Hydrophobilization of Lysozyme by Genetic Combination with Polyproline at the Carboxyl Terminal

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Polyproline, which is an amphiphilic polypeptide, was incorporated into lysozyme by the recombinant DNA technique. The hydrophobicity of lysozyme increased with the incorporation of a longer polyproline. The bactericidal activity of lysozyme against Gram-negative bacteria increased with the hydrophobicity of the enzyme.

The use of enzymes greatly expands the potential applications in organic synthesis.[1] To increase their utilization, enzymes have been suspended, cross-linked by glutaraldehyde, encapsulated in reverse micelles, immobilized on a solid support, and coated with fatty acid.[2] Attachment of synthetic polymers to enzymes has been reported useful to solubilize enzymes in organic media.[3,4] The catalytic rate was significantly enhanced by the solubilization with synthetic polymers.[4] However, site-specific modification with synthetic polymers is difficult. On the other hand, genetic engineering has an advantage in the synthesis of enzymes having a definite structure. Previously Chen and Arnold tuned the activity of subtilisin for catalysis in dimethylformamide by sequential random mutagenesis.[5] Here, lysozyme was markedly hydrophobilized by attachment of a polyproline chain at the carboxyl end of the enzyme instead of chemical modification with synthetic polymers, and although it was not soluble in organic media, one of the activities was enhanced.

The plasmid containing human lysozyme[6] was kindly provided by Takeda Chemical Industries, Ltd. The oligonucleotide encoding polyproline was synthesized by the solid phase method and was inserted into the plasmid as shown in Figure 1.[7] The plasmid encoding lysozyme-polyproline was expressed in *Saccharomyces cerevisiae*. The lysozyme carrying a polyproline chain consisting of 10, 20, or 30 residues at the carboxyl end was secreted because the plasmid contained a signal sequence.[6]

The hydrophobicity of lysozyme was monitored by hydrophobic chromatography. The elution time was longer with increasing length of proline chain as shown in Figure 2. This indicates that the hydrophobicity of polyproline-modified lysozyme increases to that resembling polyproline with the increase of proline content.

The activities of human lysozyme were affected by polyproline incorporation (Table 1). The bactericidal activity of lysozyme against the Gram-positive bacteria was reduced with increase of polyproline length. The activity directly corresponded to the hydrolytic activity. The polyproline incorporation should induce some conformational change.

On the other hand, the bactericidal action of lysozyme against Gram-negative bacteria increased with the polyproline length. Ibrahim et al.[8] reported that the bactericidal action of lysozyme against Gram-negative bacteria was enhanced by the

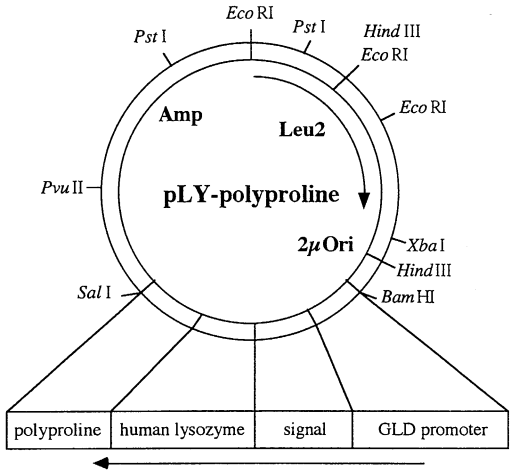

Figure 1. Expression vector (pLY-polyproline) for secretion of human lysozyme carrying polyproline at the carboxyl end. The glyceraldehyde 3-phosphate dehydrogenase (GLD) promoter and the signal peptide from chicken lysozyme were used to express and secrete the human lysozyme.

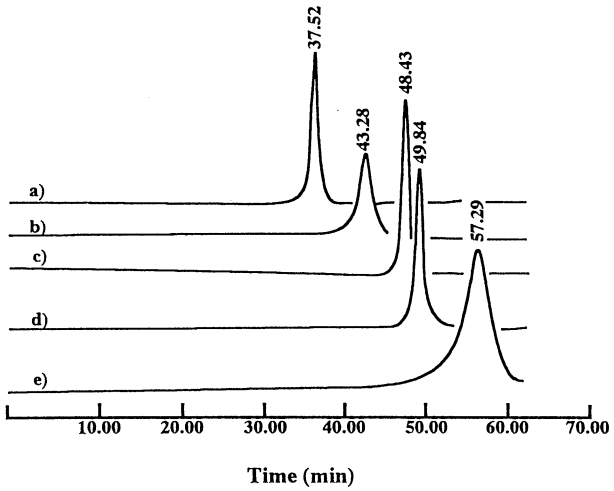

Figure 2. Analysis of mutant lysozymes by hydrophobic chromatography using TSKgel phenyl-5PW (Tosoh, Japan) (linear gradient from 0.5 M phosphate-buffered solution (pH 7.0) + 2.0 M ammonium sulfate solution to 0.5 M phosphate-buffered solution (pH 7.0) over 60 min at the rate of 1.0 ml/min). a) lysozyme, b) lysozyme carrying 10 proline residues, c) lysozyme carrying 20 proline residues, d) lysozyme carrying 30 proline residues, and e) polyproline (MW=8000).

