. Typically, about 75% of the enzyme activity was recovered in the periplasmic fraction and 25% was detected in the spheroplast homogenate obtained on sonication (data not shown). The specific activity of the enzyme in the periplasmic fraction was found to be as high as 51.2 U/mg protein, demonstrating that PEP accounts for the majority of periplasmic proteins in *E. coli*. These results were further confirmed by SDS-PAGE of the whole cell lysate, and the spheroplast and periplasm fractions (Fig. 1). It was clearly demonstrated that most of the proteins appearing in the whole cell lysate lane remained in the spheroplast fraction, and that smaller amounts of proteins including PEP were released into the periplasmic fraction by the osmotic shock. The distribution of PEP between the spheroplast and periplasmic fractions was well in accord with the above enzyme assay results, proving secretion of the expressed PEP into the periplasm. Since the purity of the enzyme in the crude extract had been very high, PEP was purified to homogeneity from the periplasmic fraction (Fig. 1, lane 4) by only a single step of chromatography. The periplasmic fraction was loaded on a CM-cellulose column. After extensive washing of the column, the enzyme was eluted with a linear gradient of NaCl as a single peak. The amount of the enzyme was 7,200 U/liter culture with a specific activity of 124 U/mg protein, showing an extremely high yield of 58.1 mg/liter culture.

The purified enzyme was subjected to amino acid sequence analysis, which revealed that the N-terminal amino acid sequence was A-Q-N-S-N-X-L-K-Y-P. This sequence showed that the expressed enzyme in *E. coli* lacked the first 19 amino acid residues constituting the putative signal peptide.

Development of a Directed Evolution System—We devel-

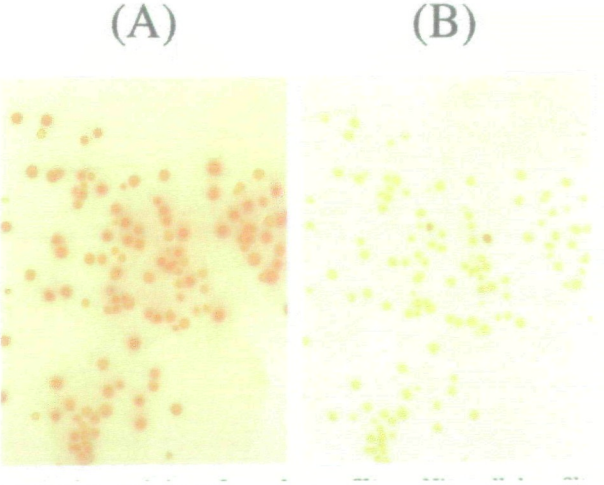

Fig. 2. **Active staining of membrane filters.** Nitrocellulose filters which had adsorbed PEP expressed by the first-generation bacterial library derived from the wild-type gene were stained as described under "MATERIALS AND METHODS" without (A) or after heat treatment at 50°C (B).

oped a procedure for directed evolution in which random mutagenesis and selection were iterated to improve the thermostability of PEP. Random mutation was generated by error-prone PCR (*22*). The 2.3-kb DNA fragment of pUK-FPEPb encoding PEP was amplified with a pair of 25-nucleotide primers, 5′-GAGCGGATAACAATTTCACACAG-GA-3′ and 5′-AATCTTCTCTCATCCGCCAAAACAG-3′, which are complementary to the regions immediately flanking the *Eco*RI and *Pst*I sites. The error-prone PCR conditions for random mutagenesis were optimized to introduce a limited number of base substitutions (*i.e.*, one or at most only a few) into the PEP gene. The effects of several factors decreasing the fidelity of *Taq*DNA polymerase were examined, *i.e.*, addition of $MnCl_2$ or DMSO, alteration of the $MgCl_2$ concentration, and an imbalance of the deoxynucleotide pool. The PCR was carried out separately under fourteen sets of conditions including the basic ones and all the products were used to generate a mutation library (see "MATERIALS AND METHODS").

