PRODUCTION, STRUCTUAL ANALYSIS AND FUNCTIONAL DEVELOPMENT OF USEFUL PROTEINS

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PREFACE

The advent of gene technology in the 1970s has led to the flourishing of the biotechnology industry. In the 1980s, the attention was focused on biotechnology as a result of the enormous potential of recombinant DNA technology and genetic engineering. This expansion in commercial activity is based largely on the ability to produce proteins in heterologous cell systems at industrial scale. Although the conventional mutation and screening methods are still used to increase the productivity of already existing activities in microbial cells, recombinant DNA technology allows creation of novel activities that can be directed toward the synthesis of huge amount of products normally produced at lower levels by other organisms. The rapid developments in recombinant DNA techniques have resulted in the identification and isolation of various novel genes involving several known and unknown functions. There is almost invariably a need to express the gene in a heterologous cell system in order to produce: 1) material for structure-function studies, 2) diagnostic reagents (e.g. monoclonal or polyclonal antibodies), 3) material for *in vivo* activity testing.

Several alternative systems for the expression of foreign genes have been developed, an extension of *in vivo* genetic manipulation or intergenic gene transfer, in mammaliam cells, insect cells, fungal cells, bacterial cells, and transgenic animals or plants. The choice of expression system for a given gene depends upon the properties of the encoded protein (e.g. protein modifications needed for biological activity) and the objective of the study (e.g. structure-function analysis or production of diagnostic reagents). Other important considerations for the investigation are the available facilities, time and cost involved in generating the amounts of recombinant protein required.

The application of recombinant DNA technology to the production of useful gene products was focused initially on the research or the commercial level in *Escherichia coli*. *E. coli* has become an industrial microorganism since the

beginning of the modern fermentation industry. How has *E. coli* been converted to a producer of useful products? Many examples of the production of heterogeneous proteins in recombinant microorganisms have been reported. However, the productivity of heterogeneous proteins differs considerably from protein to protein even in the same host, which is *E. coli*.

The most widely used and convenient system for the production of foreign proteins is based on the simple prokaryote, *E. coli*. The advantages of this system are: 1) ease of gene manipulation, 2) availability of commercial products and reagents, including gene expression vectors, 3) ease of producing quantities of protein (up to a few gram in simple shake-flask culture), 4) speed of culture, and 5) adaptability of the system, thereby allowing solution to expression problems.

Expression of any foreign gene in *E. coli* begins with the insertion of the gene into an expression vector. Many forms of expression vector are available and they usually consist of: 1) a plasmid origin of DNA replication, 2) antibiotic selectable marker, 3) strong promoter and transcriptional terminator separated by a multicloning site (expression cassette), and 4) DNA sequences encoding a ribosomal-binding site.

When plasmids are used as vectors, plasmid stability, copy number, and effect on growth of the host are very important considerations. Several genetic menipulations are known to increase plasmid stability. Mutation of the host strain to lose both uptake of an amino acid and auxotrophy for that amino acid and insertion of the amino acid biosynthetic genes on the plasmid result to the plasmid-dependent growth of the host strain even in complex medium containing the amino acid. Plasmid-free segregants die under these conditions.

There is very little difference between the various strong promoters now available (pTac, λpL, λpR, T7) except in the method of transcriptional regulation. pTac and T7 expression systems are controlled by the chemical

inducer isopropyl-β-D-thiogalactoside (IPTG), whereas the λpL and λpR promoters by a temperature shift. Gene expression can also be controlled through the use of inducible copy-number vector. The dual-origin vector relies upon a temperature switch to increase plasmid copy number from five to 200 copies/cell, which induces foreign gene expression. Examples showing the use of these vectors is given below.

The translation of foreign mRNA remains a significant problem associated with gene expression in *E. coli*. The basic rules for achieving translation of mRNA are simple: 1) complementarity between the Shine-Dalgano (SD) sequence and the 3'end of the 16S ribosomal RNA, 2) a 6-10 bp spacing between the SD and the initiation codon (AUG), 3) an AT-rich base composition between the SD and the AUG. Regions of internal complementarity within the mRNA, paticularly those involving the SD and AUG should be avoided, since uncomplementarity in these regions significantly reduces the frequency of translational initiation. In instances where translation is a problem, a variety of approaches is available to overcome this obstacle.

However, we still sometimes encounter problems or limitations in the efficient expression and production of heterologous protein products in *E. coli*. This maybe partly due to the disadvantages of *E. coli* for foreign gene expression which are: 1) inability to carry out post-translational modifications typical of eukaryotic cells and 2) the tendency of foreign proteins to form insoluble inclusion bodies when expressed intracellularly.

Recent advances have shown that it is possible to engineer the *E. coli* host cell genetically to carry out at least a kind of post-translational modification, which is myristoylation of a foreign protein. This result suggests that other post-translational modifications can be carried out. The problem of protein insolubility can be addressed either by refolding the denatured protein *in vitro*

and varying the conditions of expression (e.g. temperature), or secreting into the periplasm or culture medium.

Accumulation of foreign protein intracellularly may be affected by proteolysis. This is particularly relevant to small peptides or to protein fragments. Two approaches have been used to overcome this problem, namely the use of host strains with reduced protease levels and the production of fusion proteins through the in-phase translational fusion of the gene sequence to the C-terminus of a highly expressed homologous protein. Genetically engineered proteins can be produced with special tails to facilitate purification. Tails can be ion-exchange, hydrophobic and covalent, metal chelate, affinity, and for acid precipitation. The fusion protein product is often purified using the property of the homologous fusion protein partner and cleaved to release the peptide or protein fragment of interest. Fusion vectors have been designed to produce unique chemical or protease cleavage sites between fusion partner and protein for this purpose. The fusion protein approach has an additional advantage, in that it also overcomes the problem of translational initiation, since the N-terminus of the fusion protein is a highly expressed homologous protein.

Proteins play central roles in all life processes, catalyzing biochemical reactions with remarkable specificity and serving as key structural elements in all cells and tissues. As enzymes, they catalyze both the digestion of food stuffs and the construction of new macromolecules. As collagen, actin, myosin, and intermediate filaments, they control the structure and motion of cells and organisms. Antibodies protect us against disease. Membrane proteins regulate ion transport and intercellular recognition. Repressors and activators regulate gene expression. Polymerases replicate genes, and histones help package DNA into chromosomes. They are involed in every aspect of life (e.g. catalysis, structure, motion. recognition, and regulation) and indispensable to all life.

The properties of many enzymes have been altered by genetic means. Mutagenesis and random screening of microorganisms over the years have led to the change in optimum pH, thermostability, feed back inhibition, carbon source inhibition, substrate specificity, Vmax, Km, and Ki. Such information is exploited now by the more rational technique of protein engneering. Single change in amino acid sequences have yielded similar types of changes in a large variety of enzymes. Today it is no longer necessary to settle for an enzyme's natural properties since these can be altered to suit the needs of the investigator or the process.

The ability to modify some proteins has increased our understanding of a variety of important biologically relevant processes, including protein stability, catalysis, and substrate binding. We need theoretical approaches to better understand these properties. With the appearance of fast and affordable computers, it is now practicable to study complex macromolecular systems with computer simulation methods.

In recent decades, tremendous progress has been made in understanding protein structure and function. The amino acid sequence of insuline was reported in the early 1950s and the three-dimensional structure of myoglobin was elucidated in 1960s. Genetic engineering was born in 1973. Progress in gene synthesis and genetic engineering have made it possible, in principle, to construct any desired amino acid sequence, which started the epoch of protein engineering in the early 1980s.

Protein engineering is a more recent development which has important implications for the biotechnology industry. Clearly, the capability of designing novel proteins broadens the opportunity to identify new products with altered function or improved stability or potency. Since the introduction of recombinant DNA methodology, genes can be removed from their nomal environment in an intact genome and isolated as DNA fragments on cloning vectors. The availability of purified genes *in vitro* in microgram amounts has

dramatically expanded the potential for inducing mutations. In controlled environment using test tube, it is now possible to alter, efficiently and systematically, the sequence of nucleotides in a segment of DNA. The new methods of *in vitro* mutagenesis are divided into three broad categories: 1) methods that restructure segments of DNA, 2) localized random mutagenesis, 3) site-directed (oligonucleotide-directed) mutagenesis. This classification emphasizes the practical aspects of each method's application. Analogues can be produced by recombinant DNA technology: 1) hybrid proteins can be produced by *in vitro* recombination of hybrid genes, 2) analogue polypeptides can be produced by chemical synthesis of DNA that contain triplets encoding a different amino acid that is present in the natural polypeptides, 3) a cloned gene can be randomly mutated before insertion into the vector, 4) site-specific mutagenesis can be employed using DNA polymerase to prepare mutant synthetic oligonucleotide primers on single-stranded DNA templates, 5) deletions or insertion of a few nucleotides in length can be carried out with restriction enzymes, and genes can be truncated by insertion of stop codons.

Protein engineering should have tremendous theoretical and practical implications. It can be used both to explore fundamental questions about protein folding, structure, and function, and to design useful protein for medical and industrial applications. A flow chart of protein engineering is as follows: 1) cloning of the gene of an enzyme , 2) determination of the nucleotide sequence and/or amino acid sequence of the enzyme, 3) biochemical characterization of the enzyme, 4) determination of three-dimensional structure of the enzyme, 5) molecular design of the enzyme, 6) alteration of the gene of the enzyme by site-directed mutagenesis, 7) characterization of the mutant enzyme, and back to step 5). Since protein engineering is an interdisciplinary research field, the cooperation among biologist, chemist, and physicist is essential for its success.

In the area of human therapeutics a number of so-called second-generation recombinant products are now in development (e.g. humanized monoclonal antibodies and modified tissue plasminogen activators). Second-generation peptide products include modifications of current products to alter specificity of targets, distribution in tissues, pharmacokinetics, side effects, and stability. These modifications will be achieved by recombinant DNA technology through removal of proteinase recognition sites and alteration of receptor-binding domains. Analogues will be especially useful to disturb human bioregulatory systems in the area of pharmacology. The pharmaceutical industry is using recombinant DNA techniques to prepare analogues for testing as receptor binding inhibitors and as effectors of activity. A commercial motive is the patentability of such products. Third-generation peptides will fuse coding sequences of different genes. In some cases it remains to be seen whether the risk of immunogenicity associated with the use of a modifed protein is offset by improved efficiency, but in general it seems that protein engineering is destined to have a real impact on drug discovery.

In the near future, it must be possible to alter the specific activity, substrate specificity, and optimum pH of enzymes, increase the thermal stability of proteins, and design proteins that can be used in nonaqueous solvents. In addition to modification of existing proteins, the possibilities to design entirely novel peptides and proteins with useful properties seem endless, since the amino acid sequences found in existing proteins represent an infinitesimal fraction of all possible amino acid sequence.

In this thesis, the aspect on the productivity of homologous and heterologous gene products are discussed in detail. In chapter I, I demonstrated an example of the genetic design of chimeric *E. coli* as an overproducer of mammalian proteins. In chapter II, the other example of overproduction of membrane-bound protein is shown. In chapter III and chapter IV, I deal with an example of

extracellular protein, analysis of structure-function relationships, and the engineering of starch-processing enzymes by recombinant DNA technology.

The aim of this investigation is to put into a real-world context the recombinant microorganisms used in the area of human therapeutics, conventional food industry, and industrial processes. I discuss current problems and how to overcome the limitations associated with the practical use of recombinant microorganism, as well as strategies for creating a supermicrobe.

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Chapter I Expression of a Gene for Human Metallothionein: Genetic Design of a Gene for Human Metallothionein II and Its Expression as an Active Fusion Protein in *Escherichia coli*

Introduction

Metallothioneins (MTs) are low-molecular-weight, cystein-rich proteins that have been found in a broad range of eukaryotic apecies and in many different tissues (1, 2). These proteins bind a variety of heavy metals, such as Cd, Zn, Cu, Hg, Co, Pb, Ni, Fe, Bi, Sn, Au, and Ag, with extremely high affinity through coordination bonds to their cysteins residues (1, 2). Mammalian MTs are present as two major forms, MT-I and -II, and both have been cloned (3-5). The structure of the MT-II from rat has been determined both by X-ray crystallography (6,7) and by NMR spectroscopy (8).

Since MT proteins are small, MT seems to be an ideal model protein with which to investigate the nature and function of the binding of heavy metals to proteins. As part of our investigations of the structure and function of MTs, the monkey gene for MT-II was expressed as a native protein and MT protein was synthesized in *Escherichia coli* (9). Subsequently, a variety of MT genes from different sources were cloned and expressed both directly and as fusion proteins (10-13). However, the rates of production of these MTs in *E. coli* were low compared with those of other eukaryotic gene products. The high level of cysteine residues, which account for one third of the total amino acids in MT, might be responsible for the low efficiency of the production of MT in E. coli. Thus, the production of MT in *E. coli* also provides an excellent model system for studies of the expression of SH-rich proteins.

In this report, we describe the design, expression, and production of an hMT-II protein fused to β-galactosidase. The fusion protien was formed in *E. coli* and purified to homogeneity. The fused protein had the ability to bind heavy metal ions when the protein was renatured after extraction from inclusion bodies. Thus, the fusion protein has the potential to serve as a tool in the engineering of proteins that have binding affinity for the ions of many different heavy metals.

Materials and methods

Materials — All reagents were of the higher grade available commercially and were used witjout further purification. Restriction endonucleases, T4 DNA ligase, T4 polynucleotide kinase, and Klenow enzyme were purchased from Takara Shuzo Co. Ltd. (Kyoto) or Toyobo Co. Ltd. (Osaka). DNA sequencing was performed using the sequenase version 2.0 Kit (United States Biochemical corporation, Cleveland, OH, USA). [α-^{32}P]-labeled dCTP (650 Ci/mmol) and [^{109}Cd]Cl_2 (164 mCi/mg) were obtained from ICN Biochemical Inc. (Irvine, CA, USA) and New Egland Nuclear (Boston, MA, USA), respectively. Metallothionein II from horse and rabbit and pure β-galactosidase from *E. coli* (native β-gal) were purchased from Sigma Chemical Co. Ltd. (St. Louis, MO, USA).

Bacterial strains and plasmids — *E. coli* JM109 (14) was used in this study. The cells were cultured in LB medium that contained ampicillin (100 μg/ml). The vectors constructed in this study were derivatives of pUEX2 (15) which was purchased from Amersham (Amersham, UK). pUEX2 is a derivative of pEX2 (16) and contains a thermolabile repressor, cI857, the lambda P_R promoter, and a *cro-lacZ* gene. It is basically the same as our previous construct pRK1 (17) except that the gene for galactokinase has been replaced by the gene for β-galactosidase.

Synthesis of the gene — DNA oligonucleotide were synthesized on a DNA synthesizer Cyclone^{TM} (Bio-Search, Burlington, MA, USA) and subsequently purified by HPLC. Phosphorylation and annealing of complementary single-stranded DNA fragments were perfomed by mixing 0.2 μmol of each strand of DNA in a 20-μl reaction mixture that contained 66mM Tris-HCl (pH 7.6) 6mM $MgCl_2$, 10 mM dithiothreitol, 1mM adenosine triphosphate and 10 units of T4 polynucleotide kinase. The mixture was incubated at 37°C for 60 min, at 65°C for 10 min and then allowed to cool at 12 °C for 4h. Ligation of the fragments

and manipulations of DNA were performed as described by Maniatis *et al.* (18).

Gene expression and preparation of inclusion bodies — *E. coli* JM109 (100 ml) harboring an expression vector was cultured in LB medium at 28°C. At the mid-logarithmic phase, the cell culture was shifted to 42°C for 1 h and then incubated 37°C for a further 16 h. The cells were harvested by centrifugation at 8,000 x *g* for 10 min at 4°C. The cells were washed with 10 ml of 30 mM Tris-HCl buffer (pH 8.0) that contained 30 mM NaCl and then they were suspended in 10 ml of the same buffer.

The cell suspension was placed in an ice bath and then disrupted by sonication (model W-385, Heat Systems Ultrasonics, Farmingdale, N. Y.) at 1-s intervals for a total time of 10 min at 200 watts output. The supernatant was removed by centrigugation at 8,000 x *g* for 10 min at 4°C. The pellet was washed twice with the same buffer and homogenized with a 2% solution of Triton X-100 that contained 10 mM EDTA (pH 8.0) by sonocation. The homogenate was incubated at 4°C for 16 h. The supernatant was removed by centrifugation at 8,000 x *g* for 10 min at 4°C. The pellet was washed twice with 30 mM Tris-HCl buffer (pH 8.0) that contained 30 mM NaCl. Protein concentrations were determined by using the Bio-Rad protein assa kit (19) or measuring absorbance at 280 nm.

Electrophoresis on an SDS-polyacrylamide gel — Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out by the method of Laemmli (20). A separation gel containing 7.5% (w/v) acrylamide was used. Samples were dissolved in sample buffer [25 mM Tris-HCl buffer (pH 6.5) that contained 1% SDS, 5% glycerol, 0.01% bromphenol blue, and 1% 2-mercaptoethanol]. Proteins separated on the gel were stained with Coomassie Brilliant Blue (CBB) R-250. The densitometric scanning of the CBB-stained gel was performed with a digital densitimeter (DMU-33C; Toyokagaku, Tokyo, Japan).

Blotting of proteins and metal-binding assays — Proteins that had been separated by SDS-PAGE were transferred electrophoretically to a nitrocellulose membrane by the method of Towbin *et al.* (21). In order to reduce proteins on the gel after electrophoresis, the gel was incubated in 25 mM Tris, 192 mM glycine buffer (pH 8.3) that contained 0.1% SDS and 5% 2-mercaptoethanol at room temperature for 30 min. The gel was washed with electrode buffer [20 % (v/v) methanol, 25 mM Tris, and 192 mM glycine (pH 8.3)]. Transfer of the proteins was carried out in a transfer chamber (Marisoru Co. Ltd. , Tokyo) at a constant current of 120 mA for 18 h at room temperature with the same electrode buffer.

The assay of the binding of [^{109}Cd] to proteins on the nitrocellulose – membrane was based on the Aoki *et al.* (22). The membrane was soaked in 100 ml of 10 mM Tris-HCl buffer (pH 7.4) for 1 to 2 h at room tempetature, and then it was incubated in 10 ml of 10 mM Tris-HCl buffer (pH 7.4) that contained 1 μCi/ml [^{109}Cd]Cl_2, 0.1 mM $ZnCl_2$ and 0.1 M KCl for 10 min at room temperature. Proteins that had bound the radioactive cadmium were detected by autoradiography.

Spot transfer estimation of the binding affinity for heavy metals of proteins — Proteins, namely fused hMT-II protein, standard metallothionein II from horse and rabbit, and pure β-galactosidase were spotted on a nitrocellulose membrane and the spots were dried at room temperature. The membrane with dried proteins was incubated in a reducing solution of 25 mM Tris-HCl buffer (pH 8.3), 192 mM glycine, 0.1% SDS, and 5% 2-mercaptoethanol at room temperature for 3 to 5 min. The membrane was incubated in 20 mM glycine-HCl (pH 2.3) for 3 to 5 min and rinsed twice for 3 to 5 min with 10 mM Tris-HCl buffer (pH 7.4) for 3 to 5 min. The membrane was subsequently soaked in 10 mM Tris-HCl buffer (pH 7.4) that contained 0.1 M KCl, 0.2 μCi/ml [^{109}Cd]Cl_2 and 1mM unlabeled salts of heavy metals for 30 min at room temperature. The membrane was rinsed three times with 10 mM Tris-HCl buffer (pH 7.4) that

contained 0.1 M KCl for 5 to 10 min and dried: excess buffer was absorbed with filter paper, and the membrane was allowed to dry at room temperature. In order to visualize the transferred protein, the nitrocellulose membrane was soaked in a 0.1% solution of amido black in 30% (v/v) methanol and 10% acetic acid for several minutes and destained with 50% methanol and 10% acetic acid. Proteins that had bound radioactive cadmium were detected by autoradiography.