Table 1. Hydrolytic activity and bactericidal action of lysozymes against Gram-positive and Gram-negative bacteria

	Number of proline residues	Bactericidal action (%)		Hydrolytic activity (%)[11]
		Gram-positive[9]	Gram-negative[10]	
Wild-type		100	100	100
Mutant	10	81	—	79
	20	38	115± 2	33
	30	11	206±15	12

hydrophobic pentapeptide (Phe-Phe-Val-Ala-Pro) at the carboxyl terminus. The hydrophobic domain perturbated the unique cell envelopes of Gram-negative bacteria. The hydrophobilization of lysozyme by incorporation of polyproline also should enhance the bactericidal activity.

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7 Primer 5'-GGTGTTGCCCGGGTCGACCCGGTCGAC-3' was used to introduce a *Sma*I endonuclease site instead of stop codon of human lysozyme in pLY plasmid by site-directed mutagenesis. Four primers 5'-AATTCTCAGTC-ATCCCGGGGGGCCCACCACCACCAC-3', 5'-CACC-ACCACCACCACCATAATAGGTCGACCGACCTGTG-CTCA-3', 5'-TGGTGGTGGTGGTGGTGGGCCCCC-CGGGATGACTGAG-3' and 5'-AGCTTGAGCACAG-GTCGGTCGACCTATTATGGTGGTGGTGG-3' were annealed to form a double strand DNA fragment coding 10 residues of proline and the stop codon. Subsequently, the DNA fragments were annealed in the presence of pUC19 digested with *Eco*RI and *Hind*III and were ligated to construct the pPR10 plasmid. The pPR10a plasmid coding 10 residues of proline without the stop codon was constructed similarly. The pPR20 plasmid coding 20 proline residues was constructed by insertion of the proline region in the pPR10a plasmid into the upstream of pPR10 plasmid. The pPR30 plasmid was also constructed similarly. The synthesis of plasmid was confirmed by electrophoresis. The pLY was digested with *Sma*I and *Sal*I endonucleases. The pPR10, pPR20 and pPR30 plasmids were digested with *Apa*I-blunting-*Sal*I endonuclease. Subsequently, pLY-polyproline, which is the plasmid DNA coding the lysozyme carrying polyproline, was constructed by the ligation reaction. Lysozymes carrying various lengths of polyproline at the carboxyl end were expressed in *Saccharomyces cerevisiae* AH22 (MATa, *his3*, *leu2*) with pLY-pPR10, pLY-pPR20, or pLY-pPR30 plasmid. The synthesis of proteins was confirmed by electrophoresis and mass spectroscopy. The fundamental techniques are described in the following paper. T. Kanai, H. Atomi, K. Umemura, H. Ueno, Y. Teranishi, M. Ueda, and A. Tanaka, *Appl. Microbiol. Biotechnol.*, **44**, 759 (1996).

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9 The suspension of *Micrococcus lysodeikticus* cells (1 mg/ml) was mixed with 100 μl of lysozyme solution (10 μg/ml) in 0.1 M potassium phosphate buffer (pH 6.2). The mixture was incubated at 25 ℃ for 3 min. The decrease in absorbance at 450 nm was measured. The activity of wild-type lysozyme was taken as 100%.

10 The suspension of *E. coli* DH5α cells (10^4 cells/ml) was mixed with lysozyme (final concentration, 1.2 μg/ml) in 50 mM phosphate buffer (pH 7.0). The mixture was incubated at 37 ℃ for 60 min and a diluted portion (50 μl) was picked out and plated onto LB agar plates. After incubation of the plates at 37 ℃ overnight, the number of colonies was counted. The activity of wild-type lysozyme was taken as 100%.

11 The activity of lysozyme was determined by hydrolysis of *p*-nitrophenyl-tri-N-acethyl-β-chitotrioside. The substrate (6 mM) was dissolved in 0.5 ml of 40 mM citric acid buffer solution (pH 5.08) containing 20% of dioxane. The solution was incubated at 40℃ for 3 min followed by addition of lysozyme solution. The absorbance of *p*-nitrophenol released was monitored at 405 nm. The activity of wild-type lysozyme was taken as 100%.