Active staining of filter membranes utilizing a synthetic peptide substrate was employed for screening of the thermostable mutant clones. *E. coli* harboring the expression plasmid of PEP was plated out on nitrocellulose filters to obtain colonies. After replica filters had been subjected to cell lysis and washing, the expressed PEP was adsorbed on the filter membranes with retention of their activity. Therefore, colonies expressing PEP were visualized as red spots on the filter membranes upon staining with *Z*-Gly-Ala-Pro-β-naphthylamide and Fast Garnet GBC sulfate salt, because the hydrolysis product of the synthetic substrate, β-naphthylamine, reacts with Fast Garnet GBC to yield an insoluble product (Fig. 2a). When active staining was carried out after heat-treatment of replica filters, only colonies producing the thermostable enzyme were stained, other colonies only giving a faint yellow trace (Fig. 2). The clones corresponding to the positive colonies were then isolated from the original filters.

Screening for Thermostable Mutants—The DNA fragment amplified by error-prone PCR was digested with *Eco*RI and *Pst*I, and then subcloned into the original vector fragment to give the first-generation gene library. *E. coli* JM109 was transformed with the gene library to generate a bacterial library comprising about 80,000 bacterial colonies. After lysis of the bacterial cells and blocking, the membranes were washed in phosphate buffer at 52°C for 15 min. After active staining, the most thermostable clone among the visible colonies, designated as PEP-227, was selected and isolated.

The plasmid containing the PEP-227 gene (pUK-FPEP-227) was used as a template for the second cycle to give a library consisting of about 40,000 colonies. Screening of the second generation was performed similarly except for the screening temperature (57°C), and a clone expressing the most heat-stable PEP (PEP-361) was selected. In the third cycle the mutagenesis and screening were repeated once again starting from the expression plasmid encoding PEP-361 (pUK-FPEP-361), and from among 50,000 clones the mutant PEP, PEP-407, was isolated. The plasmid encoding PEP-407 (pUK-FPEP-407) was isolated for DNA sequencing. The temperature for the heat treatment was gradually increased (61°C in the third cycle) as the mutagenesis and screening cycle proceeded.

Characterization of Thermostable Mutants—Expression plasmids of the thermostable mutants, pUK-FPEP-227, pUK-FPEP-361, and pUK-FPEP-407, were subjected to DNA sequencing to determine the amino acid substitutions that had been introduced into these clones. It was found that a single or only a few base substitutions had been introduced during each mutagenesis, and it was also confirmed that the mutations had accumulated in the gene during sequential cycles of mutagenesis and screening (Table I).

The thermostabilities of the wild-type and evolved enzymes were evaluated in terms of T_{50}, the temperature at which the enzyme loses 50% of its activity in 30 min. Each purified enzyme solution was incubated at various temperatures ranging from 46 to 69°C for 30 min and then the residual activity was measured (Fig. 3). While T_{50} of the wild-type was estimated to be 53.5°C, that of thermostable mutants PEP-227, 36l, and 407 was shifted upward to 56.5, 59.5, and 60.5°C, respectively.

To evaluate the improvement of thermostability more quantitatively, the enzyme solutions were also incubated at 60°C, and the residual activity after various time intervals was measured and plotted semilogarithmically as functions of the incubation time (Fig. 4). The residual activity of the wild-type enzyme and the three mutants showed a fairly

TABLE I. **Base substitutions identified in mutant genes encoding thermostable mutants of PEP and the resulting amino acid substitutions.**

pUK-FPEP-227	pUK-FPEP-361	pUK-FPEP-407
199→C	199G→C	199G→C
$(E67Q)_{GAA\rightarrow CAA}$	(E67Q)	(E67Q)
	442G→A	442G→A
	$(A148T)_{GCA\rightarrow ACA}$	(A148T)
	1955G→T	1955G→T
	$(G652V)_{GGT\rightarrow GTT}$	(G652V)
	1980T→C	1980T→C
	$(silent)_{TCT\rightarrow TCC}$	(silent)
		208T→C
		$(F70L)_{TTT\rightarrow CTT}$

The numbering of nucleotides and amino acid residues starts at the initiation codon of the PEP gene from *F. meningosepticum*.