Autoradiography — The membrane was completely dried with a hair drier and exposed to X-ray film (X-Omat AR; Kodak) under an intensifying screen at -80°C for 16 to 48 h.

Results

Design of the synthetic gene for hMT-II — The hMT-II protein consists of 61 amino acid residues of which 20 are cysteine residues. Of the 20 cysteine residues, 18 are encoded by the UGC codon (4, 23). Because of this unusually large number of UGC codons for cysteine, suitable tailoring of a synthetic gene for hMT might be expected to increase its translation in *E. coli.* (17). Thus, I designed a gene for hMT-II in which an equal number of the two codons, UGC and UGU, for cysteine were present separately, since two codons, UGC and UGU, were used equally in *E. coli.* Then I synthesized a 190-bp oligonucleotide for hMT-II. The DNA sequence for the synthesis was based on the amino acid sequence of human metallothionein (24) and the nucleotide sequence of human metallothionein II (4, 23). The design of the primary nucleotide sequence also adhered to the criteria suggested by Itakura *et al.* (25): first, amino acid codons known to be favored in *E. coli* were used; second, the fragments were designed to eliminate undesirable inter- and intramolecular pairing; and third, GC-rich followed by AT-rich sequences were avoided. I designed the gene with the following additional features: I, in order to generate the hMT-II insert from the plasmid vector, the gene included suitable sites for restriction endonucleases; II, the initiation codon AUG was placed adjacent to the natural NH_2-terminus of hMT-II; and III, two nonsense codons (UGA, UAG) were added just after the C-terminal codon. The final construct of hMT-II after annealing is shown in Fig. 1. The sequence of the synthetic gene was confirmed by nucleotide sequening using a plasmid-based primer.

Since the accumulation of SH-rich protein, namely, hMT-II, might inhibit cell growth, the temperature-controlled expression vector pUEX2 was used (15), which includes the gene for the lambda *cI857* repressor, that allows induction of *cro-lacZ* gene from the lambda P_R promoter upon a shift in temperature. The hMT-II gene was inserted 27 bp upstream from the C-terminus of *lacZ*

β-gal | hMT-II

cleavage by formic acid

E F P D K M D P N C S C A A G D S C T C

— GAATTCCC CGATAAAATGGATCCGAACTGCAGCTGTGCAGCTGGTGACTCCTGCACCTGT

— CTTAAGGG GCTATTTTACCTAGGCTTGACGTCGACACGTCGACCACTGAGGACGTGGACA

Bam HI *Pst* I

A G S C K C K E C K C T S C K

GCCGGCTCTTGCAAATGTAAAGAATGCAAATGTACTAGTTGCAAG

CGGCCGAGAACGTTTACATTTCTTACGTTTACATGATCAACGTTC

Nae I

K S C C S C C P V G C A K C A

AAAAGCTGCTGTTCCTGCTGTCCGGTGGGCTGCGCAAAATGTGCG

TTTTCGACGACAAGGACGACAGGCCACCCGACGCGTTTTACACGC

Q G C I C K G A S D K C S C C

CAGGGCTGCATCTGTAAAGGCGCTAGCGACAAGTGCAGCTGTTGC

GTCCCGACGTAGACATTTCCGCGATCGCTGTTCACGTCGACAACG

A END END

GCTTGATAGGATCCTCTAGAG

CGAACTATCCTAGGAGATCTCAGCT

Bam HI *Sal* I

cI857

Amp

P_R

pZMT001

6.90 kb

lac Z

Sal I

Sma I

Fig. 1. Construction of the pZMT001 expression vector. Eight synthetic oligonucleotides were synthesized corresponding to both strands of the gene for human metallothionein II. After annealing, the monomer was isolated, subcloned into pUC vector, and then inserted into the *Sma* I-*Sal* I site of pUEX2 by ligation to generate a 6.9-kb plasmid (pZMT001).

and, as a result, we expected to synthesize hMT-II as a β-gal-hMT-II fusion protein. The resultant plasmid was named pZMT001 and was used to transform *E. coli* JM109 (Δ(l*ac-proAB*)/F'[*lacZ*ΔM15], Ap^S) to Ap^r.

Expression of the recombinat hMT-II — *E. coli* cells cerying plasmid pUEX2 or pZMT001 were incubated at 28°C. At the midlogarithmic phase of growth, the temperature was shifted to 42°C to inactivate the *cI857* repressor and then kept at 37°C for 16 h. The extracts were analyzed by SDS-PAGE. A band of a 122-kDa protein was observed in the analysis of the cell extract from *E. coli* JM109 that carried pZMT001 after SDS-PAGE (Fig. 2a, lane 3). This protein is the same size as predicted from the sum of the 116 kDa of β-galactosidase and the 6 kDa of hMT-II. The induced cells carrying the plasmid vector pUEX2 only accumulated a 116 kDa protein, the size of which corresponds to that of β-galactosidase (Fig. 2a, lane 2). Without either plasmid, neither of these proteins was formed (Fig. 2a, lane 1).

Densitometric analysis revealed that the β-gal-hMT-II fusion protein accouned for more than 24% of the total protein in the cell extract (Fig. 2c, ▽). This value indicates that the recombinant protien is synthesized at a rate of about 2 g/*l* culture broth. The fusion protein was present in an insoluble form and could be purifed more than 95% by washing the inclusion bodies with a solution of Triton X-100 only (Fig. 2b). A minor band of a 220-kDa protein was observed in the analysis of the crude extract and the purified sample from *E. coli* JM109 that carried pZMT001, but not in the extract of cells carrying pUEX2. This band may be a dimer of the fusion protien.

Binding of cadmium to the fusion protein — To test the ability of the β-gal-hMT-II fusion protien (95% pure) to bind heavy metals, a blotting method was used (22). Radioactive [^{109}Cd] was used to demonstrate the binding by the h-MT-II moiety of the fused protein of heavy metals, since it is known that hMT-II can bind Cd^{2+} ions (26). After SDS-PAGE (Fig. 3a), the protein was transferred electrophoretically to a nitrocellulose membrane. The membrane

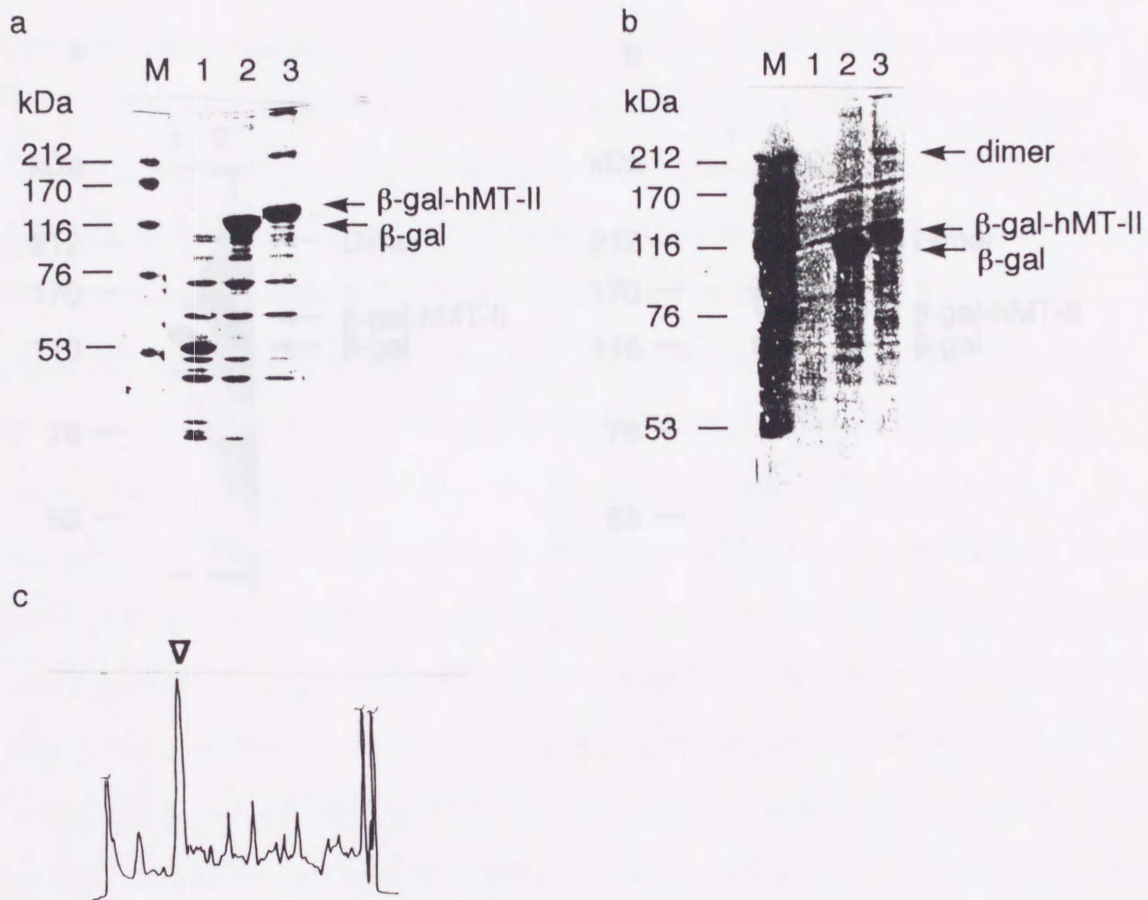

Fig. 2. SDS-polyacrylamide gel electrophoresis of recombinant fused matallothionein. Proteins on the gel were stained with Coomassie Brilliant Blue (CBB) after electrophoresis. (a) Crude extract; (b) samples after washing inclusion bodies with a solution of Triton X-100. Lane M, molecular mass standards: 212 kDa (myosin), 170 kDa (α-macroglobulin), 116 kDa (β-galactosidase), 76 kDa (transferrin), 53 kDa (glutamate dehydrogenase). Lane 1, *E. coli* JM109; Lane 2, *E. coli* JM109 (pUEX2); Lane 3, *E. coli* JM109 (pZMT001). (c) Densitometric scanning of 640 nm of the CBB-stained gel (a, lane 3). The β-gal-hMT-II fusion protein (indicated by the arrow) represents 24% of the total protein in the transfomed *E. coli* cells.

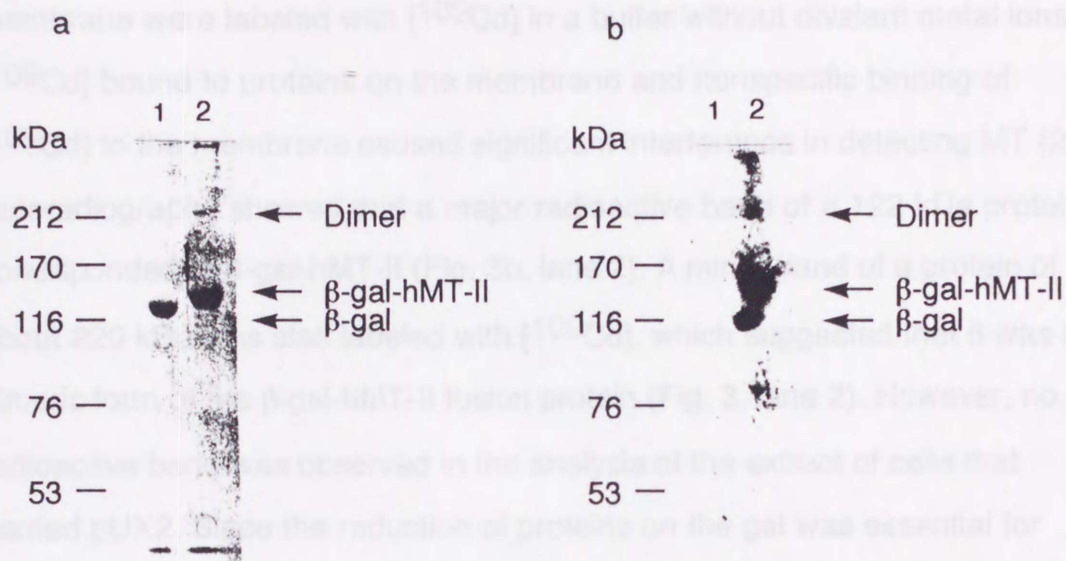

Fig. 3. Identification of [$^{109}Cd^{2+}$]-binding proteins in vitro. Cultures in *E. coli* JM109 containing the plasmid were grown in the LB medium and the purified proteins were used for assays (see Materials and methods). (a) Each protein, 10 mg, was subjected to SDS-PAGE and the gel was stained with Coomassie Brilliant Blue. (b) An autoradiograph of the nitrocellulose membrane to which proteins were transferred after electrophoresis. A dark spot near the β-gal was an artificial contaminant of radioactive Cd. Molecular mass standards were the same as those described in the legend of Fig. 2. Lane 1, *E. coli* JM109 (pUEX2). Lane 2, *E. coli* JM109 (pZMT001).

was incubated in a buffer that contained 0.05 μM [^{109}Cd]Cl_2 and 0.1 mM $ZnCl_2$. Addition of Zn^{2+} ions into the [^{109}Cd]-containing buffer was found to be essential for detection of hMT by this technique because it reduced the nonspecific binding of [^{109}Cd]. When proteins on the nitrocellulose membrane were labeled with [^{109}Cd] in a buffer without divalent metal ions, [^{109}Cd] bound to proteins on the membrane and nonspecific binding of [^{109}Cd] to the membrane caused significant interference in detecting MT (22). Autoradiography showed that a major radioactive band of a 122-kDa protein corresponded to β-gal-hMT-II (Fig. 3b, lane 2). A minor band of a protein of about 220 kDa was also labeled with [^{109}Cd], which suggested that it was the dimeric form of the β-gal-hMT-II fusion protein (Fig. 3, lane 2). However, no radioactive band was observed in the analysis of the extract of cells that carried pUX2. Since the reduction of proteins on the gel was essential for generation of reproducible results in terms of the radioactive bands of the fusion protien, endogenous Cd^{2+} ions were probably removed from hMT-II during the reduction process (22). These results indicate that Cd^{2+} ions can bind to the fused hMT-II protein. The fusion protein seemed to be at least partially renatured and was, thus able to bind Cd^{2+} ions.

In order to produce hMT-II from the fusion protein, the fused protein has to be cleaved to remove the β-galactosidase moiety. Formic acid, acting at the aspartyl-proline bond, has been used for the production of hMT-II (27). Samples (2 μg of β-gal-hMT-II and recombinant β-galactosidase) treated with 70% formic acid and 6M guanidine at 37°C for 48 h were subjected to SDS-PAGE, and the metal-binding assay with [^{109}Cd] was performed. Weak and smeared spots of [^{109}Cd]-binding protein that corresponded to a protein of about 17 kDa from β-gal-hMT-II were observed, but no radioactive spot was observed from recombinant β-galactosidase (data not shown).

Affinity for heavy metal ions by the fusion protein — To determine the extent of renaturation of the fusion protein, standard metallothionein and the purified

β-gal-hMT-II protein that was 95% pure were diluted and spotted on two nitrocellulose membranes. One part of the membrane was stained with amido black (Fig. 4a) and the other was subjected to labeling with [^{109}Cd] (Fig. 4b). About 40 μg of β-gal-hMT-II protein (25-fold dilution of about 1000 μg of protein) were labeled with [^{109}Cd]. The intensity of the band corresponded to the intensity of a band of 2 μg of standard MT-II from rabbit of horse (25-fold dilution of about 10 μg of protein) (Fig. 4). These results indicate that, in terms of binding of [^{109}Cd], one mole of β-gal-hMT-II is equivalent to one mole of standard MT-II which binds 7 atoms of Cd. β-Galactosidase (Sigma) and recombinant β-galactosidase from cells that carried pUEX2 did not bind any radioactivity. Since the percentage of renaturation of the fusion protein was not known, it was impossible to quantify the specific activity determined in the presence of Cd^{2+} ions.

The affinity of the fused hMT-II protein for heavy metals was qualified. The membranes spotted with samples of diluted proteins were incubated with 0.01 μM [^{109}Cd]Cl_2 in the presence of various heavy metals ions at 1 mM. Darkening of the Cd-binding protein after addition of 1 mM Zn^{2+} ions to the [^{109}Cd]-containing buffer was observed, but not after a similar addition of 1 mM Cu^{2+} or Co^{2+} ions (Fig. 4d-f). The fused hMT-II had a higher affinity for Cd^{2+} ions than for Zn^{2+} ions, as did the standard horse MT-II protein (Fig. 4d and h). By constrast, both the fused hMT-II and horse MT-II failed to bind Cd^{2+} ions in the presence of 1 mM of Cu^{2+}, Co^{2+}, or Ag^{+} ions (Fig. 4e-g). These results illustrate that the affinities of hMT-II for Cu^{2+}, Co^{2+} and Ag^{+} ions are higher than those for Cd^{2+} and Zn^{2+} ions, as is the case for horse MT-II (28, 29). We observed small black spots of β-gal-hMT-II and β-gal, and high background in the presence of Ag^{+} ions. Ag^{+} ions do not seem to inhibit non-specific binding of [^{109}Cd] to proteins and nitrocellulose membrane that is affected by divalent metal ions (22).

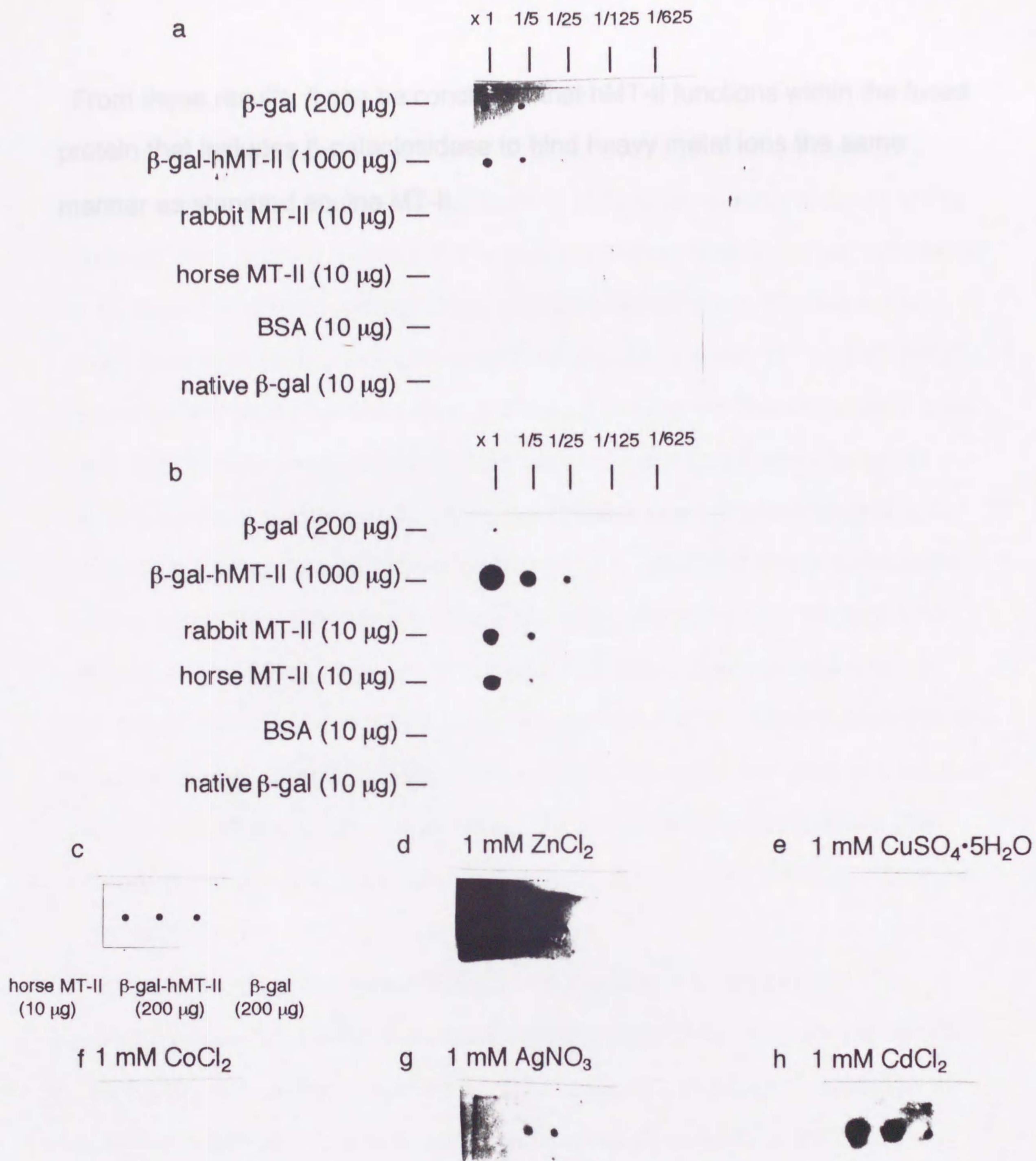

Fig. 4. Effects of heavy metal ions on binding of [$^{109}Cd^{2+}$] ions to the hMT-II fusion protein. The purified fusion protein was dotted on a nitrocellulose membrane as described in Materials and methods. The membrane was incubated in a solution of 0.2 mCi / ml [$^{109}Cd^{2+}$] in the presence of heavy metal ions and then rinsed with 10 mM Tris-HCl buffer. Panels (a) and (c), protein stained with amido black; panels (b) and (d)-(h), autoradiograph after 16 h and 48 h, respectively. The membrane was labeled with [$^{109}Cd^{2+}$] in buffer that contained 1 mM $ZnCl_2$ (b, d), 1mM $CuSO_4 \cdot 5H_2O$ (e), 1 mM $CoCl_2$ (f), 1 mM $AgNO_3$ (g), or 1 mM $CdCl_2$ (h).