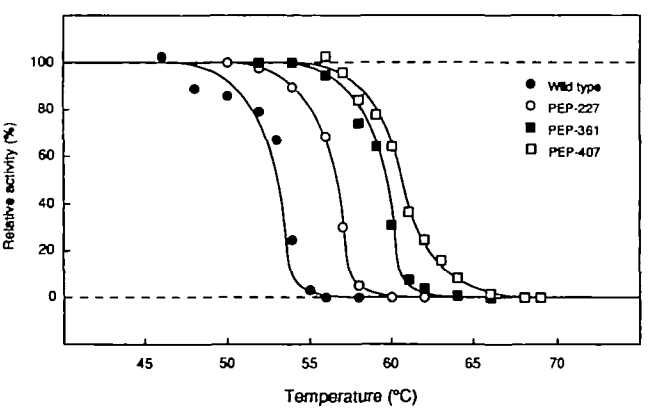

Fig. 3. **Thermal inactivation curves for the wild-type and thermostable mutants of PEP.** A solution of the wild-type PEP (solid circles), PEP-227 (clear circles), PEP-361 (solid squares), or PEP-407 (clear squares) was diluted to 0.2 mg/ml in 20 mM sodium phosphate (pH 7.0) and then incubated at various temperatures ranging from 46 to 69°C for 30 min. The residual activity was measured and plotted as a function of temperature.

TABLE II. **Thermostability and kinetic parameters of the wild-type enzyme and thermostable mutants.**

	T_{50} (°C)	Rate constant of inactivation (min^{-1})	$t_{1/2}$ (min)	Specific activity (U/mg)	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1} \cdot mM^{-1}$)
Wild-type	53.5	4.3×10^{-1}	0.70	110	0.084	210	2.6×10^3
PEP-227	56.5	7.1×10^{-2}	4.2	110	0.10	200	2.0×10^3
PEP-361	59.5	1.3×10^{-2}	23	84	0.091	150	1.6×10^3
PEP-407	60.5	7.2×10^{-3}	42	94	0.097	170	1.7×10^3

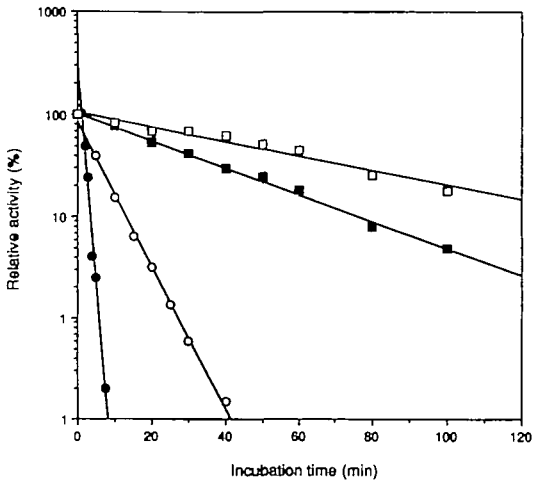

Fig. 4. **Thermal inactivation of the wild-type and thermostable mutants of PEP.** A solution of the wild-type PEP, PEP-227, PEP-361, or PEP-407 was diluted to 0.2 mg/ml in 20 mM sodium phosphate (pH 7.0) and then incubated at 60°C, the residual activity being measured after various intervals. The symbols for the enzymes are the same as in Fig. 3.

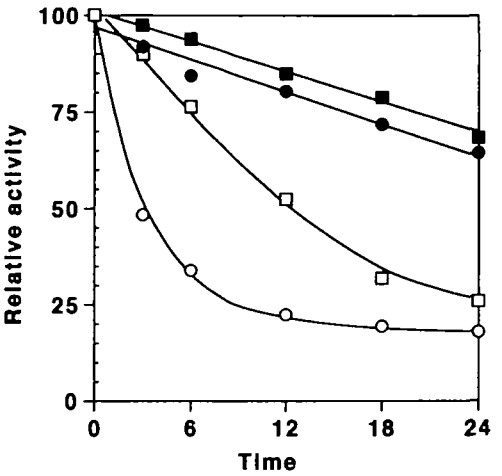

Fig. 5. **Stability of the wild-type and thermostable PEP in 50% glycerol.** Solutions of the wild-type and PEP-407 enzymes were incubated at 30°C in the absence (solid circles for wild-type and solid squares for PEP-407) or presence of 50% glycerol (clear circles for wild-type and clear squares for PEP-407). The concentration of PEP for each sample was 0.08 μM, as described by Togame *et al.* (*21*). Ten-microliter aliquots were removed at the times shown, and remaining activity was determined from the initial rate toward Z-Gly-Pro-pNA as described under "MATERIALS AND METHODS."