From these results, it can be concluded that hMT-II functions within the fused protein that includes β-galactosidase to bind heavy metal ions the same manner as standard equine MT-II.

Discussion

The rate of production of gene products that contain large numbers of SH groups in *E. coli* is often limited because of the toxicity of such proteins to the bacterial cells and the stability of the proteins themselves which sometimes fail to undergo the correct folding. In the previous experiments, Murooka *et al.* succeeded in the direct expression of the cDNA for monkey MT-II in *E. coli* (9). However, the yield of protein was less than a few mg per liter of cultured cells. Several efforts to overproduce MT proteins in *E. coli* and *Saccharomyces cerevisiae* were subsequently made, but no high level of expression of MTs from various sources has been reported (10-12, 30). Thus, the production of MTs in fused form with other proteins has been attempted (13, 31, 32). The gene coding for the copper metallothionein of *Neurospora crassa* was expressed as a fusion product at the NH_2-terminus of β-galactosidase and the fused protein was produced as a soluble from. However, the yield of the fused protein was 16 times lower than that of the non-fused β-galactosidase (32). Moreover, no protein of the size expected for the fused protein was observed after SDS-PAGE.

Romeyer *et al.* synthesized DNA that encoded for human hepatic metallothionein and they expressed the gene as a fusion protein with Ara'B. The fusion protein accounted for approximately 8% of the total protein in *E. coli.* The Ara'B-hMT fusion protein produced by *E. coli* had a half-life of approximately 50 min (2, 13). Jacobs *et al.* cloned the gene for hMT in an expression vector, pUA7, including the promoter-operator and regulatory sequences of the *ara* operon of *Salmonella typhimurium* and part of the 5'-coding and all of the 3'-noncoding regions of the *lpp* from *E. coli.* The fusion protein was produced as a stable protein. The ability of the fused protein to bind heavy metals was characterized, but it was not demonstrated that the hMT-II moiety of the fused protein was renatured to the same degree as the native MT (31).

In the present study, a synthetic DNA encording hMT-II was designed and fused with the gene for a large protein (β-galactosidase). Moreover, the overproduction of the hMT-II fused protein in *E. coli* was successfully achieved. Upon heat induction of the cl857-P_R promoter system, the fusion protein was produced to a level equal to more than 24% of total cellular protein. Since the hMT-II gene contains an usually large number of UGC codons, for 18 of a total of 20 cysteine residues it was postulated that the substitution of codons suitable for expression in *E. coli* might increase the translation of the gene in *E. coli*. However, the stability of the hMT-II protein was so high after fusion with β-galactosidase that the codon usage was not evaluated. Now we are examining the direct expression of hMT-II to investigate the effects of codon changes. The fused hMT-II was stabilized as an insoluble protein that formed inclusion bodies in *E. coli* cells. The insoluble β-gal-hMT-II protein was easily solubilized by incubation treatment with SDS and 2-mercaptoethanol which genetated the reduced form. Some of the reduced β-gal-hMT-II protein formed dimers on SDS-PAGE, as does native MT (22). The fused hMT-II protein was easily detected with standard protein-specific dyes or by labeling with [^{109}Cd], whereas the native MT obtained by direct expression is attacked by proteases and has been difficult to detect after SDS-PAGE because of its small size. Furthermore, we clearly demonstrated that the hMT-II moiety of the fusion protein is functional in the same hierarchy with respect to binding of heavy metals ions (Cu^{2+}> Cd^{2+}> Zn^{2+}) as native hMT-II from the horse (33). We also succeeded in regeneration of hMT-II from the fusion protein, and the regenerated hMT-II protein migrated in a gel with the same mobility as that of rabbit liver MT and horse kidney MT (34), and bound the radioactive cadmium (unpublished results). We are presently studing how many atoms of heavy metals the fusion protein binds and whether the fusion protein binds heavy metals in solution.

The present results suggest that any barriers to the production of a protein with many SH groups in *E. coli* may not involve the inhibitory effect of any specific structural feature of the protein, but may rather involve the localization or stability of the protein within the cells (13, 31). With increased understanding of structure-function relationships, we should be able to create novel forms of MT that have an altered specific affinity for given metal ions.

Summary

A synthetic DNA coding for human metallothionein II (hMT-II) was designed for efficient expression in *Escherichia coli* and cloned into a vector, pUEX2. Upon induction by heating, the gene for hMT-II was expressed as a fusion protein accounted for approximately 24% of the total protein in the cells and accumulated in the cells as an insoluble form. The fused hMT-II protein with a molecular mass of approximately 122 kDa was purified to homogeneity by washing the inclusion bodies with a solution of TritonX-100. The insoluble protein was easily solubilized in the presence of 2-mercaptoethanol and sodium dodecyl sulfate without any need for denaturants, such as urea or guanidine hydrochloride. The fused hMT-II protein bound to radioactive cadmium. A competition experiment with radioactive cadmium and various heavy metals showed that the hMT-II fusion protein had a higher affinity for Cu^{2+}, Co^{2+}, Ag^{+} ion than Cd^{2+} and Zn^{2+} ions. Thus, we secceeded in over expression of hMT-II as an active form in *E. coli*.

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Chapter II Production and Purification of Monoamine Oxidase: Production, Purification, and Characterization of a new type of Monoamine Oxidase containing Cu from *Klebsiella aerogenes*

Introduction

Monoamine oxidases catalyze the oxidative deamination of monoamines according to the following reaction:

$$RCH_2NH_2 + O_2 + H_2O \text{ -------->} RCHO + NH_3 + H_2O_2$$

Monoamine oxidase from mitochondria of animal organs (1-5) and plasma (6-9), from pea seedings (10), from various microorganisms (11, 12), and the cell membranes of bacteria (13) has been characterized. The enzyme has a broad substrate specificity and palys a major role in the metabolism of biogenic amines, such as the neurotransmitters of the central nervous system.

The monoamine oxidase in *Klebsiella aerogenes* is induced by tyramine and catecholamines, and it is specific for tyramine and dopamine (14). Thus, the enzyme from *K. aerogenes* W70 has been classified as a tyramine oxidase (EC 1.4.3.9) (15). Tyramine oxidase in *K. aerogenes* is encoded by the *tynA* gene, and the synthesis of the enzyme is subjected to catabolite and ammonium repression (14, 16). Expression of the *tynA* gene results in derepression of the *ats* operon, which is closely linked to *tynA* (17, 18) and is composed of the arylsulphatase (*atsA*) gene and the positive regulator, The *atsB* gene (19).

Since the tyramine oxidase from *K. aerogenes* binds tightly to the cell membrane and has not been solubilized in an active form (14), characterization of the enzyme and determination of the mechanism of monoamine oxidization remain to be performed. These studies will require the pure enzyme. During the course of our studies on the genetic regulation of the synthesis of the tyramine oxidase, we cloned the structural gene for the tyramine oxidase of *K. aerogenes* W70 into a multicopy plasmid. This cloning resulted in overproduction of the enzyme in a soluble form (20), thus rendering possible both the purification and the characterization of the enzyme. We found that the pure enzyme was highly specific for dopamine, β-phenylethylamine and tryptamine, in addition to tyramine and contained

copper but not FAD as a prosthetic group. From these results, we classify the enzyme from *K. aerogenes* as a monoamine oxidase (EC 1.4.3.6) instead of a tyramine oxidase (EC 1.4.3.9), and hence the gene encoding this enzyme was renamed from *tynA* to *maoA* (20).

Materials and methods

Materials — All reagents were of the highest commercially available grade and were used without further purification. Horseradish peroxidase (Grade II) was purchased from Boehringer Mannheim Yamanouchi Co. Ltd. (Tokyo). DEAE-Toyopearl 650S, DEAE-Toyopearl 650M, Butyl-Toyopearl 650M, and Toyopearl HW55S were purchased from Tosoh Co. Ltd. (Tokyo). Iproniazid phosphate salt and tyramine hydrochloride were purchased from Sigma Chemical Co. Ltd. (St. Louis, MO). Clorgyline hydrochloride and deprenyl hydrochloride were purchased from Research Blochemicals Inc. (Natick, MA). [^{3}H]-Tyramine hydrochloride (20 Ci/mmol) was purchased from New England Nuclear Research/DuPont (Boston, MA).

Bacterial strains and plasmids — The bacterial strain used in this study was *K. aerogenes* W70 (prototroph)(21), which produces monoamine oxidase (14). pTO58, a plasmid containing a gene for monoamine oxidase and conferring kanamycin resistance (Km^r) was constructed previously (20).

Medium and culture conditions — *K. aerogenes* W70 harboring pTO58 was subcultured in 500 ml of K medium (22) in a 5-liter flask. Xylose (0.5%) and 1mM NH_4Cl were used as sources of carbon and nitrogen, respectively. Tyramine (1 mM) was used as inducer of monoamine oxidase (14). Kanamycin (50 μg/ml) was used in precultures. The subculture was inoculated into a jar fermenter that contained 30-liter of K medium. The incubation was carried out at 28°C for 16 h with aeration. The cells were harvested by centrifugation at 4,000 x *g* for 30 min, washed with 0.1 M potassium phosphate buffer (pH 7.4), and frozen at -80°C.

Assay of enzymatic activity and quantitation of protein — Monoamine oxidase activity was determined by a colorimetric assay that was based on a coupled oxidation reaction, with measuremant of the monoamine oxidase-dependent formation of hydrogen peroxide. The method was essentially the same as that employed for assays of amine oxidase in fungi (11) but tyramine

and *o*-dianisidine were used as substrate and indicator, respectively. The assay mixture was composed of 2.6 ml of 0.1 M citrate-phosphate buffer (pH 7.4) that contained 0.6 mM *o*-dianisidine, 0.02% (w/v) horse radish peroxidase, and 5 mM tyramine. No inhibition of the activity of the monoamine oxidase from *Klebsiella* by *o*-dianisidine was observed at concentrations up to at least 1.2 mM. The mixture was allowed to stand at 30°C for 5 min and the reaction was started by the addition of 0.1 ml of enzyme solution and incubated at the same temperature for 20 min. The reaction was stopped by the addition of 0.3 ml of 1 M citric acid, and the mixture was centrifuged when the preparation of enzyme became impure. The absorbance at 435 nm was recorded on a Beckman DU64 spectrometer against a reagent blank without addition of enzyme solution. One unit of enzymatic activity was defined as the amount that catalyzed the formation of 1 μmol of H_2O_2 in 1 min at 30°C, which was caluculated using a molar absorption coefficient of 2230 $M^{-1}cm^{-1}$. Monoamine oxidase was also assayed by a radiometric procedure with [^{3}H]-tyramine (14). Protein content was quantitated by the method of Lowry *et al.* (23) with bovine serum albumin as a standard. The elution of proteins was monitored by measuring absorbance at 280 nm.

Purification of the enzyme — All the purification steps were carried out at 4°C.

Step 1. Preparation of crude extract. The cells (about 177 g wet wt/20 *l* culture) of *K. aerogenes* W70 harboring pTO58 were disrupted by sonication. Intact cells and debris were removed by centrifugation and the supernatant was used as the crude enzyme solution.

Step 2. Ammonium sulfate fractionation. The precipitate obtained from the supernatant between 35% to 65% saturation of ammonium sulfate was dissolved in 50 mM potassium phosphate buffer (pH 7.4), and dialyzed against the same buffer.

Step 3. Column chromatography on DEAE-Toyopearl 650S. The dialyzed solution (120 ml) was applied to a column (4.4 cm i.d. x 18 cm) of DEAE-Toyopearl 650S which had been equilibrated with 50 mM potassium phosphate buffer (pH 7.4) and eluted with a gradient of NaCl from 0 to 0.5 M in the same buffer (pH 7.4). The active fractions were pooled and concentrated by the addition of solid ammonium sulfate.

Step 4. Column chromatography on Butyl-Toyopearl 650M. The precipitate obtained by centrifugation was dissolved in 50 mM potassium phosphate buffer (pH 7.4) and dialyzed against the same buffer. Solid ammonium sulfate was added to the dialysate to 35% saturation. The concentrated solution of enzyme (45 ml) was applied to a column (2.3 cm i.d. x 24.5 cm) of Butyl-Toyopearl 650M which had been equilibrated with 0.1 M potassium phosphate buffer (pH 7.4) that contained solid ammonium sulfate to 35% saturation. The enzyme was eluted with a gradient of ammonium sulfate from 35% to 0% in the same buffer. The active fractions were pooled and concentrated by the addition of solid ammonium sulfate.

Step 5. Column chromatography on DEAE-Toyopearl 650M. The precipitate obtained by centrifugation was dissolved in 50 mM potassium phosphate buffer (pH 7.4) and dialyzed against the same buffer. The dialyzed enzyme solution (23 ml) was applied to a column (1.9 cm i.d. x 35 cm) of DEAE-Toyopearl 650M which had been equilibrated with same buffer and eluted with a gradient of NaCl from 0 to 0.5 M in the same buffer. The active fractions were pooled and concentrated by the addition of solid ammonium sulfate.

Step 6. Gel filtration. The precipitate obtained by centrifugation was dissolved in 50 mM potassium phosphate buffer (pH 7.4). The enzyme solution (1 ml) was loaded to a column (2.6 cm i.d. x 96 cm) of Toyopearl HW55S which had been equilibrated with same buffer and eluted with same buffer. The active fractions were collected and used for the characterization of the enzyme.

Electrophoresis — SDS-polyacrylamide gel electrophoresis (SDS-PAGE; 7.5% acrylamide) was carried out according to the method of Laemmli (24), and the bands of protein were stained with Coomassie Brilliant Blue R-250.

Molecular weight determination — The molecular weight of the purified enzyme was determined by gel-filtration on a column (2.6 cm i.d. x 96 cm) of Toyopearl HW55S by the method of Andrew (25) and SDS-PAGE.

Spectrophotometric determination — Enzymatic reaction and protein concentrations were measured in a Beckman DU64 spectrophotometer. The absorption spectra were recorded with an EPSON sp-500 recording spectrophotometer.

Metal determination — The matal content of the enzyme was determined spectrophotometrically. The final sample of enzyme was prepared with metal-free distilled water and reagents. The enzyme was dialyzed for 24 h against 0.01 M potassium phosphate buffer (pH 7.0) that contained 0.1 mM dithiothreitol and 1 mM EDTA. The dialyzed enzyme at a concentration of 1-1.5 mg of protein per ml was analyzed with a Nippon Jarrell-Ash Model AA-845 atomic absorption spectrophotometer.

Computer analysis — The amino acid sequence were analyzed with GENETYX programs (SDC Software Development Co., Ltd., Tokyo, Japan).

Results

Purification of monoamine oxidase from *K. aerogenes* W70 (pTO58) — The overall purification procedure is summarized in Table 1. After ammonium sulfate fractionation and chromatography on a series of columns, the enzyme was purified 10-fold with a yield of 9.1%. The purity of the enzyme was analyzed by SDS-PAGE and the final preparation of enzyme was found to be homogeneous (Fig. 1, lane 2).

Molecular weight — The molecular weight of monoamine oxidase was determined by gel filtration on a column of Toyopearl HW55S and SDS-PAGE. The enzyme gave a single peak and was eluted at a position near that of bovine serum albmin (68,000 Da). Its molecular weight was estimated to be 79,000 (Fig. 2). The purified enzyme gave a single band that corresponded to a molecular weight of 79,000 on SDS-PAGE (Fig. 1, lane 2). Since the value is agree with that deduced from the predicted amino acid sequence (26), the enzyme appears though to consist of a single polypeptide chain.

Effects of pH — Enzyme activity was determined in buffers of different pHs. The enzyme showed maximum activity at pH 6.0 when examined in the presence of 0.1 M potassium phosphate buffer, and 50% of the maximum activity was obtained at pH 5.5 and pH 7.0 . The enzyme was stable at pH 4.0, but lower activity was recorded after a 2-h incubation at pH 10.5 and 4°C.

Effects of temperature — The effects of temperature on the activity and stability of the enzyme were examined. The optimal temperature for activity was 50°C, and 50% of the maximum activity was observed at 35°C and 55°C. The enzyme was stable up to 45°C but 50% of the activity was lost after a 20-min incubation at 50°C.

Substrate specificity — The activity of purified monoamine oxidase was examined in the various amines (Table 2). The enzyme exhibited high activity when tyramine, β-phenylethylamine, dopamine, and tryptamine were tested as substrates; moderate activity towards serotonin and histamine. Other

Table 1. Purification of monoamine oxidase from *Klebsiella aerogenes* W70 (pTO58)

Fraction	Total activity (U)	Tatal protein (mg)	Specific activity (mU/mg)*	Yield (%)
Crude extract	115.2	20160	5.7	100
Ammonium sulfate fraction	58.9	9480	6.2	51.5
DEAE-Toyopearl 650S	21.5	2220	9.7	18.7
Butyl-Toyopearl 650M	14.0	468	29.9	12.2
DEAE-Toyopearl 650M	13.1	264	49.6	11.4
Toyopearl HW55S	10.5	178	59.0	9.1

* For defination of units, see text.

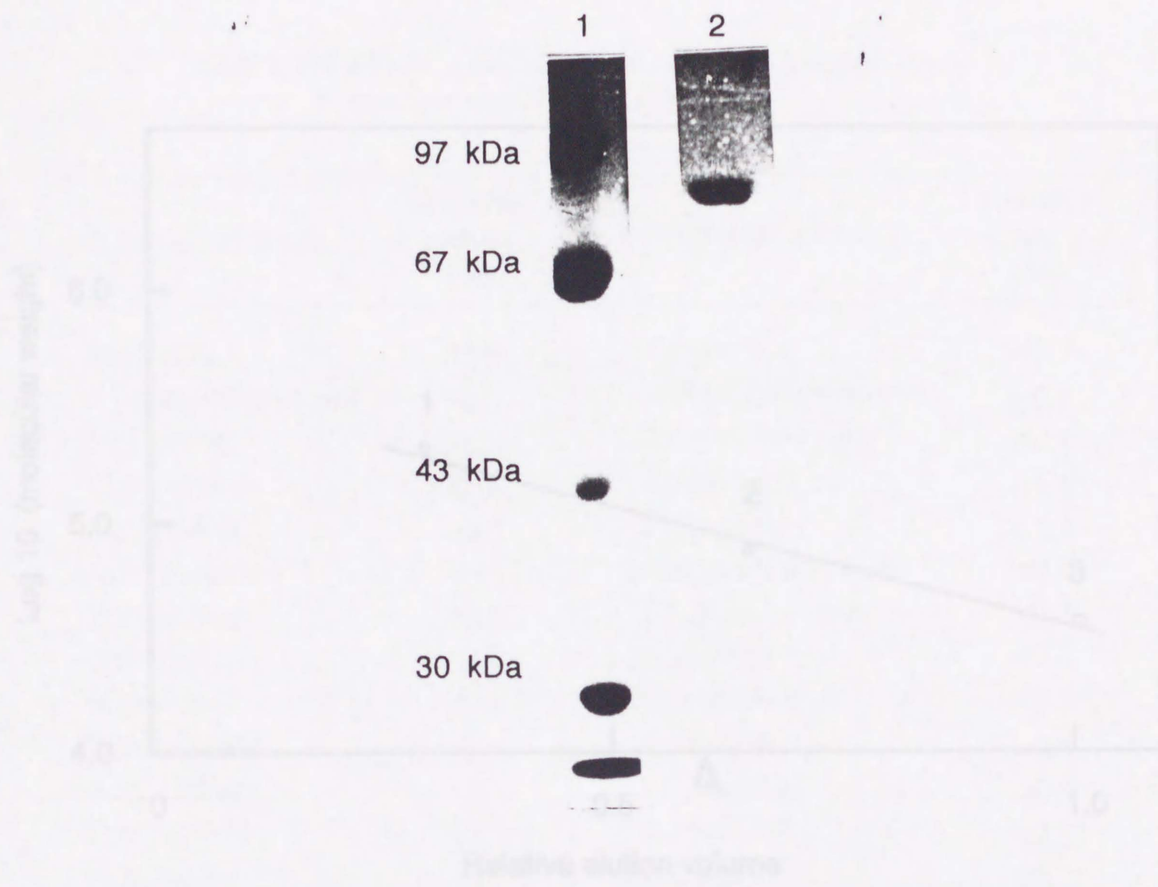

Fig. 1. Results of sodium dodecyl sulfate-polyacrylamide gel electropforesis of monoamine oxidase. Proteins were stained with Coomassie Brilliant Blue. Lane 1, Molecular weight standards: phosphorylase (94 kDa); bovine serum albumin (67 kDa); ovalbumin (43 kDa); carbonic anhydrase (30 kDa); lane 2, fraction from Toyopearl HW55S.

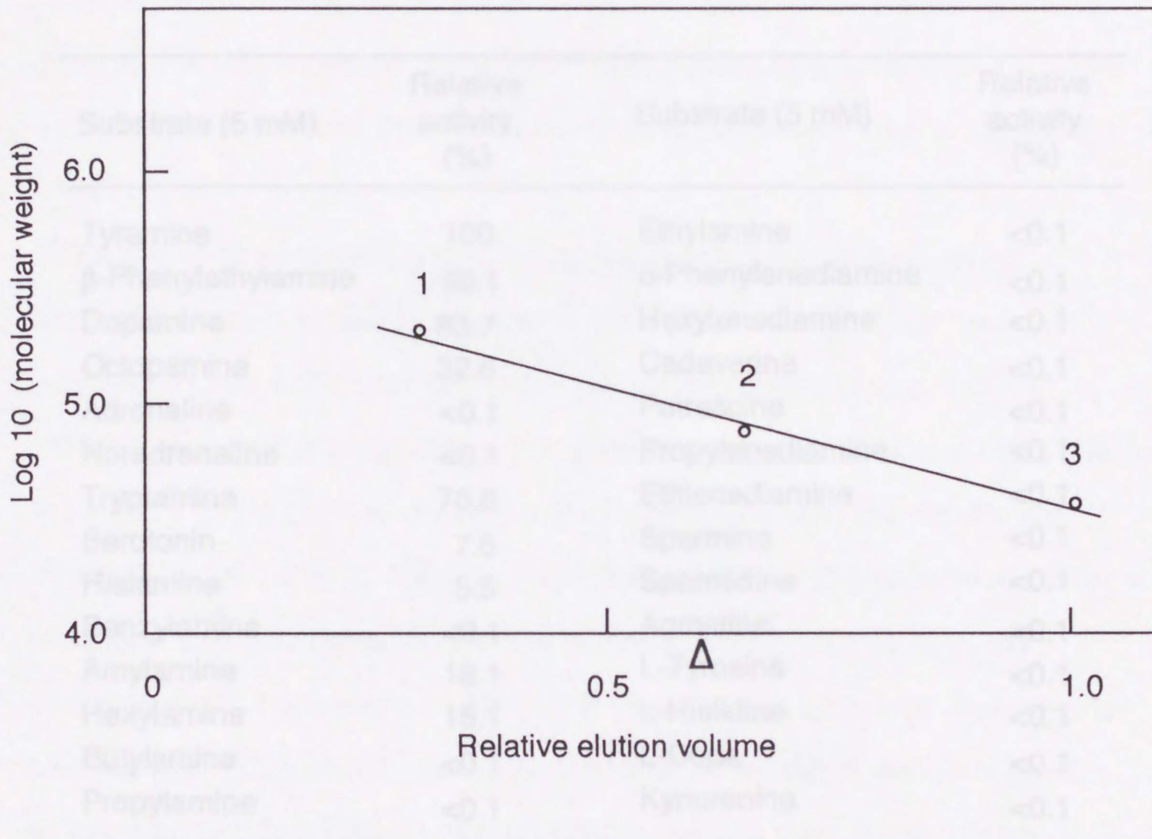

Fig. 2. Relation between elution volumes of proteins and their molecular weights during gel filtration on Toyopearl HW55S. 1, Aldolase (158 kDa); 2, bovine serum albumin (67 kDa); 3, chymotrypsinogen A (25 kDa). The symbol Δ shows the position of elution of the monoamine oxidase.

Table 2. Substrate specificity of monoamine oxidase from *K. aerogenes* W70 (pTO58)*

Substrate (5 mM)	Relative activity (%)	Substrate (5 mM)	Relative activity (%)
Tyramine	100	Ethylamine	<0.1
β-Phenylethylamine	89.1	o-Phenylenediamine	<0.1
Dopamine	83.7	Hexylenediamine	<0.1
Octopamine	32.6	Cadaverine	<0.1
Adrenaline	<0.1	Putrescine	<0.1
Noradrenaline	<0.1	Propylenediamine	<0.1
Tryptamine	76.6	Ethlenediamine	<0.1
Serotonin	7.6	Spermine	<0.1
Histamine	5.5	Spermidine	<0.1
Benzylamine	<0.1	Agmatine	<0.1
Amylamine	18.1	L-Tyrosine	<0.1
Hexylamine	15.1	L-Histidine	<0.1
Butylamine	<0.1	L-Dopa	<0.1
Propylamine	<0.1	Kynurenine	<0.1

* The enzymatic activity was determined under the standard assay conditions described in Materials and methods. The reaction mixture consisted of 5 mM of a given substrate, 0.1 M citrate-phosphate buffer (pH 5.6), 0.02% (w/v) horse-radish peroxidase, 6.6 mM o-dianisidine and 13.6 mU of the purified monoamine oxidase in a total volume of 3.0 ml. Oxidation of tyramine is taken as 100%; other activities are given relative to that value.

monoamines, such as benzylamine and kynurenine, which are known as typical substrates for animal amine oxidases (4, 7), were not oxidized at all. Diamines, polyamines, and amino acids were also not oxidized. Thus, this enzyme can be classified as a monoamine oxidase rather than a tyramine oxidase (14, 15). However, the pattern of substrate specificity of the enzyme from *K. aerogenes* was different from those of other monoamine oxidase (9, 11, 27).

Kinetics — The reaction catalyzed by the purified enzyme exhibited Michaelis-Menten kinetics. The Km and Vm values for tyramine were calculated to be 0.282 mM and 0.551 (U/mg of protein), reapectively.

Effects of chemical reagents — The effects of various compounds at 1 mM on the activity of the purified enzyme were investigated, and the results are summarized in Table 3. Among competitive inhibitors of monoamine oxidase, clorgyline inhibited the activity of the enzyme more markedly than did deprenyl. From this result, we classify the enzyme as an A type monoamine oxidase (28). The enzyme was also inhibited by isoniazid and iproniazid, which are specific inhibitors of monoamine oxidase (29). Even though clorgyline and deprenyl are substrate analogues, they bind to coenzyme FAD irreversibly after a long incubation with monoamine oxidase (30). Several monoamine oxidase have been reported that contain FAD or pyrooloquinoline quinone (PQQ) as a prosthetic group (1,2). Thus, we examined the absorption spectra (Fig. 3A) of the purified monoamine oxidase from *K. aerogenes*. However, no characteristic shoulder was observed, and the enzyme exhibited an absorption maximum at about 280 nm. The enzyme did not generate any particular peaks specific for FAD (31, 32, 33) and PQQ (34), neither in its absorption spectrum nor in its fluorescence spectrum (34, 35; data not shown). The enzyem from *K. aerogenes* was markedly inhibited by carbonyl reagents (Table 3). By contrast, tyramine oxidase from *Sarcine lutea* (11) is not inhibited by carbonyl reagents, such as hydrazine, hydroxylamine, and

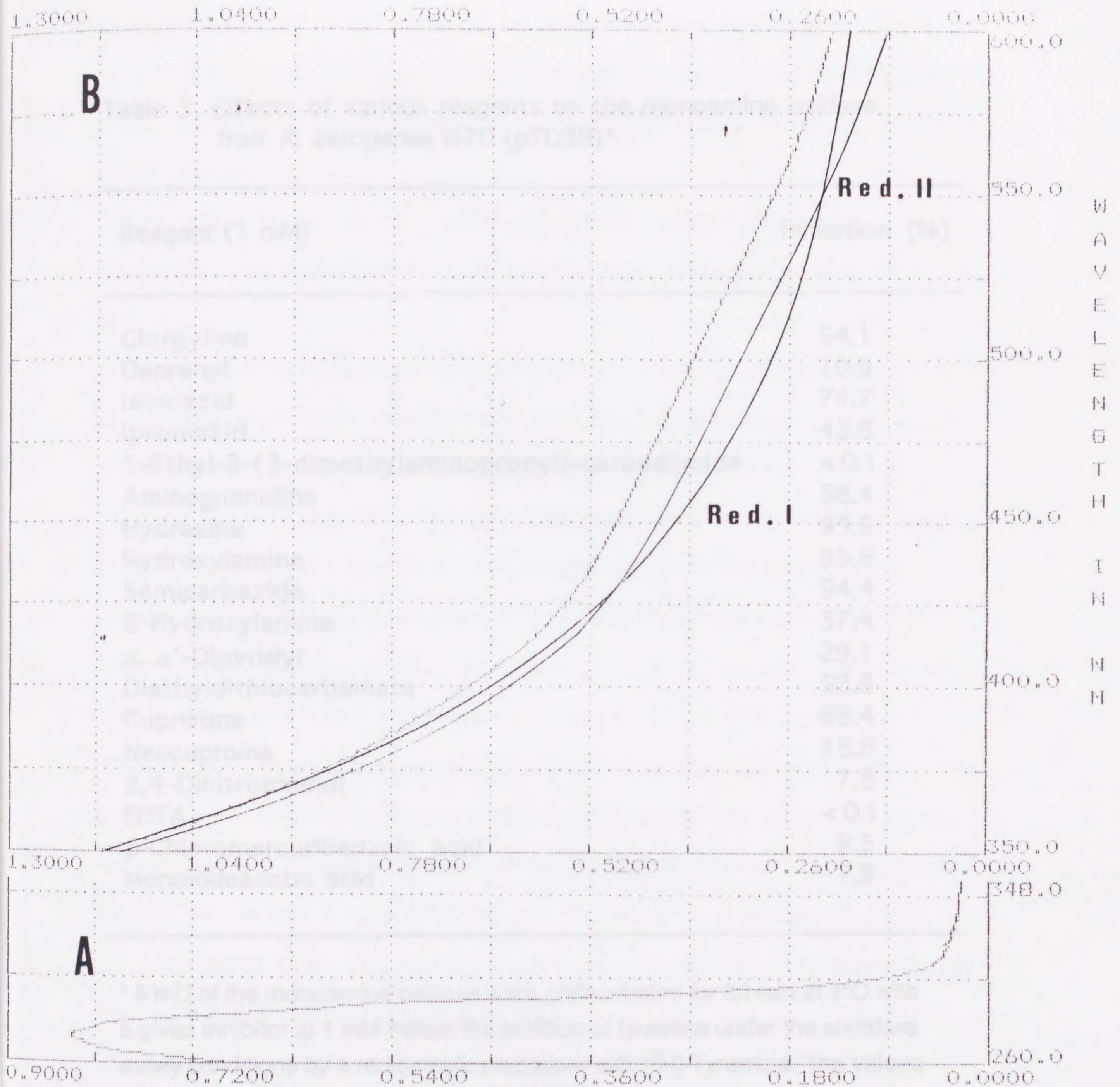

Fig. 3. Absorbance spectra of the monoamine oxidase from *K. aerogenes*. Purified enzyme with a specific activity of 59 mU/mg was used at concentrations of 1.5 (mg/ml) (A) and 259 (mg/ml) (B) in 50 mM potassium phosphate buffer (pH 7.4). Reduced form I (Red. I) was obtained by the addition of tyramine (final concentration 0.1 mM) to the purified enzyme under anaerobic conditions. Reduced from II (Red. II) was obtained by the addition of sodium ditionite (final concentration 16 mg/ml) to the purified enzyme under anaerobic conditions.

Table 3. Effects of various reagents on the monoamine oxidase from *K. aerogenes* W70 (pTO58)*

Reagent (1 mM)	Inhibition (%)
Clorgyline	54.1
Deprenyl	10.9
Isoniazid	74.7
Iproniazid	45.8
1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide	< 0.1
Aminoguanidine	58.4
Hydrazine	95.5
Hydroxylamine	95.6
Semicarbazide	94.4
8-Hydroxylamine	57.4
α, α'-Dipyridyl	29.1
Diethyldithiocarbamate	52.9
Cuprizone	88.4
Neocuproine	15.8
2,4-Dinitrophenol	7.8
EDTA	< 0.1
p-Chloromercuribenzoic acid	9.5
Monoiodoacetic acid	7.9

* 9 mU of the monoamine oxidase were preincubated for 30 min at 4°C with a given inhibitor at 1 mM before the addition of tyramine under the standard assay conditions by a radiometric procedure with [^{3}H]-Tyramine. The values indicate the amount of the activity remaining after incubation with each compound as a percentage of the activity in absence of the compound.

semicarbazide, which are known to be potent inhibitors of diamine oxidase (27). The moderate inhibition of the enzyme by 8-hydroxyquinoline and other metal-chelating reagents indicates that the activity of the enzyme may be metal-dependent.

Identification of the metal ion — Effects of metal ions at 1 mM on the enzymatic were investigated (Table 4). The enzyme was activated by Cu^{2+} ions and completely inhibited by Hg^{2+} ions. These results suggest that the enzymatic activity is dependent on the Cu^{2+} ions. Nara *et al.* (3) demonstarted that copper is a metallic constituent of animal monoamine oxidases. Yamada *et al.* (36) also demonstrated that copper is a constituent of the amine oxidase from *Aspergillus niger*. Next, we tested the possibility of involvement of metal ions in the oxidative processes, using a spectrophotometry revealed that copper was present in the enzyme. The purified enzyme contained 10 g of copper per 79,000 g of protein. The same ammount of copper in the enzyme was found even when the cells were incubated with 0.3 mM $CuSO_4$. Furthermore the iron, manganase, cobalt, and zinc were also examined. However, the only metal present in significant quantity was copper. Thus, we reexamined the absorption spectra of the purified enzyme with concentrated sample. A shoulder at about 480 nm was noticed in the spectrum (Fig. 3B). This absorption band was partially bleached by the addition of tyramine and of excess sodium dithionite under anaerobic conditions.

Search for homology — The amino acid sequence deduced from the nucleotide sequence of the *maoA* gene, which encodes the structural gene for monoamine oxidase from *K. aerogenes* W70, revealed that the mature protein conteins 725 amino acids residues and has a molecular weight of 80,445 (20). We searched for homologies between the amino acid sequence of monoamine oxidase from *K. aerogenes* and those of other amine oxidases. We found that only peroxisomal amine oxidase from *Hansenula polymorpha* (37) showed some homology (Fig. 4). However, the sequences of the amino

Table.4. Effects of metal ions on the monoamine oxidase from *K. aerogenes* W70 (pTO58)*

Metal (1 mM)	Relative activity (%)
None	100
$CuSO_4 \bullet 5H_2O$	123
$CuCl_2$	107
$CoCl_2 \bullet 6H_2O$	102
$ZnCl_2$	100
$CdSO_4$	99
$NiCl_2 \bullet 6H_2O$	98
$MnCl_2 \bullet 4H_2O$	101
$BaCl_2 \bullet 2H_2O$	97
LiCl	98
$MgCl_2 \bullet 6H_2O$	95
$CaCl_2$	99
$Pb(NO_3)_2$	87
$HgCl_2$	0
$CuSO_4 \bullet 5H_2O$ (2mM)	183
$CuCl_2$ (2mM)	130

* Monoamine oxidase (12 mU) was preincubated for 30 min at 4°C with a given metal salt at 1 mM before the addition of tyramine under the standard assay conditions by a radiometric procedure with [^{3}H]-Tyramine. Oxidation in the absence of metals is given as 100%; other activities are given relative to that value.

acids of the two enzymes exhibit 27.9% homology. In neither sequence we could find the amino acid sequence Gly-X-Gly-X-X or Ser-Gly-Gly-Cys-Tyr, which are highly conserved in the flavin adenine dinucleotide-binding region of flavoproteins of a number of oxidases and dehydrogenases (38, 39).

```
MAO    MANGLKFSPRKTALALAVAVVCAWQSPVFAHGSEAHMVPLDKTLQEFGADVQWDDYAQMF

AMO                                           MERLRQIASQATAASAAPA

MAO    TLIKDGAYVKVKPGAKTAIVNGKSLDLPVPVVMKEGKAWVSDTFINDVFQSGLDQTFQVE

AMO    RPAHPLDPLSTAEIKAATNTVKSYFA-GKKISFNTVTLREPARKAYIQWKEQGGPL-PPR
       ...***..**.***. *...**.  .   ...*....*.**.. * ..   **.*. .**
MAO    KRPHPLNSLSAAEISKAVTIVKAAPEFQPNTRFTEISLHERDKAAVWAFALQGTPVDAPR

AMO    LAYYVILEAGKPGVKEGLVDLASLSVIETRALETVQPILTVEDLCSTEEVIRNDPAVIEQ
        *  *.. .**. . *..***.. ....  ...... .. ..*. *....* *... ...
MAO    TAD-VVMLDGKHVI-EAVVDLQNKKILSWTPIKGAHGMVLLDDFVSVQNII-NTSSEFAE

AMO    CVLSGIPANEMHKVYCDPWTIG-YDERWG--TGKRLQQALVYYRSDEDDSQYSHPLD-FC
        **.    ..  **  .* *.* .*.. *   . ** ... *   ..... ..**.. .
MAO    -VLKKHGITDPGKVVTTPLTVGFFDGKDGLQQDARLLKVVSYLDTGDGNY-WAHPIENLV

AMO    PIVDTEEKKVIFIDIPNRRRKVSKHKHANFYPKHMIEKVGAMRPEAPPINVTQPEGVSFK
       ..** *.**.* *.  ..   *... ..  *     .  .   *.. *...*.*** ...
MAO    AVVDLEAKKIIKIE-EGPVIPVPMEPRP--Y-----DGRDRNAPAVKPLEITEPEGKNYT

AMO    MTGNVMEWSNFKFHIGFNYREGIVLSDVSYNDHGNVRPIFHRISLSEMIVPYGSPEFPHQ
       .**....* *..**. .* * * .**.*.***.*. *....  **..******.*.
MAO    ITGDTIHWQNWDFHLRLNSRVGPILSTVTYNDNGTKRQVMYEGSLGGMIVPYGDPDVGWY

AMO    RKHALDIGEYGAGYMTNPLSLGCDCKGVIHYLDAHFSDRAGDPITVKNAVCIHEEDDGLL
        *  ** *.** * .*.*.  * *  .    **. ..* .*.*.*. .** * *  .*
MAO    FKAYLDSGDYGMGTLTSPIVRGKDAPSNAVLLDETIADYTGKPTTIPGAVAIFERYAGPE

AMO    FKHSDFRDNFATSLVTRATKLVVSQIFTAANYEYCLYWVFMQDGAIRLD------IRLTG
       .** . ...  ... *   .***. * *..**.* . *** ..*.* .*        ..*
MAO    YKHLE-MGK--PNVSTERRELVVRWISTVGNYDYIFDWVFHDNGTIGIDAGATGIEAVKG

AMO    ILNTYILG-DDEEAGPWGTRVYPNVNAHNHQHLFSLRIDPRIDGDGNSAAACDAKSSPYP
       .*.. . . ...*....** . .*. . .***....*.*  .**..*. .* *.. .* .
MAO    VLAKTMHDPSAKEDTRYGTLIDHNIVGTTHQHIYNFRLDLDVDGENNTLVAMDPEVKPNT

AMO    LGSPENMYGNAFYSEKTTFKTVKDSLTNYESATGRSWDIFNPNKVNPYSGKPPSYKLV--
        *.* .   ... ..  *... ...  .....* *   ... .. .   * * **...
MAO    AGGPRT---STMQVNQYTIDSEQKAAQKFDPGTIR---LLSNTSKENRMGNPVSYQIIPY

AMO    --STQCPPLLAK--EGSLVAKRAPWASHSVNVVPYKDNRLYPSGDHVPQWSGDGVRGMRE
         .*. ..  **   .. . .*. . .. . *..*. .  **.*.. *..*.... *. .
MAO    AGGTHPAATGAKFAPDEWIYHRLSFMDKQLWVTRYHPTERYPEGKY-PNRSAHDT-GLGQ

AMO    WIGDGSENIDNTDILFFHTFGITHFPAPEDFPLMPAEPITLMLRPRHFFTENPGLDIQPS
       .  *... ....*.... * *.** . .*..*.**.*    .*.*..**.*.*.*.
MAO    YAKDDESLTNHDDVVWITT-GTTHVARAEEWPIMPTEWALALLKPWNFFDETPTLGEKKK

AMO    YAMTTSEAKRAVHKETKDKTSRLAFEGSCCGK
```

Fig. 4. Comparison of amino acid sequences of monoamine oxidase from *K. aerogenes* (MAO) and peroxisomal amino oxidase from *Hansenula polymorpha* (AMO). Identical amino acid (*) and chemically similar amino acids (•) are indicated.