good linear correlation with the incubation time, indicating that the inactivation processes obeyed first-order kinetics. The inactivation rate constants were estimated from the slopes of the plots. Then, the values for the half-life of the activity, $t_{1/2}$, *i.e.* the periods in which the enzyme loses 50% of its activity, were calculated using the rate constants and used for evaluating the thermostability. The $t_{1/2}$ values for the wild-type enzyme, PEP-227, 361, and 407, as well as the inactivation rate constants, are summarized in Table II. The results demonstrated large improvements in the thermostability of the mutant enzymes, especially for PEP-407, which showed a 60-fold increase in $t_{1/2}$ compared with the wild-type enzyme.

The thermostability of PEP-407 under the conditions used for *in vitro* amidation of LH-RH (*21*) was examined. Solutions of the wild-type and PEP-407 enzymes were incubated at 30°C with or without 50% glycerol. With 50% glycerol, the wild-type enzyme rapidly lost more than half of its activity in 3 h, while both the wild-type and PEP-407 enzymes were rather stable without glycerol. However, the thermostability of PEP-407 in 50% glycerol was greatly increased in comparison with that of the wild type, more than 12 h being taken for it to lose half of its activity. This confirmed that directed evolution can improve the thermostability of the enzyme and efficiently increase its utility for peptide coupling reactions.

DISCUSSION

For directed evolution of proteins, three procedures are essential: an efficient expression system, mutagenesis to generate an appropriate library, and screening for the desired property of the target proteins. First, we attempted to established an efficient expression system for PEP in *E. coli*. Having constructed several kinds of plasmid and optimized the conditions for their expression, we established an excellent expression system in which the enzyme was secreted into the periplasm without induction with IPTG. The enzyme was highly purified using simple steps of osmotic shock and purification involving only column chromatography. The final yield of 58.1 mg/liter culture was much greater than that (1.25 mg/liter) with the *E. coli* system reported by Diefenthal *et al.* (*23*).

At the beginning of this study, as we had no structural information about the thermostability of PEP from *F. meningosepticum*, we intended to introduce random mutations into the gene as a whole. The error-prone PCR conditions were optimized to introduce a single or at most only a few base substitutions into the coding region of the PEP gene. The validity of the screening was proved by the successful acquisition of thermostable PEP mutants. With three sequential cycles of directed evolution, we observed the accumulation of base substitutions in the gene, a gradual increase in the screening temperature, and the T_{50} that indicated stepwise improvement in the thermostability of the enzyme. These results showed that the directed evolution system was successful. For more quantitative evaluation of the thermostability, we calculated the half-life of the activ-

ity, $t_{1/2}$, *i.e.* the period in which the enzyme loses 50% of its activity, because the residual activity of all the enzymes studied here showed a fairly good linear correlation with the incubation time at 60°C when plotted semilogarithmically as a function of the incubation time. Using the $t_{1/2}$ values for the wild-type, PEP-227, 361, and 407 enzymes, we also observed a gradual increase in thermostability with directed evolution, as seen for T_{50}, and the final degree of improvement for PEP-407 was estimated to be 60 times compared to the wild-type enzyme in terms of $t_{1/2}$.