Discussion

The cloning, expression, and purification system described here has made possible the preparation of substantial quantities of highly purified monoamine oxidase in a soluble form. Previously, we determined the complete nucleotide sequence of the gene for monoamine oxidase (*maoA*) from *K. aerogenes* W70 (26). In this sequence, two open reading frames (ORFs), capable of coding for 36-KDa (ORF1) and 84-KDa (ORF2) proteins were found (26). To identify the product of the *maoA* gene, we determined the N-terminal sequence and amino acid composition of the purified monoamine oxidase. The first 11 amino acids from the N-terminus and the amino acid composition of the enzyme were identical to those of the predicted mature protein encoded by ORF2, as deduced from the nucleotide sequence. The leader peptide extends over 30 amino acids in the precursor, and the translated polypeptide is processed to generate the mature enzyme. Thus, the purified enzyme can be considered homogeneous.

Unlike other monoamine oxidases (1, 3, 27), monoamine oxidase from *K. aerogenes* was not inhibited by SH-reagents, such as *p*-chloromercuri-benzoate, which suggests the absence of any role for an essential cystein residue in the structure and activity of the enzyme. In fact, We found no cystein residures in our amino acid analysis or in the peptide sequence deduced from the nucleotide sequence of *maoA* gene (26). Patterns of inhibition of the enzyme by SH-reagents and carbonyl reagents suggest that the mechanism of the reaction catalyzed by the monoamine oxidase of *K. aerogenes* is like that of diamine oxidase (7, 27). We showed that the monoamine oxidase from *K. aerogenes* contains a metal, copper, as an essential cofactor, as do some other amine oxidases (3, 36). The activity of the purified enzyme was stimulated about 20% by Cu^{2+} ions but not to any significant extent by other metals tested. The copper was present in the cupric state. This conclusion is based on the fact that direct determination of copper in the enzyme accounted

for all the copper, and on the fact that the enzyme was inhibited by cuprizone and diethyldithiocarbamate, which are specific for cupric copper, and not by neocuproine, which is specific for cuprous copper. Finally, the native enzyme was shown to contain 10 g of copper per 79,000 g of protein. Since one mole of monoamine oxidase has only 0.16 mole of copper, the enzyme seems to be not fully saturated with the metal when released from the cell. Metal substitution may occur in this enzyme, but other metal ions did not detect in this enzyme.

The enzyme from *K. aerogenes* did not give a shoulder of absorbance centered around 460 nm, even though a FAD-dependent peak of absorbance at 460 nm was found in the case of the putrescine oxidase from *Micrococcus rubens* (33) or hepatic monoamine oxidase (38). A copper-dependent shoulder of absorbance at 480 nm (43) as seen in copper-proteins was barely observed when the concentrated samples (259 mg/ml) was used. The faint reddish color of the enzyme could be related to the shoulder around 480 nm. This absorption band was partially reduced by substrate as well as sodium dithionite (Fig. 3B). Amine oxidases can be divided into two groups, those with FAD and those with copper as the prosthetic group. Almost all diamine oxidases, such as the animal and plant diamine oxidases (40, 41), plasma amine oxidase (42), and the amine oxidase from *A. niger* (36), and few of monoamine oxidase from such as that from *K. aerogenes,* belong to the latter group. Amine oxidases of the latter group are inhibited by carbonyl reagents. The sensitivity to carbonyl reagents of these amine oxidases suggests that a carbonyl group may be located at the active site of the enzyme. Yamada *et al.* (43) reported that the prosthetic group of amine oxidase from bovine plasma was a copper complex with pyridoxal phosphate. Mondovi *et al.* (44) found that the activity of the highly purified animal diamine oxidase was increased by the addition of pyridoxal phosphate. Although this activation may be unspecific, it suggests that pyridoxal phosphate ia a coenzyme for the animal

diamine oxidase. The monoamine oxidase of *K. aerogenes* seems to be different from these pyridoxal phosphate-containing enzymes, since no phosphate-dependent peak of absorbance was observed. Recently, the amine oxidase from *Hansenula polymorpha* was isolated and the tyrosine codon of this enzyme corresponded to 6-hydroxydopa quinone (also known as topa quinone) at the active site (45). More recently, the amino oxidase from *Escherichia coli* K-12 grown on 2-phenylethylamine has been shown to have copper and topa quinone as cofactors (46). The translated nucleotide sequence of *E. coli* amine oxidase gene included the consensus sequence of topa quinone, Asn-Tyr-Asp-Tyr. A comparison of the amino acid sequence of the yeast amine oxidase and *E. coli* amine oxidase with the deduced amine oxidase sequences of the monoamine oxidase from *K. aerogenes* W70 (26) suggests that the enzyme reported here could be a copper- and topa quinone-containing monoamine oxidase (EC 1.4.3.6).

Summary

The gene for monoamine oxidase (*maoA*) from *Klebsiella aerogenes* W70 has been cloned and the enzyme was overproduced in a soluble form. Tne enzyme was purified approximately 10-fold to homogeneity. The enzyme has a molecular weight of about 79,000, which is identical to the molecular weight deduced from the necleotide sequence of the gene for monoamine oxidase, and it consists of individual monomers. The enzyme had maximum activity at pH 6.0 and 50°C when catalyzing the oxidative deamination of tyramine. The enzyme catalyzed the deamination of β-phenylethylamine, dopamine, tryptamine, and octopamine, but not of diamines, polyamines, or amino acids. The enzyme was inhibited by clorgyline, isoniazid, and carbonyl reagents, but not by 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide. The enzyme did not exhibit a typical flavoprotein spectrum, but the enzymatic activity increased linearly with increasing amounts of added copper. The purified enzyme was found to contain 10 g of copper per 79,000 g. The enzymological properties and the amino acid sequence of the enzyme deduced from the nucleotide sequence of the *maoA* gene are different from those of known tyramine or monoamine oxidase. Thus, monoamine oxidase from *K. aerogenes* seems to be a new type of monoamine oxidase.

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Chapter III Structure and Function of Pullulanase: Role of Lipid Modification on a Starch-debranching Enzyme, *Klebsiella* Pullulanase

Introduction

Pullulanase (EC 3.2.1.41) is a maltose-inducible lipid-modified enzyme produced by bacteria belonging to the genus *Klebsiella* (Bender and Wallenfels, 1961; Konishi et al., 1979). The enzyme is specifically released into the medium by Gram-negative *Klebsiella aerogenes* (Takizawa and Murooka,1984; 1985) and *Klebsiella pneumoniae* (d'Enfert *et al.*, 1987). previously, we cloned the pullulanase gene (*pulA*) from *Klebsiella aerogenes* W70 into vector of pKI212, which is stable maintained in *Klebsiella* species (Takizawa and Murooka, 1985). *Escherichia coli* cells carrying the recombinant plasmids producted three to seven-fold more pullulanase than did the wild-type strain of *K. aerogenes* W70, but most of the pullulanase remained intracellular, whereas *K. aerogenes* W70 released pullulanase entracellularly. Transfer to the plasmid into the original producing strain (W70) resulted in an approximately 40-fold increase in pullulanase production, with the intracellular enzyme level being about 100-fold higher than that of strain W70. This accumulation of the enzyme may be caused by titration of 'secretion factors' responsible for pullulanase release. These factors are encoded by 14 genes, which are linked to the *pulA* gene (Pugsley and Reyss, 1990; Reyss and Pugsley, 1990).

Pullulanase is produced as a precusor with a hydrophobic N-terminal signal peptide. The sequence of *K. pneumoniae* pullulanase is highly homologous to that of pullulanase of *K. aerogenes*. However, *K. pneumoniae* UNK5023 pullulanase (Kornacker and Pugsley, 1989) lacks a collagen-like sequence present at N-terminus of the mature *K. aerogenes* W70 enzyme. Pugsley *et al.*, (1986) and Katsuragi *et al.*, (1987) found independently that pullulanase of *K. pneumoniae* and *K. aerogenes* are modified by glycerly lipids. The lipids are covalently attached to the Cys-^{20} residue in the pentapeptide sequence, Leu-Leu-Ser-Gly-Cys, which is similar to the pentapeptides that are adjacent to the sites of post-translational modification and processing of other prolipoproteins

in *E. coli* (Inouye *et al.*, 1977). To investigate this modification, Cys-^{20} was replaced by Ser-^{20}. This prevented fatty acylation and caused slow cleavage of the signal peptide to be processed at a site within the hydrophobic part of the signal peptide. A large amount of the unmodified mutant pullulanase accumulated in the periplasmim of *K. aerogenes* W70 during the logarithmic phase of growth, but some was secreted into the medium (Murooka and Ikeda, 1989). Thus, efficient secretion and correct processing of *Klebsiella* pullulanse require the modification of lipid. In this report, we compare other biochemical properties of the two forms of the enzyme.

Materials and methods

Materials, bacterial strains and plasmids — Pullulan (M_r, 5 x 10^4), G1- to G5-α-cyclodextrins and crystalline pullulanase from *K. pneumoniae* IFO3321 were provided by Hayashibara Co. Ltd., (Okayaka, Japan). Pure collagenase from *Achromobacter iophagus* was purchased from Boehringer-Yamamouchi Co. Ltd., (Tokyo, Japan). All other reagents were standard commercial preparations. The bacterial strains used in this study were *K. aerogenes* W70 and *E. coli* C600 (*leu, thr, thi*). pMPK1, a plasmid contains the *pulA*gene, and plasmid pMPK151, in which the *pulA* gene was mutagenized (Cys-^{20} to Ser-^{20}), were constructed by Murooka and Ikeda (1989).

Production and assay of pullulanase — *K. aerogenes* W70 carrying pMPK1 or *E. coli* C600 carrying pMPK151 were cultured for 3day in minimal medium (Takizawa and Murooka, 1985). *E. coli* C600 cells without the plasmid did not produce any pullulanase (Takizawa and Murooka, 1985). The medium used for production of pullulanase contained 0.5% soluble starch for *K. aerogenes* W70 carrying pMPK1 and 0.5% pullulan for *E. coli* C600 carrying pMPK151 as a sole source of carbon. Pullulanase activity was measured as descreived by Takizawa and Murooka (1984). The release of aldehyde groups from pululan was measured by the Somogyi method as modified by Nelson (1944). One unit of activity was defined as the amount of enzyme causing liberation of 1μmole of aldehyde per min. Proteins were measured by the method of Lowry *et al.* (1951) with crystalline bovine albumin as a standard. The absorbance at 280 nm was used to monitor protein in the column effluent.

Purification of pullulanase — All the steps were carried out at 4°C.

Step 1. Preparation of subcellular fractions. Three days culture broth (about 6 l) was used. Pullulanases from the extracellular, periplasmic, and cytoplasmic fractions were perpared as described previously (Murooka and Ikeda, 1989).

Step 2. Fractination with ammonium sulfate. The crude enzyme was fractionated with 20 to 80%saturation of ammonium sulfate. The precipitate

was dissolved in a small volume of 0.02 M acetate buffer (pH 6.0) and dialyzed against the same buffer.

Step 3-1. DEAE-Toyopearl 650S column chromatography. The dialyzate was applied on DEAE-Toyopearl 650S column equilibrated wih 0.02 M acetate buffer (pH 6.0) containing 0.1 M NaCl. In the extracellular wild-type enzyme, two peaks of pullulanase activity were appeared (Fig. 1A). The two peaks (I and II) were concentrated and purified individually.

Step 3-2. Butyl-Toyopearl 650M column chromatography. For the purification of periplasmic enzyme, this step was added. The dialyzate was brought to 1.5 M saturation by the addition of ammonium sulfate and the mixture was applied onto a Butyl-Toyopearl 650M column equilibrated with 0.02 M acatate buffer (pH 6.0) containing 1.5 M saturation of ammonium sulfate. The proteins absorbed were eluted with a decreasing gradient of ammonium sulfate (1.5 to 0 M) in the same buffer.

Step 4. Gel filtration with Toyopearl HW55S. The concentrated solution was applied onto a Toyopearl HW55S column equilibrated with 0.02 M acetate buffer (pH 6.0). Elution was performed with the same buffer. The fractions with enzyme activity were collected and the solution was concentrated with a membrane filter.

Step 5. High-performance liquid chromatography. Final purification of enzyme was carried out by a high-performance liquid chromatography on a DEAE-NPR column (Toso Co. Ltd., Tokyo,Japan).

Results

Purification of the pullulanases — The wild-type and mutant pullulanases were purified about 150-fold with specific activity of 132.1 Umg^{-1} protein and about 110-fold with specific activity of 139.9 Umg^{-1} protein, respectively (Table 1). The wild-type enzyme chromatographed as two active peaks, eluting at 0.15 M (I) and 0.3 M (II) NaCl, on DEAE-Toyopearl (Fig. 1A), whereas the mutant enzyme chromatographed a single active peak eluting at 0.3 M NaCl (Fig. 1B). The two active fractions of the wild-type pullulanase were indistiguishable on sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and in enzymeatic properties (data not shown) (Table 1 shows data only for peak I).

Both purified enzymes migrated a single protein band (on SDS-PAGE) (Fig. 2). More than 90% of the extracellular wild-type pullulanase was modified with glyceryl lipids, but none of the mutant pullulanase from any cell fractions contained lipid (Murooka and Ikeda, 1989). The molecular masses of the wild-type and mutant enzymes were both determined to be 120 kDa by SDS-PAGE. However, the wild-type enzyme usually appeared as two active peaks on gel filtration (appromimately 350 kDa and 110 kDa). Since the protein with the higher molecular mass behaved as a single protein of 120 kDa on SDS-PAGE, we concluded that it probably coresponds to a trimer reported for the *K. neumoniae* FG9 pullulanase (Charalambous *et al.*, 1988). The other peak is presumably monomer, for which a mass of 117 kDa is predicted from DNA sequence data (Katsuragi *et al.*, 1987). In contrast to the wild-type pullulanase, the mutant pullulanase produced a single active peak on gel filtration. These results suggest that lipid palys a key role in facilitating trimerization of pullulanase.

Properties of the unmodified enzyme — The effects of pH and tampereture on active and stability of the mutant pullulanases were compared with those of the wild-type and the *K. pneumoniae* (*Aerobacter aerogenes*) IFO3321

Table 1. Purification of the extracellular wild-type and the periplasmic mutant pullulanases

Fraction	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)
Crude extract	1345 (1971)	1520 (1247)	0.9 (1.3)	100 (100)
Ammonium sulfate fraction (20-80%)	1088 (2063)	87.4 (148)	12.4 (13.9)	89 (123)
Butyl-Toyopearl 650M	(1597)	(31.7)	(50.4)	(96)
DEAE-Toyopearl 650S	455 (1236)	13.0 (9.6)	35.1 (129.0)	34 (74)
Toyopearl HW55S	261	3.2	81.3	19
DEAE-NPR	230 (854)	1.7 (6.0)	132.1 (139.9)	17 (51)

The extracellular wild-type and the periplasmic mutant pullulanases were prepared from *K. aerogenes* W70 (pMPK1) and *E. coli* C600 (pMPK1), respectively. Values in the parenthese represent the periplasmic enzyme, Butyl-Toyopearl 650M colume was used, whereas Toyopearl HW55S was used for the extracellular enzyme. In the extracellular wild-type enzyme, purification steps from DEAE-Toyopearl 650S show only peak (I) (Fig. 1A).

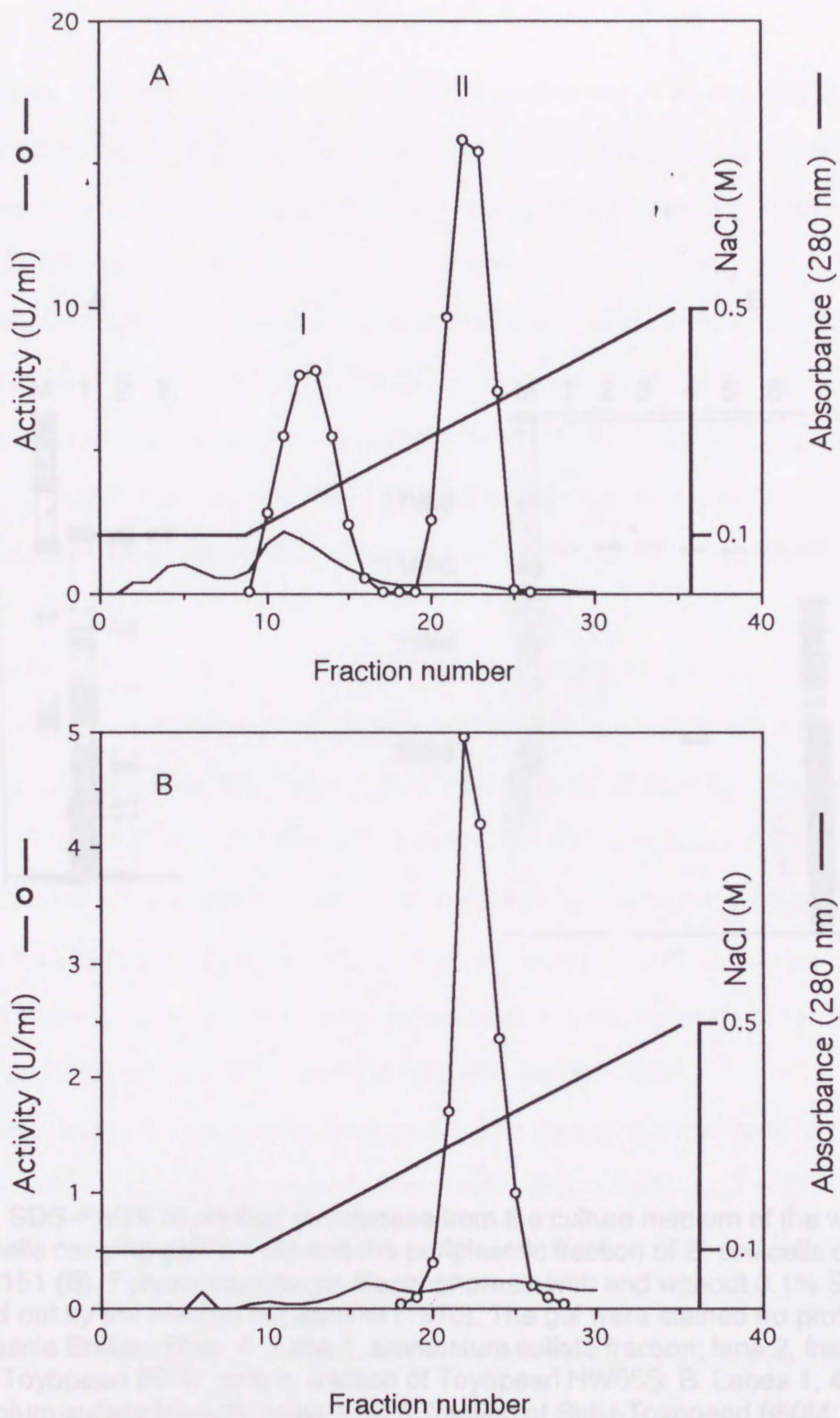

Fig. 1. Elution pattern of the extracellular wild-type pullulanase (A) and mutant-type pullulanase (B) on DEAE-Toyopearl 650S. Elution was done with a linear gradient of 0.1 to 0.5 M NaCl. The two active fractions, I and II were purified individually.

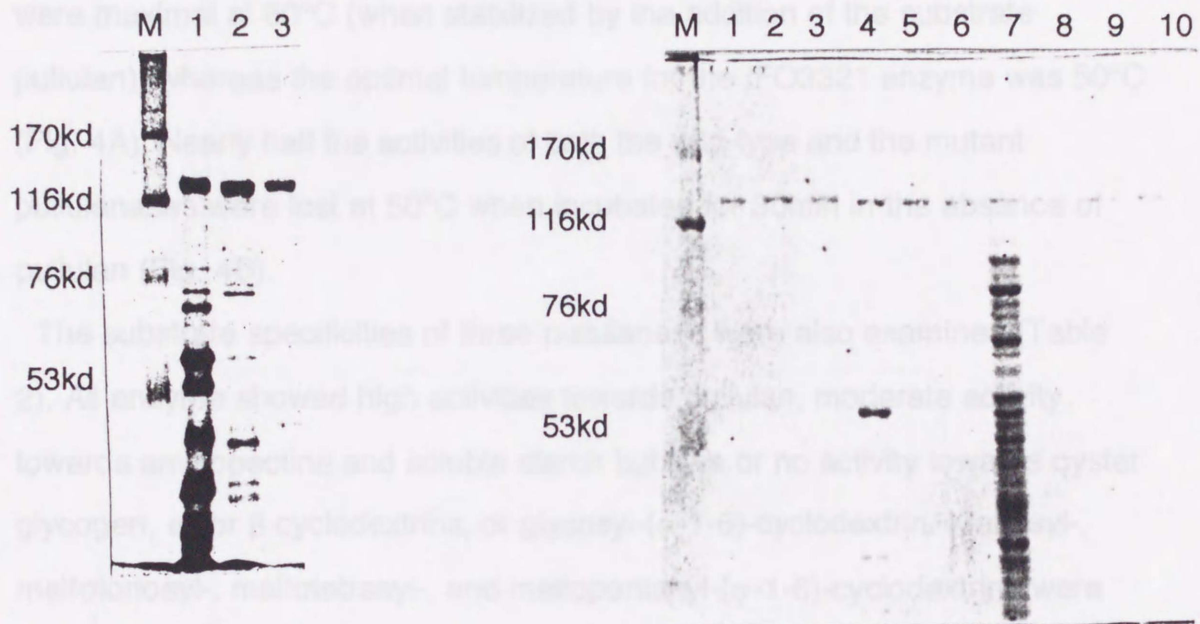

Fig. 2. SDS-PAGE of purified pullulanase from the culture medium of the wild-type W70 cells carrying pMPK1 (A) and the periplasmic fraction of *E. coli* cells carrying pMPK151 (B). Polyacrylamide gel electrophoresis with and without 0.1% SDS was carried out by the method of Laemmli (1970). The gel were stained fro proteins with Coomassie Brilliant Blue. A. Lane 1, ammonium sulfate fraction; lane 2, fraction of DEAE-Toyopearl 650S; lane 3, fraction of Toyopearl HW55S. B. Lanes 1, 4, 7, ammonium sulfate fraction; lanes 2, 5, 8, fraction of Butyl-Toyopearl 650M; lanes 3, 6, 9, fraction of DEAE-Toyopearl 650S; lane 10, fraction of DEAE-NPR. Lanes 1, 2, 3, extracellular fraction; lanes 4, 5, 6, periplasmic fraction; lanes 7, 8, 9, 10, cytoplasmic fraction.

pullulanse, which is though to be identical to that of the W70 enzyme (Ohba and Ueda,1973). The enzyme activities of these three pullulanase were maximal at pH 6.0. They were stable at lower pH but completely inactivated at pH 12.5, although the IFO3321 pululnase showed lower activity at pH 7.0 or more alkaline pH (Fig. 3). Activities of the wild-type and mutant pullulanases were maximal at 60°C (when stabilized by the addition of the substrate pullulan), whereas the optimal temperature for the IFO3321 enzyme was 50°C (Fig. 4A). Nearly half the activities of both the wild-type and the mutant pullulanases were lost at 50°C when incubated for 30min in the absence of pullulan (Fig. 4B).

The substrate specificities of three pullulanase were also examined (Table 2). All enzyme showed high activities towards pullulan, moderate activity towards amylopectine and soluble starch but little or no activity towards oyster glycogen, α- or β-cyclodextrins, or glycosyl-(α-1-6)-cyclodextrin. Maltosyl-, maltotoriosyl-, maltotetrasyl-, and maltopentasyl-(α-1-6)-cyclodextrins were hydrolyzed to similar degrees by the three enzymes. The K_m and V_{max} values of both the wild-type and mutant pullulanases for pullulan were 1.7 x 10^{-3} gml^{-1} and 556 μmoles min^{-1} mg^{-1} of protein, respectively.

The effects of various compounds on the activities of the wild-type, mutant and IFO3321 enzymes were examined. Ca^{2+}, Ba^{2+}, Fe^{2+}, Co^{2+}, Zn^{2+}, Mn^{2+}, Li^{2+}, and Mg^{2+} had no effect, but 0.1 mM $HgCl_2$ inhibited the enzyme activity completely and 0.1 mM $CuCl_2$ caused about 60% inhibition. One mM SDS and 1 mM ethylendiaminetetraacetic acid (EDTA) were not inhiibitory. Since 2-mercaptoethanol, dithiothreitol, and *p*-chloromercuribenzoate were not inhibitory, an accessible sulphydryl residue seems nonessential for the activity of pullulanase. Similar values were obtained for the three enzymes.

Physical difference between lipid-modified and unmodified pullulanases – It is known that a lipoprotein of the outer membrane of *E. coli* changes its apparent molecular weight upon heating in solutions containing SDS

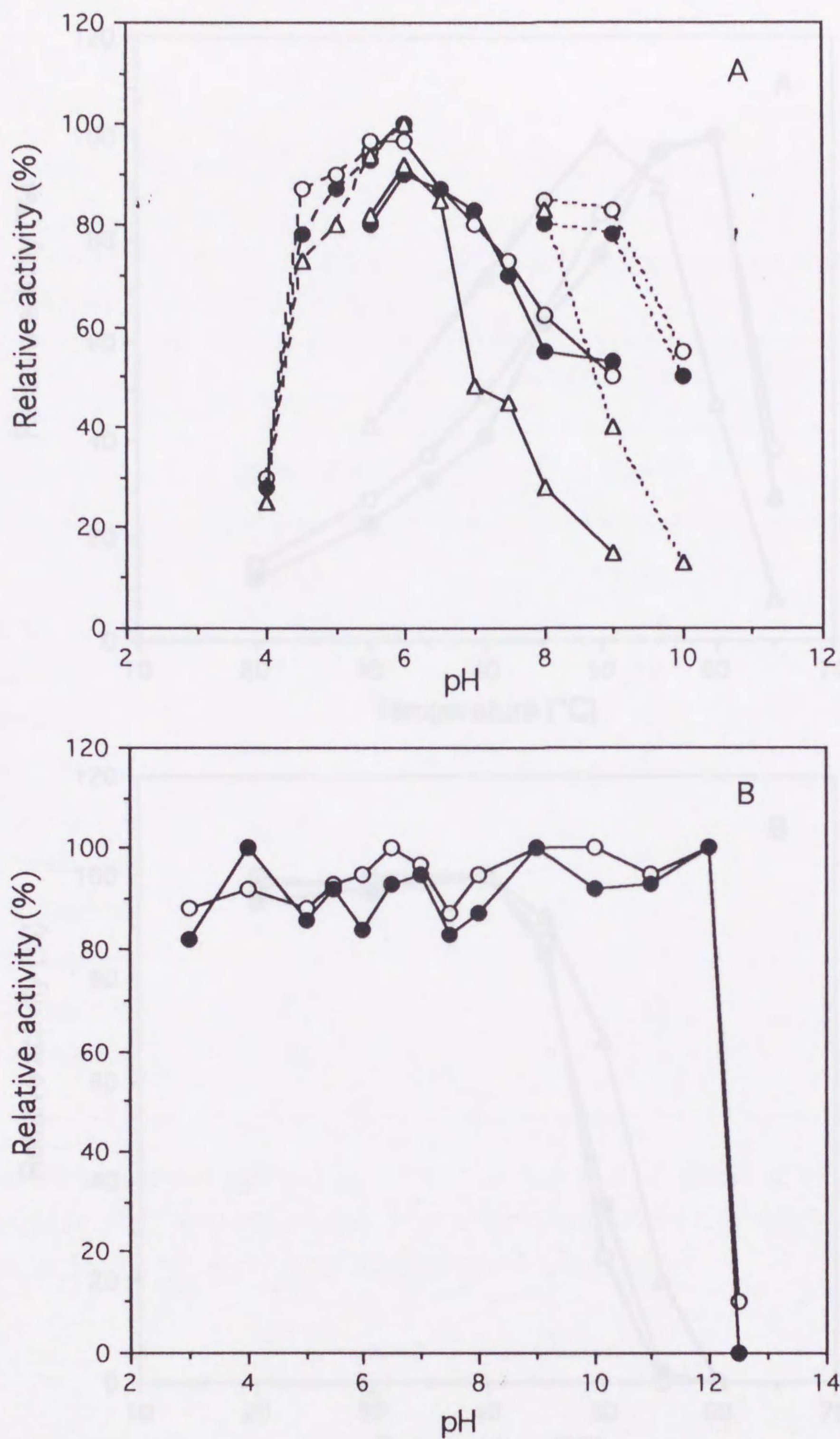

Fig. 3. Effect of pH on enzyme activity (A) and stability (B) of pullulanase.
The enzyme activity was assayed with various buffer. (— —), acetate buffer; (——), phosphate buffer; (••••••), glycine-NaOH buffer. (o), wild-type pullulanase; (●), mutant pullulanase; (Δ), crystaline IFO3321 pullulanase.
For the pH stability, the enzyme solution was incubated at 4°C for 30 min.

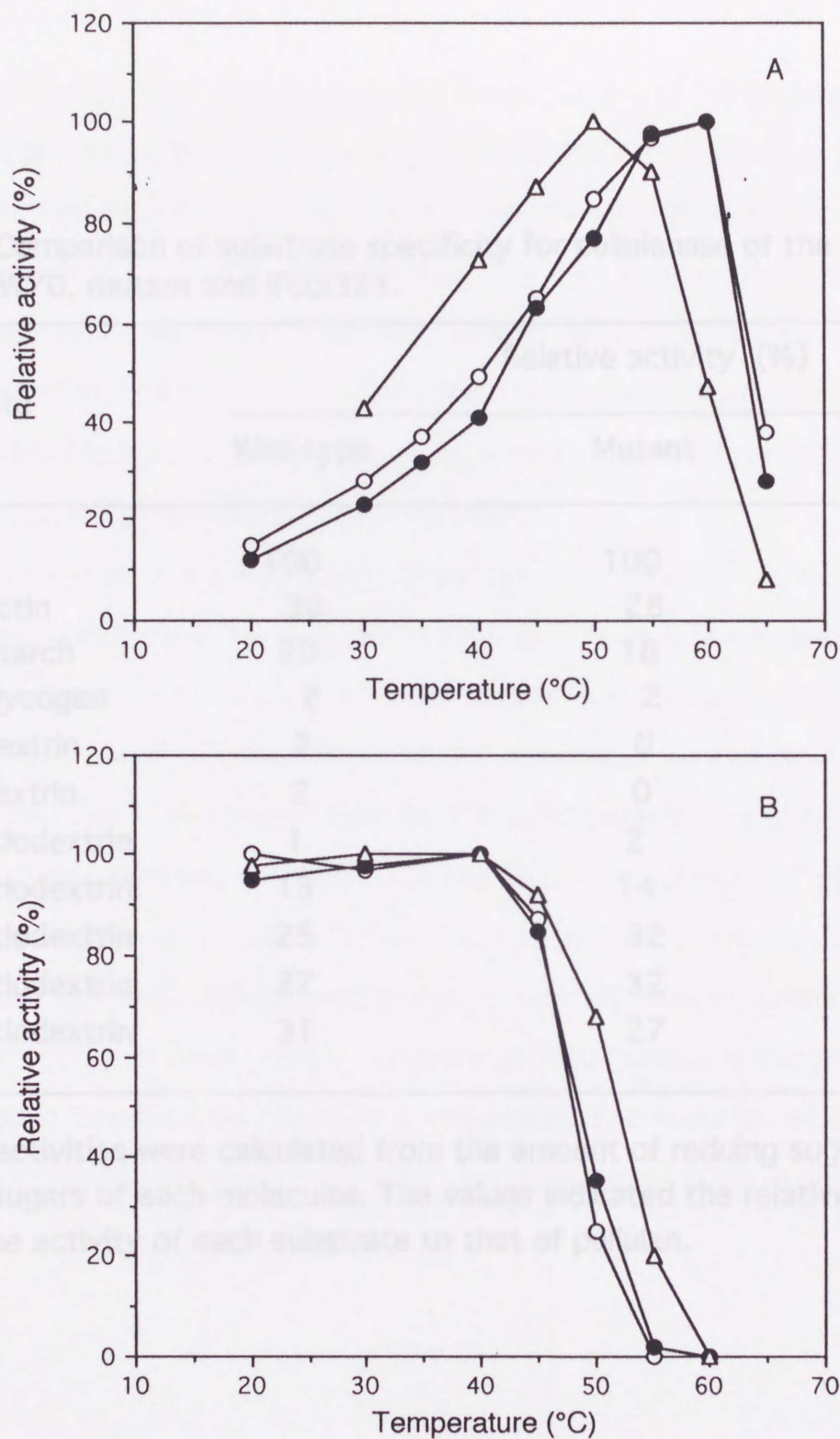

Fig. 4. Effect of temperature on enzyme activity (A) and stability of pullulanase (B). (○),Wild-type pullulanase; (●), mutant pullulanase; (Δ), crystalline IFO3321 pullulanase. For the thermstability, the enzyme solution in 0.1M acetate buffer, pH 6.0, was kept at various temperature for 30 min.

Table 2. Comparison of substrate specificity for pullulanase of the wild-type W70, mutant and IFO3321.

Substrate	Relative activity (%)		
	Wild-type	Mutant	IFO3321
Pullulan	100	100	100
Amylopectin	30	28	34
Soluble starch	20	18	26
Oyster glycogen	2	2	5
α-Cyclodextrin	2	0	0
β-Cyclodextrin	2	0	0
G1-α-Cyclodextrin	1	2	0
G2-α-Cyclodextrin	13	14	0
G3-α-Cyclodextrin	25	32	31
G4-α-Cyclodextrin	27	32	30
G5-α-Cyclodextrin	31	27	28

Enzyme activities were calculated from the amount of reduing sugars to that of total sugars of each molecules. The values indicated the relative percentage of the activity of each substrate to that of pullulan.

(Schanitman, 1973). Thus, we tested the migrations of the lipid-modified and unmodified pullulanases were heatd in SDS-PAGE. When these pullulanases were heated in SDS solution, they showed the same mobility (M_r approximately 120,000) on SDS-PAGE (Fig. 5). However,when these pullulanases were not heated, they migrated more faster and have the apparent M_r of about 60,000. The mutant pullulanase migrated more slowly than the the band of the wild-type pullulanase or the crystalline IFO3321 enzyme, which showed rather broad band.

To test whether the difference of migration rates between the wild-type and mutant pullulanases was caused by binding of lipids to the protein, both pullulanases were digested with collagenase. The pullulanase from W70 has a collagenase recognition sequence six repeated of the tripeptide (Gly-X-Pro) between Gly-^{11} and Pro-^{28} from the N-terminus of mature enzyme and is digested by bacterial collagenase in this sequence, as reported by Charalambous *et al.* (1988) for the FG pullulanase. Collagenase digestion caused unheated wild-type enzyme to migrate with the unmodified (mutant) enzyme (Fig. 5) These results suggest that the difference in migration rates between the wild-type and mutant pullulanases is caused by binding of lipid and/or the sequence of the extreme N-terminal region of the mature enzymes.

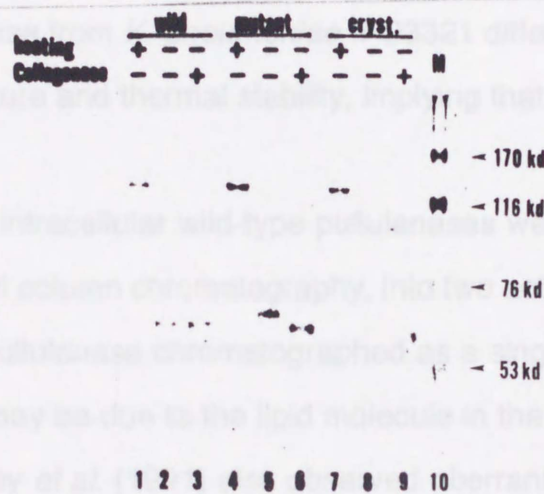

Fig. 5. Effects of heating and collagenase digestion of pullulanase on the migrations on SDS-PAGE. Lanes 1-3, wild-type pullulanase; lanes 4-6, mutant pullulanase, lanes 7-9, IFO3321 pullulanase. Lanes 1, 4, 7, heating for 5 min in the Laemmli buffer (Laemmli, 1970); lanes 2, 5, 8, without heating; lanes 3, 6, 9, collagenase digestion (0.01 U ml^{-1} of 0.02 M phosphate buffer, pH 7.2) without heating; lane 10, molecular marker.

Discussion

We have examined the effects of lipid modification of the secreted enzyme pullulanase on enzymatic properties and physical stability. No distinct differences in various properties (optimal pH and temperature, substrate specificity and affinities, and pH and tempareture stabilities) were observed between the lipid-modified and the un-modified pullulanases. However, we found that pullulanase from *K. pneumoniae* IFO3321 differs from W70 enzyme in optimal temperature and thermal stability, implying that these enzymes are not identical.