Recently, the crystal structure of porcine prolyl oligopeptidase was reported (*24*), and a substantially similar structure for the PEP from *F. meningosepticum* can be assumed from the 38.2% homology of the amino acid sequences (*12*). In the porcine enzyme, residues 428–710, which exhibit a characteristic α/β hydrolase fold, built up a catalytic domain together with the 72 residues of the N-terminal portion, and the rest of the residues form a non catalytic β-propeller domain. As seen on alignment of primary sequences, three mutations out of four accumulated in PEP-407 are clustered in the contact region between the N-terminal and C-terminal portions of the catalytic domain. E67 and F70 of the *F. meningosepticum* enzyme, which correspond to Q46 and I49 in the porcine enzyme, respectively, are located on the α1 helix, these residues facing the C-terminal portion. Residue G652 on the αE helix is also directed to the N-terminal portion. These three mutations might strengthen the interaction between the N-terminal and C-terminal portions, and stabilize the enzyme molecule. This is consistent with the consideration that the N-terminal portion provides stability by covalently linking the propeller domain to the catalytic domain (*24*). A more precise structure of the enzyme from *F. meningosepticum* is necessary to interpret the mechanism of stabilization caused by amino acid substitutions in further detail.

Stabilization of proteins through site-directed mutations often leads to decreased enzymatic activity (*25–27*) and an inverse correlation between protein stability and activity has been suspected. On the contrary, the specific activity and kinetic parameters of all the mutant enzymes in this study were comparable with those of the wild type (Table II). Amino acid substitutions introduced into the thermostable mutant enzymes did not seem to affect their enzymatic activity greatly. Together with other cases in which the stabilization of proteins was achieved without loss of their functions (*28*, *29*), our results strongly support the implication that the structure of a protein has a higher tolerance as to changes in structural amino acids than in functional ones (*27*).

Finally, we confirmed the stabilization of the thermostable enzyme in the presence of glycerol, which we used for the *in vitro* peptide coupling reaction. In enzymatic peptide synthesis reactions, water-miscible organic solvents are often used at high concentration to increase the yield by shifting the equilibrium toward the coupling reaction (*30*). However, the hydrolytic activity of PEP from *F. meningosepticum* is markedly inhibited in the presence of co-solvents, even at rather low concentrations (~10%) (*31*). We previously reported that the *in vitro* amidation reaction of LH-RH was effectively catalyzed by PEP from *F. meningosepticum* (*21*). In the previous study, we examined the effects of several organic solvents, including 1,4-dioxane, dimethylformamide (DMF), dimethylsulfoxide, glycerol, ethylene glycol and ethanol, and confirmed their inhibitory activity against PEP. All of them except ethylene glycol and glycerol inhibited PEP activity even at 10%, and stopped the coupling reaction before the equilibrium state had been attained. Only glycerol had a very low inhibitory effect on PEP, and was effective for improving the conversion of the PEP-catalyzed coupling reaction at high concentration (50%). Therefore, the stability of PEP with a high concentration of glycerol is important for judging the versatility of the enzyme. As shown in Fig. 5, PEP-407 was more stable than the wild-type enzyme in the presence of 50% glycerol, whereas the wild-type enzyme rapidly lost more than half of its activity in 3 h. Both the wild-type and PEP-407 enzymes showed only a slight difference in stability in the absence of glycerol. It was suggested that denaturation of the enzyme molecule was at least partly involved in the loss of PEP activity in 50% glycerol, and that thermostabilization of the enzyme molecule also had a beneficial effect on stability in the presence of organic solvents. A lower substrate/enzyme ratio can be applied in kinetically controlled peptide reactions, and might also be applicable to those that are controlled thermodynamically if further stabilization of PEP could be achieved. The inhibitory effect of DMF at a low concentration below 20% was also examined, but no difference was observed between the wild-type and PEP-407 enzymes (data not shown). Therefore, inhibition of PEP activity by organic solvents at low concentration is considered to be due to a mechanism different from denaturation of the enzyme molecule, although further study of this aspect is necessary. The stabilization of the enzyme and prolongation of its half life with directed evolution indicate that thermostabilization using this approach is very promising for broadening the utility of PEP in peptide reactions. Recently, the cloning and overexpression of PEP from the hyperthermophilic archaeon, *Pyrococcus furiosus*, was reported (*14*, *32*). The stability of the PEP from *P. furiosus* is very high, but the optimum temperature of the enzyme is between 80 and 90°C, and thus it needs adaptation to a lower temperature for use at 30°C. PEP from *F. meningosepticum* stabilized by directed evolution, which shows high activity at 30°C, would be more applicable for peptide synthesis because it can be used under milder conditions.

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