Extracellular and intracellular wild-type pullulanases were usually separable by DEAE-Toyopearl column chromatography, into two active fraction (Fig. 1A). Since the mutant pullulanase chromatographed as a single peak (Fig. 1B), this phenomenon may be due to the lipid molecule in the wild-type pullulanase. Pugsley *et al.* (1991) also observed aberrant migration of wild-type pullulanase in gel when the crude samples were not heated to 100°C. We now report that the lipid-modified and unmodified pullulanases migrate differently in SDS-PAGE when the enzymes are not heated prior to loading on the gel. The presence or absence of reducing agents such as β-mercapto-ethanol did not affect electrophoretic mobility. It is possible that heating causes unfolding of pullulanase with consequent increased binding of SDS. This alteration of migration in SDS-PAGE probably accounts for the variation in estimates of M_r of pullulanase (143,000 according to Eisel *et al.*, (1972) and 70,000 to 80,000 according to Ohba and Ueda (1973)). Furthermore, we also confirmed the finding by Charalambous *et al.* (1988) that pullulanase exists as non-covalently associated homotrimers. Three monomers of pullulanase may associate to form a collagen triple-helical structure that serves to stabilize the trimer in a manner analogous to the structure of a number of mammalian 'collagen-like' proteins (Charalambous *et al.*, 1988) However, since the mature enzyme, which retains the collagen-like sequences but lacks the fatty

acids, migrates as a monomer upon gel filtration without collagenase traetment, both the collagen-like sequence of the pullulanase polypeptide and the N-terminal fatty acids must be required to stabilized the trimeric form.

Summary

Klebsiella pullulanase is a lipoprotein synthesized as a precursor with a signal peptide,which is processed by lipoprotein signal peptidase. To clarify the role of lipid modification of pulullanase, we purified lipid-modified wild-type and the unmodified (mutant) pulullanases and compared thier properties. The K_m and V_{max} values of both pulullanases for pullulan were the same. The optimal pH and temparature, the stabilities over pH and temparature ranges, the specificity of substrates, and the patterns of inhibition of the lipid-modified and the unmodified pulullanases were also the same. However, we found that the wild-type pullulanase formed trimers whereas the unmodified enzyme did not, and that the migrations of the two enzymes on sodium dodecyl sulphate electrophoresis were different when the samples were applied on the gel without heating. The results presented in this paper and in previous work show that the correct processing and translocation of pullulanase in *K. aerogenes* require modification of lipid. However, the enzymatic properties and physical stabilities of pullulanase were not affected by the lipid modification.

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Chapter IV Functinal Development of Pullulanase:

Random mutagenesis of Pullulanase from *Klebsilella aerogenes* for Studies of the Structure and Function of the Enzyme

Abbreviations: α-CD, α-cyclodextrin (cyclohexaamylose); β-CD, β-cyclodextrin (cycloheptaamylose).

Introduction

Pullulanase [EC 3.2.1.41], which was first isolated by Bender and Wallenfels (*1*) from *Klebsiella aerogenes* (formerly *Aerobacter aerogenes*), is one of the starch-debranching enzymes that are widely distributed in plants (*2*) and bacteria (*3-6*). This enzyme hydrolyzes α-(1-6)-glucosidic linkages and is commonly used in the starch industry for the production of dextrose and high-fructose corn syrup at high temperature. Two initial steps are involved in the process: first, liquefaction, which requires a very thermostable α-amylase; and second, saccharification, in which pullulanase in addition to glucoamylase or β-amylase generates dextrose with a yield of greater than 94%. For the latter step, pullulanase should be compatible with the other enzymes in terms of pH optimum and thermostability. This requirement for the adaptability of pullulanase attracted our interest to the structure and function of this enzyme.

Extensive studies have been reported of pullulanases from *K. aerogenes* (*7-11*) and *Klebsiella pneumoniae* (*12, 13*). The genes for pullulanase (*pulA*) from *K. aerogenes* W70 (*14, 15*) and *K. pneumoniae* (*12*) have been cloned and sequenced, but most studies have focused on the secretory pathway in Gram-negative bacteria (*16, 17*). Little information about the structure and function of pullulanases has been reported (*13*). Additional pullulan-hydrolyzing enzymes (α-amylase-pullulanases) that hydrolyze both α-(1-4) and α-(1-6)-glucosidic linkages have recently been described (*18-22*). Plant *et al.* (*23*) suggested that the same active center might be involved in the dual activity toward α-(1-4)- and α-(1-6)-glucosidic linkages. Kuriki and Imanaka (*24*) reported the existence of four highly conserved regions in α-amylases, pullulanases, isoamylases [EC 3. 2. 1. 68], cyclodextrin glucanotransferases [EC 2. 4. 1. 19], and neopullulanases that hydrolyze not only the α-(1-4)-glucosidic linkages but also the α-(1-6)-glucosidic linkages of several branched oligosaccharides. These four conserved regions most likely constitute the active center of each enzyme. Kuriki *et al.* (*25*) also reported that one active center in neopullulanase from *Bacillus*

stearothermophilus participated in the dual hydrolytic activity toward α-(1-4)- and α-(1-6)-glucosidic linkages.

To date, two eukaryotic α-amylases, namely, Taka-amylase A of *Aspergillus oryzae* (*26*) and α-amylase from pig pancreas (*27*), have been studied by X-ray crystallography. However, no three-dimensional structure of any pullulanase has been reported. A rational approach to enzyme design involving site-directed mutagenesis is inefficient in the absence of detailed structural information or when the molecular basis for the property of interest is poorly understood. In such cases, random mutagenesis combined with selection or screening can be a useful alternative for generating both the desired improvements and a data base for future rational approaches to protein design. Random mutagenesis has been used to enhance or alter various features of enzymes, such as thermal stability (*28-33*), stability to alkali (*34*), and substrate specificity (*35*), as well as to restore the catalytic activity of an enzyme damaged by site-directed mutagenesis (*36*).

Here we report seven variants of pullulanase that were generated by random mutagenesis and selected by screening at high temperature. Characterization and localization of the amino acid substitutions provided information useful for further improvements in the enzyme's function.

Material and methods

Materials and Chemicals — Restriction endonucleases, T4 DNA ligase, AMV reverse transcriptase, and M13 primer were purchased from Takara Shuzo Co. Ltd. (Kyoto) or Toyobo Co. Ltd. (Osaka). Pullulan (average molecular mass, 99,8 kDa) was provided by Hayashibara Co. Ltd. (Okayama). α-CD and β-CD were gifts from Dr. J. Abe (University of Kagoshima). Reactive Red 120 was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Reactive Red 120-derivatized pullulan (PRR) was prepared by the method of Rinderknecht *et al.* (*37*). All other reagents were standard commercial preparations.

Bacterial Strain, Plasmid, and Phages — The following bacterial strain, plasmid, and phages were used; *Escherichia coli* JM109, pUC18,and M13mp18 and mp19 (*38*).

Manipulation of DNA — Procedures for purification of DNA, restriction enzyme digestion, ligation, agarose-gel electrophoresis, and transformation of competent *Escherichia coli* cells were carried out as described by Sambrook *et al.* (*39*). DNA sequencing was carried out by the dideoxy chain-termination method (*40*).

Random Mutagenesis — A convenient expression vector for pullulanase, pMP10 was constructed with a 2.3-kb *Eco*RI-*Aat* II fragment that consisted of the origin of replication and the gene for ampicillin resistance from pUC18, and a 5.5-kb *Eco*RI-*Aat* II fragment that contained the structural gene for pullulanase (*pulA*) from pMPK1 (*16*). The *pulA* gene was divided into four fragments of similar length (Fig. 4), and each fragment was subcloned into phage vectors M13mp18 (coding strand) and mp19 (noncoding strand). Random mutagenesis of the cloned DNA was performed by the method of Myers *et al.* (*41*). In brief, single-stranded DNA of the hybrid plasmid was treated at 20°C with either hydrazine (for 5–10 min), formic acid (for 5–10 min) or sodium nitrite (for 1–1.5 h). The mutagenized single-stranded DNA was annealed with the M13 primer and a duplex was generated by reverse transcriptase with dNTPs. The duplex DNA was digested with appropriate restriction endonucleases. The resulting fragments that contained part of *pul*A

were inserted into pMP10. *E. coli* JM109 was transformed with the hybrid plasmids and selection was performed after plating on LB medium (*39*) that contained agar and ampicillin (100 μg/ml).

Screening for Mutant Pullulanases — Fresh transformants were replicated on MM agar medium to monitor production of pullulanase (*42*). Cells were grown at 37°C for two days and incubated at 70°C for 1 h until the wild-type pullulanase activity was lost. The plates were overlaid with PRR (0.5 %) soft agar. Expression of mutated *pulA* genes that were functional in *E. coli* was detected as haloes around individual colonies, whereas colonies of cells with an inactivated pullulanase do not form haloes (*43*). The positive clones were purified by repeated streaking on MM agar plates to confirm the expression of active pullulanase. Mutations generated by random mutagenesis were identified by DNA sequencing.

Production and Purification of Pullulanases — *E. coli* JM109 cells carrying a plasmid that included a wild-type or mutated *pul*A gene were cultured in MM medium. Wild-type and mutant pullulanases were purified as described by Yamashita *et al.* (*44*).

Assay of Pullulanase Activity — Pullulanase activity was measured by a modification of the method of Takizawa and Murooka (*42*). A 1.4-ml reaction mixture consisting of enzyme and a 0.5% solution of pullulan in 0.1 M acetate buffer (pH 6.0) was incubated for 10 min at 50°C and the release of aldehyde groups from pullulan was measured by the method of Somogyi (*45*) and Nelson (*46*). One unit of activity was defined as the amount of enzyme that caused the liberation of 1 μmol of aldehyde groups per min. For the kinetic analysis of hydrolysis of pullulan, initial rates were determined by linear regression analysis, and the kinetic parameters were calculated from Eadie-Hofstee plots (*47*). Protein contents were measured by the method of Lowry *et al.* (*48*) with crystalline bovine serum albumin as the standard. The absorbance at 280 nm was used to monitor protein in column effluents.

Results

Screening for and Isolation of Mutant Pullulanases — In the present experiment, when individual transformants (a total of about 50,000 colonies) were analyzed on the plates, seven clones were positive for pullulanase activity after incubation at 70°C for 1 h. All of them were obtained from the formic acid treatment (about 20,000 transformants). Plasmid DNAs were isolated from these clones.

The DNA sequences of both strands of each fragment from the seven plasmids were determined. Table 1 summarizes the base substitutions in the gene for pullulanase that were caused by chemical mutagenesis. Among 17 base substitutions in seven kinds of mutant pullulanase, one was a transition and 16 were transversions. Of the 16 transversions, 14 were transversion from a purine to a pyrimidine. In this experiment, we mutagenized both the coding and the noncoding strands of the fragment using M13 mp18 and mp19, respectively. This procedure may have increased the variety of mutant genes. Of 17 base substitutions generated during mutagenesis, 13 resulted in amino acid replacements, while the other four did not (Table 1). None of the seven mutant pullulanases showed any change in secondary structure calculated according to Chou and Fasman (*49*) (data not shown).

Amino Acid Substitutions Clustered in a Single Region — The clones that formed haloes on PRR plates were isolated and cells were cultured in MM medium. Crude preparations of enzymes from these cultures were prepared and assayed for pullulanase activity at 70°C. No activity was found in cells with either the wild-type or the mutant enzymes at 70°C. The increased thermostability of the mutant enzymes was confirmed by measuring the residual activity after heat treatment of solutions of crude enzyme at 50°C for 30 min (Table 1). Under these conditions, the residual activity of the wild-type enzyme was 18.0% of the initial activity. Most mutant pullulanases had higher residual activity than that of the wild-type enzyme, whereas other mutant enzymes had the same or slightly lower activity than that of the wild-type enzyme, although they had been selected for

Table 1. Base changes induced by chemical mutagenesis and deduced amino acid substitutions encoded by the mutant genes for pullulanase and specific activities of the purified wild-type and mutant pullulanases.

Strain	Phage	Nucleotide change	Amino acid substitution	Residual activity (%)	Specific activity (U/mg)
Wild type				18.5	67.5
I-27	M13mp18	GTG to TTG ATT to TTT	Val 441 Leu Ile 600 Phe	31.3	44.0
I-35	M13mp18	GCG to TCG ACC to ATC	Ala 515 Ser Thr 617 Ile	19.4	23.7
I-56	M13mp19	GAC to GTC AAG to TAG	Leu 409 Gln Phe 562 Ile	43.7	109
I-62	M13mp19	GGC to GTC GAC to GTC CAG to CAT CGG to CGT	Pro 403 Gln Leu 482 Gln Val 492 Val Ala 493 Ala	23.4	33.4
I-75	M13mp19	CGC to CTC CGG to CGT TCA to TCT	Ala 159 Glu Ala 165 Ala Ser 332 Arg	15.9	22.4
I-92	M13mp18	CAG to CTG ACG to TCG	Gln 317 Leu Thr 484 Ser	37.4	23.9
I-97	M13mp18	GCC to GCA TTC to ATC	Ala 265 Ala Phe 562 Ile	32.7	11.6

Nucleotide changes are shown in the strand used for chemical mutagenesis with the mutagens, as described in the text. M13mp18 and mp19 phages carried the coding and noncoding strands of the *pulA* gene, respectively; the coding strand is the DNA strand whose nucleotide sequence is the same as that of the mRNA for the enzyme. Amino acid sequences run from the NH_2-terminus. The values indicate the amount of activity remaining after heating at 50°C for 30 min as a percentage of the activity after incubation at 4°C for 30 min. The activities were measured in duplicate and the two determinations were in agreement to within 20% under the conditions described in Materials and methods.

thermal stability of the enzyme on PRR plates. The amino acid substitutions were all located within only one of four fragments of the *pulA* gene and no substitutions were detected in any other fragment. Among 12 kinds of amino acid substitution, 10 were substitutions of noncharged amino acids. Substitutions of two amino acid residues in mutant I-75 resulted in introduction of charged amino acids. In the mutant I-56, Phe562 was replaced by Ile562 as in mutant I-97, in addition to the replacement of Leu409 by Gln409.

Characterization of Mutant Pullulanases — Wild-type and mutant pullulanases in the cytoplasmic space were purified about 100-fold. Each purified enzyme migrated as a single protein band during SDS-PAGE by the method of Laemmli (*50*). The molecular mass of each enzyme was determined to be 120 kDa (data not shown). The specific activities of the purified enzymes were measured (Table 1). The following analyses were performed with purified enzyme samples at a final concentration of pullulanase of 1 U/ml .

The optimum pH and pH stability of the seven mutant pullulanases were compared with those of the wild-type enzyme. The optimum pH of the wild-type and all the mutant pullulanases was pH 6.0 (data not shown). The mutant enzymes also displayed pH stability similar to that of the wild-type enzyme (*44*).

Although six of the seven mutant pullulanases had the same optimum temperature (55°C) for activity as that of the wild-type enzyme, the I-97 mutant pullulanase had maximum activity at 60°C (Fig. 1). To test the thermal stability of the mutant enzymes, residual activities were measured after incubation at various temperatures for 30 min. The residual activity of the I-27, I-56, I-92, and I-97 mutant pullulanases ranged from 4 to 10% after incubation at 55°C for 30 min, but no activity was detected in the case of the wild-type and the I-35, I-62, and I-75 mutant pullulanases (Fig. 2). Since the kinetics of irreversible deactivation of an enzyme reflect its thermodynamic stability (*51*), the time courses of irreversible thermal inactivation of the mutant pullulanases were determined (Fig. 3). The rates of thermal inactivation of the I-27, I-56, I-92, and I-97 mutant pullulanases

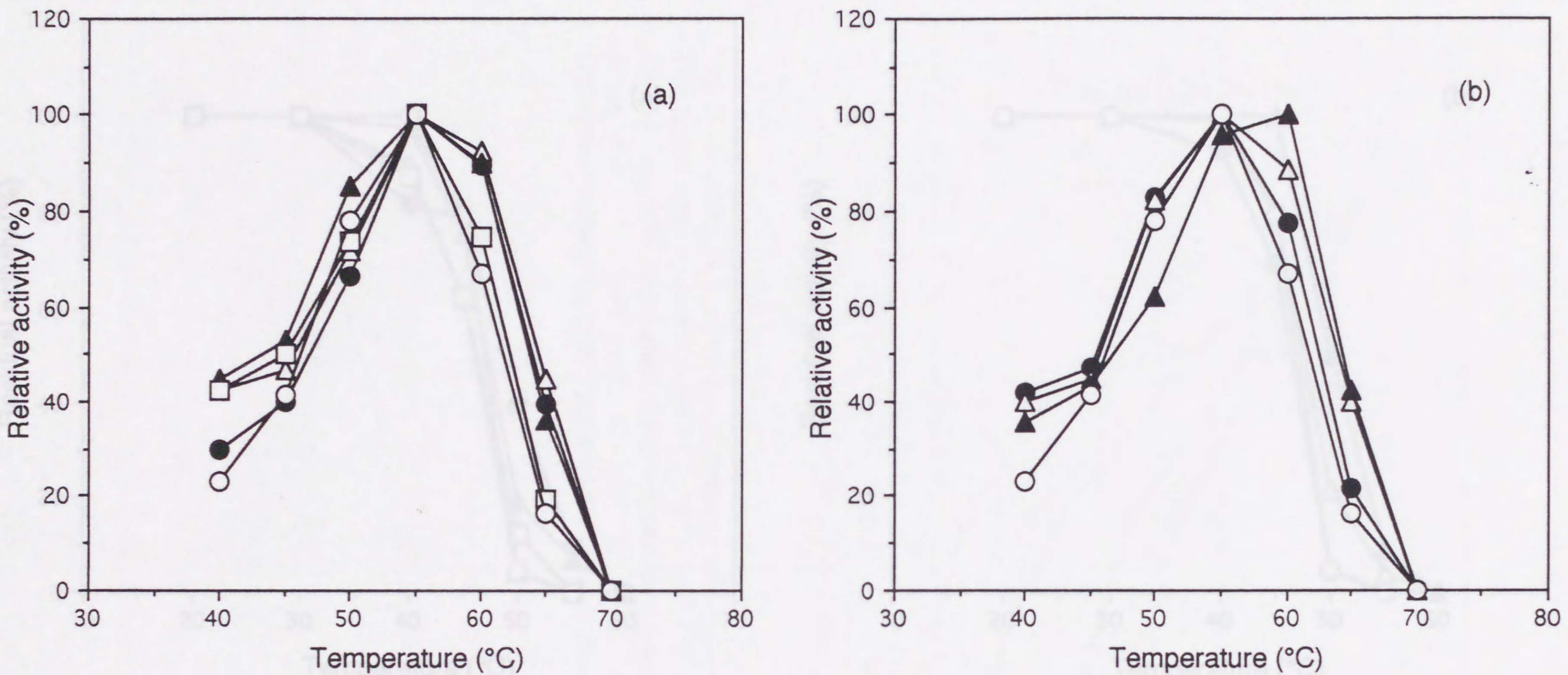

Fig. 1. Temperature-activity profiles of wild-type and mutant pullulanases.
(a) ○ , Wild type; ● , I-27; Δ , I-35; ▲ , I-56; □ , I-62.
(b) ○ , Wild type; ● , I-75; Δ , I-92; ▲ , I-97.
Data points represent the means of three experiments.

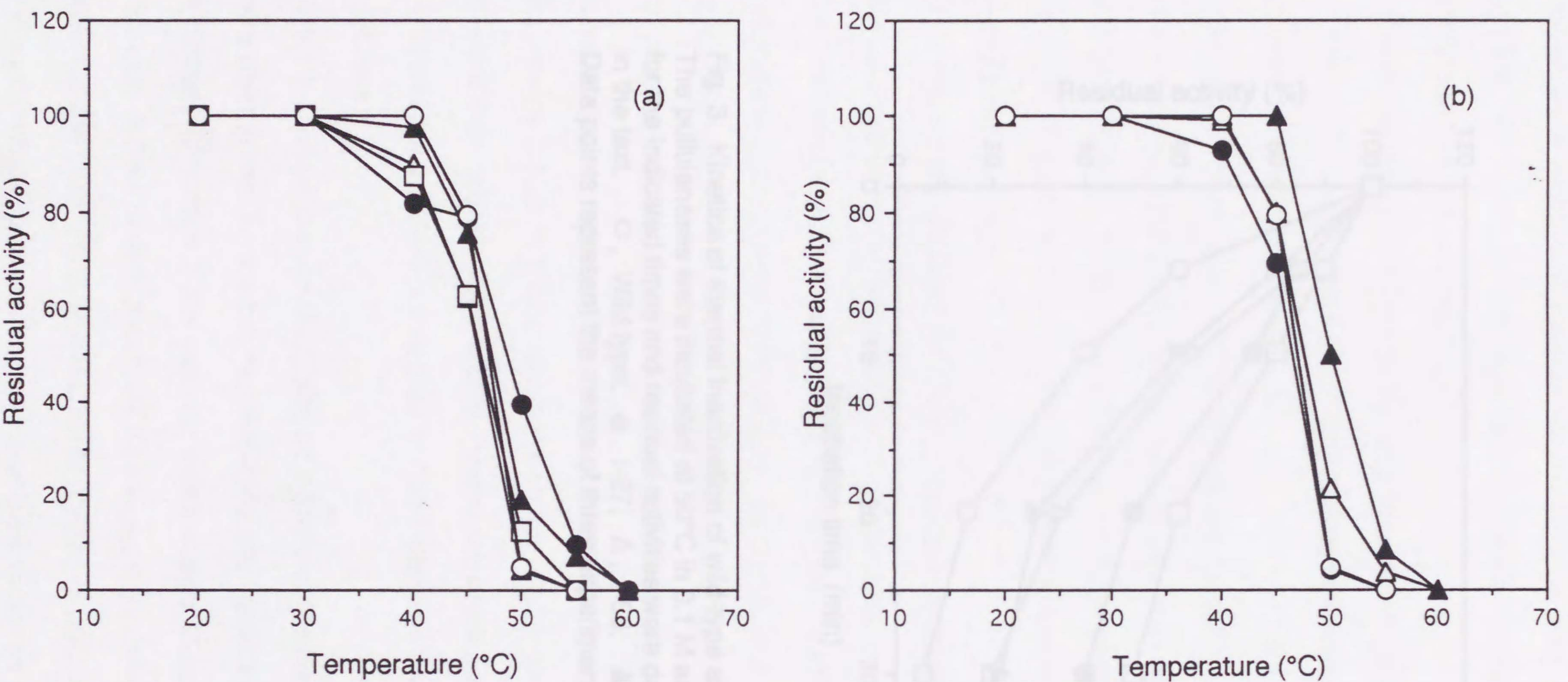

Fig. 2. Thermal stability of the enzymatic activity of wild-type pullulanase and mutant pullulanases. The enzyme in 0.1 M acetate buffer (pH 6.0) was incubated at various temperatures for 30 min. The residual activity was measured as described in the text. (a) ○ , Wild type; ● , I-27; Δ , I-35; ▲ , I-56; □ , I-62. (b) ○ , Wild type; ● , I-75; Δ , I-92; ▲ , I-97. Data points represent the means of three experiments.

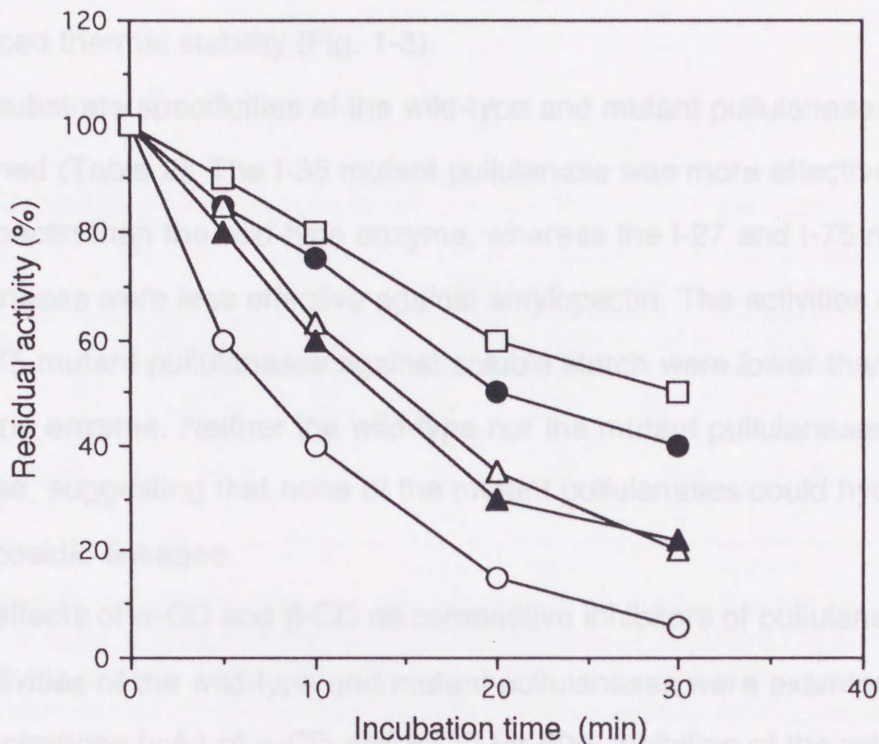

Fig. 3. Kinetics of thermal inactivation of wild-type and mutant pullulanases. The pullulanases were incubated at 50°C in 0.1 M acetate buffer (pH 6.0) for the indicated times and residual activities were determined as described in the text. ○, Wild type; ●, I-27; △, I-56; ▲, I-92; □, I-97. Data points represent the means of three experiments.

were found to be two to four times lower than that of the wild-type enzyme. These results show that these mutants are more stable than the wild-type enzyme and that the I-97 mutant pullulanase has an altered optimum temperature and enhanced thermal stability (Fig. 1-3).

The substrate specificities of the wild-type and mutant pullulanases were examined (Table 2). The I-35 mutant pullulanase was more effective against amylopectin than the wild-type enzyme, whereas the I-27 and I-75 mutant pullulanases were less effective against amylopectin. The activities of I-35, I-62, and I-75 mutant pullulanases against soluble starch were lower than that of the wild-type enzyme. Neither the wild-type nor the mutant pullulanases hydrolyzed amylose, suggesting that none of the mutant pullulanases could hydrolyze α-(1-4)-glucosidic linkages.

The effects of α-CD and β-CD as competitive inhibitors of pullulanase (*47*) on the activities of the wild-type and mutant pullulanases were examined (Table 3). Concentrations (w/v) of α-CD and β-CD for 50% inhibition of the wild-type pullulanase activity were determined to be 0.01% and 0.0004%, respectively. At a concentration of 0.01% α-CD, five of the mutant pullulanases, but not I-56 and I-97, were inhibited about 8 to 15% more than the wild-type enzyme. These mutant pullulanases were also inhibited about 15 to 37% more than the wild-type enzyme by 0.0004% β-CD. Since the I-35 and I-75 mutant pullulanases were different from the wild-type enzyme in the substrate specificity and inhibition ratio, these mutant pullulanases appear to have altered affinity for the substrate and inhibitors.

Effects of Mutations on the Catalytic Activity of Pullulanase — Kinetic parameters were determined for the purified wild-type and mutant pullulanases (Table 4). No significant difference in the catalytic rate constant (k_{cat}) between the mutant and wild-type enzymes was observed. The k_{cat} values were all within a range of ± 50%. On the other hand, the Michaelis constants (K_m) of the I-27, I-35, I-62, I-75, and I-92 mutant pullulanases were two to seven times greater than that of the

Table 2. Comparison of the substrate specificities of wild-type and mutant pullulanases.

	Relative activity (%)							
Substrate	Wild type	I-27	I-35	I-56	I-62	I-75	I-92	I-97
Pullulan	100	100	100	100	100	100	100	100
Amylopectin	42.9	33.9	52.3	44.8	40.2	38.7	43.8	41.1
Soluble starch	19.8	19.8	16.5	22.1	16.4	16.9	25.4	23.3
Amylose	0	0	0	0	0	0	0	0

Enzymatic activities were calculated from the amount of released reducing sugars relative to that of total sugars in each molecule. The activities were measured in duplicate and the two determinations were in agreement to within 15% under the conditions as described in Materials and methods. The values indicate the relative activity as a percentage with each substrate compared to that with pullulan.

Table 3. Effects of α- and β-CDs on pullulanase activity of the wild-type and mutant enzymes.

Enzyme	Residual activity (%)		
	A	B	C
Wild type	100	50.0	50.0
I-27	100	39.2	34.8
I-35	100	37.8	16.4
I-56	100	53.8	53.1
I-62	100	37.8	25.5
I-75	100	34.7	12.7
I-92	100	42.2	18.9
I-97	100	48.6	57.6

A, Pullulan 0.5%; B, pullulan 0.5% + α-CD 0.01%; C, pullulan 0.5% + β-CD 0.0004%.

The activities were measured in duplicate and the two determinations were in agreement to within 20% under the conditions described in Materials and methods.The values indicate the activity remaining in the presence of inhibitor as a percentage of the activity without any inhibitor.

Table 4. Comparison of kinetic constants for the reactions of wild-type and mutant pullulanases with pullulan.

Enzyme	k_{cat} (s^{-1})	K_m (μM)	k_{cat} / K_m ($M^{-1} \cdot s^{-1} \cdot 10^{-6}$)	$\Delta\Delta G^{**}$ ($kcal \cdot mol^{-1}$)
Wild type	33 (100)[a]	45 (100)[a]	0.73 (100)[a]	0
I-27	47 (142)	97 (216)	0.48 (66)	0.27
I-35	18 (55)	313 (696)	0.06 (8.2)	1.60
I-56	31 (94)	35 (78)	0.89 (122)	− 0.13
I-62	49 (148)	130 (289)	0.38 (52)	0.42
I-75	35 (106)	167 (371)	0.21 (29)	0.80
I-92	32 (97)	87 (193)	0.37 (51)	0.44
I-97	34 (103)	31 (69)	1.10 (151)	− 0.26

Results are means of results obtained with 10 concentrations of substrate. Kinetic parameters, namely, k_{cat}, K_m, were determined in duplicate and the two determinations were in agreement to within 10% under the conditions described in Materials and methods.
[a] The values in parentheses are percentages based on value for the wild-type enzyme.

wild-type enzyme, whereas those of the I-56 and I-97 mutant pullulanases were lower than that of the wild-type enzyme. The higher K_m values of the I-35 and I-75 mutant pullulanases were probably the cause of the change in substrate specificity, as seen in Table 2, and the extent of inhibition by α-CD and β-CD, as seen in Table 3.

The specific activities of mutant pullulanases showed similar behavior to the percentages of catalytic efficiency (k_{cat} / K_m) that based on the value for the wild-type enzyme, except for the I-35 and I-97 mutant enzymes (Tables 1 and 4).The specific activities of the I-35 and I-97 mutant enzymes were four times higher and nine times lower than that of the wild-type enzyme, respectively. The I-56 and I-97 mutant pullulanases showed 1.2-fold and 1.5-fold increases in catalytic efficiency (k_{cat} / K_m), respectively, compared to the wild-type enzyme (Table 4). Such improvements were a result of their lower Km values (decreased by a factor of 1.3 and 1.5 compared to the wild type) but not due to the change in the k_{cat} values (decreased by a factor of 1.1 and 1.0).

The effects of amino acid substitutions on the catalytic efficiency can also be quantified in terms of the changes in activation energy ($\Delta\Delta G^{**}$) for pullulan hydrolysis (*52*). The changes in activation energy calculated from the relationship, $\{\Delta\Delta G^{**} = - RT\ln [(kcat / Km)_{mutant} / (kcat / Km)_{wild\text{-}type}]\}$, are shown in Table 4. The effect of the amino acid substitutions in I-56 and I-97 was to decrease the activation energy (relative to the wild type) by 0.13 and 0.26 kcal / mol (1 cal = 4.184 J), respectively, whereas the mutant I-35 pullulanase was associated with a marked increase in $\Delta\Delta G^{**}$.

Discussion

In this report we have described a method of random mutagenesis for creating functional pullulanases with altered enzymatic properties. Key features of the approach are the incorporation of random amino acid substitutions by mutagenesis and a technique for appropriate selection of or screening for the desired variants. We tried to identify mutations that might increase the thermal stability of pullulanase. Temperature-sensitive or -resistant mutations provide one way of identifying residues and interactions that contribute to the folding and stability of a protein. The screen for mutants described here is based on a plate enzyme assay for the thermal stability of pullulanase. A large number of enzymes including protease, amylase and protein can be assayed on Petri plates. The susceptibility of a protein to thermal stability might be detected by using a sensitive colored substrate. Consequently, the method that we have outlined here may be applicable for obtaining useful variants of other enzymes. After we had analyzed about 50,000 mutagenized colonies, we obtained seven kinds of mutant gene. Five mutant pullulanases had altered catalytic properties. The amino acid substitutions caused an increase in K_m values while having a less significant effect on k_{cat} values. The amino acid substitutions of the five mutants were distributed on the NH_2-terminal side of the four conserved regions (Fig. 4). A significant change in activity with substrates, such as amylopectin and soluble starch, and in response to competitive inhibitors, such as α- and β-CD, was observed for the I-35 and I-75 mutant pullulanases, and may also be associated with the increased Km value. In the I-75 mutant pullulanase, amino acids were changed from hydrophobic or neutral residues to hydrophilic residues. The mechanism of the effect on catalytic function of these amino acid substitutions may involve hydrophobic interactions.

Iwamoto *et al.* (*47* and personal communication) suggested that the pullulanase of *K. aerogenes* has an additional substrate-binding site, with low affinity for pullulan, over and above the conserved binding regions found in α-amylases. A

Fig. 4. Positions of amino acid substitutions in mutant pullulanases. Amino acid sequences are shown from the mature NH_2-terminus. Each rectangle indicates one of four conserved regions in members of the amylase family. Amino acid on the left rectangle are numbered from the mature NH_2-terminus of pullulanse. Arrows indicate restriction sites and the fragment of the *pulA* gene that was mutagenized. The wild-type enzyme consists of a total of 1,083 amino acid residues.

deletion pullulanase, with 122 amino acids deleted from the NH2-terminus, had the same activity as the wild-type enzyme (Takizawa, N., personal communication). In our preliminary experiments, a deletion pullulanase, with 133 amino acids deleted from the NH2-terminus, had lower activity than that of the wild-type enzyme. The amino acid substitutions caused by the present random mutation started at the 159th amino acid from the NH2-terminus in the mutant I-75 and ended at the 617th amino acid in the mutant I-35. These results suggest that the NH2-terminal region of the pullulanase of *K. aerogenes* is important for the catalytic function.

Nakajima *et al.* (*53*) found that the amino acid sequences of α-amylases, irrespective of origin, have four highly conserved regions. Each α-amylase contains an eight fold $(\beta/\alpha)_8$ barrel-supersecondary structure, that is, a barrel of eight parallel β-strands surrounded by eight α-helices (*26*). Although all α-amylases have the same catalytic function, their amino acid sequences are quite varied. We found that the pullulanase of *K. aerogenes* also has a conserved eight fold $(\beta/\alpha)_8$ barrel structure as indicated by its amino acid sequence (*15*). The molecular mass of pullulanase is at least twice those of α-amylases, and the pullulanase of *K. aerogenes* has a particularly long NH2-terminal region beyond the four conserved regions. No amino acid sequences or secondary structure in pullulanase and α-amylases were found to be homologous except for those in the four conserved regions. Effective amino acid substitutions generated by random mutagenesis were located on the NH2-terminal side of the four conserved regions. The colonies with enzymes containing these four conserved regions (Fig. 4, *Kpn*I-*Sma*I) that had been randomly mutated could not grow in MM medium (data not shown). These colonies may produce an inactive pullulanase. Two amino acid residues (His607 and Asp677) of pullulanase of *K. aerogenes* in the four conserved regions were individually replaced by site-directed mutagenesis to probe their roles in catalysis (54). None of the mutant pullulanases had any activiy, although the enzymes were detected on SDS-PAGE. The mutations I600F

in mutant I-27 and T617I in I-35 are very close to the predicted substrate-binding site of pullulanase (Fig. 4). The Km values of the I-27 and I-35 mutant pullulanases were higher than that of the wild-type enzyme. These results seem to support the hypothesis that the pullulanase of *K. aerogenes* has active sites similar to those of α-amylases with the four conserved regions.

One type of mutant pullulanase exhibited increased thermal stability. Although the screening process after random mutation was designed to enhance the thermal stability of the enzyme, not all of the seven mutant pullulanases exhibited enhanced thermal stability. Since *E. coli* does not secrete pullulanase into the medium, bacterial colonies selected on PRR plates probably protected pullulanase from thermal inactivation. A distinct enhancement of the thermo-stability of this pullulanase was seen upon the replacement of Phe562 by Ile in the I-97 mutant pullulanase. In general, a globular protein is stabilized by hydrophobic residues in its interior (*55*). Further work is in progress to define the catalytic sites of pullulanase. To investigate the effect of particular amino acid residues at position 562 on the thermostability of the enzyme, we plan to replace the Phe residue at position 562 of pullulanase with all possible amino acid residues by site-directed mutagenesis.

Summary

To study the structure and function of pullulanase from *Klebsiella aerogenes*, a method involving random mutagenesis of the entire gene for pullulanase was used. Out of 50,000 clones screened at high temperature, seven genes for mutant proteins were identified by DNA sequencing. The amino acid substitutions in the seven mutant proteins were clustered on the NH2-terminal side of the four conserved regions found in α-amylases. These mutant pullulanases were classified into two types: those whose catalytic activity was altered and those whose thermal stability was increased. The results presented here and in previous reports suggest that pullulanase from *Klebsiella aerogenes* has similar active sites to those of α-amylases with the four conserved regions, as well as another substrate-binding site closer to the NH2-terminus. The plate assay method used for isolation of thermostable variants may be applicable to the generation of useful variants of other enzymes.

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GENERAL DISCUSSION (CONCLUSION)

In this thesis, the author presented the results of the study on the productivity of homologous and heterologous gene products, analysis of structure-function relationships, and functional development. The conclusions of this work are summarized as follows.

(1) An example of heterologous expression was chosen as a project to produce a sulfhydryl (SH)-rich protein, human metallothionein II (hMT-II) and illustrate approaches in the genetic design of *Escherichia coli* that can act as overproducer. The gene of hMT-II was cloned into a vector, pUEX2 and expressed as a fusion protein between β-galactosidase and hMT-II. The fusion protein accounted for approximately 24% of the total protein in the cells. The fused hMT-II protein was bound to cadmium. Thus, the successful development of chimeric cells that overproduced an active human protein was achieved.

(2) The practical aspect was focused on growing a recombinant *Klebsiella aerogenes* strain in fermentation culture and purifying the recombinant protein product. The gene for membrane-bound monoamine oxidase (*maoA*) from *K. aerogenes* W70 was cloned and the enzyme overproduced in a soluble form. The enzymological properties and the amino acid sequence of the enzyme deduced from the nucleotide sequence of the *maoA* gene were different from those of known tyramine or monoamine oxidase. The enzyme is a new type of monoamine oxidase that requires Cu and Topaquinone as cofactors. The purified enzyme was crystallized and the structure analyzed by X-ray

(3) To analyze secretion and structure-function relationships, an extra-cellular protein and starch-processing enzyme, pullulanase of *K. aerogenes*, was used. First, to clarify the role of lipid modification of pullulanase, lipid-modified wild-type and the unmodified pullulanases were purified and their properties compared. The enzymatic properties and physical stabilities of pullulanase were not affected by the lipid modification. The modification of

lipid on pullulanase in *K. aerogenes* was a signal of correct processing and translocation similar to glycosylation typical of eukaryotic cells.

(4) To analyse the structure and alter the function of pullulanase, a method involving random mutagenesis was used. Seven genes for mutant proteins were identified. These mutant pullulanases were classified into two types: those with altered catalytic activity and those with increased thermal stability. The pullulanase from *K. aerogenes* has active sites similar to those of α-amylases with four conserved regions, as well as other substrate-binding site closer to the NH2-terminus.

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