Application of Genetic Engineering to Useful Enzyme Production from Bacteria and Plant Cells

遺伝子工学的手法による細菌及び植物細胞由来の有用酵素生産

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General Introduction

The earth was first condensed from an interstellar cloud about 4. 6 billion years ago. The primitive atmosphere was formed as various gases. Many of essential building blocks of life (sugars, bases, and amino acids) could have assembled spontaneously from the gasses. When the earth was 1 to 1.5 billion years old, the first cell seemed to be evolved from the prebiotically synthesized building blocks of life. In an attempt to simulate possible conditions on the primitive earth, ten different amino acids as well as hydrogen cyanide (HCN) and various aldehydes were generated from CH_4, NH_3, H_2O and H_2 by high voltage sparks. In addition, the various sugars and riboses (a part of RNA) were synthesized from formaldehyde (H_2CO). The chemists and molecular biologists have established that the many forms of life (organisms) must share a common ancestor, since all cells are so similar as the molecular level.

From the indirect proof for the origin of life, cell was constructed by the earth. The cell has an extremely sophisticated mechanism for physiological (biological) reaction. The efficiency, reliability and specificity of the biological reaction are superior to those of chemical reaction. Although the whole reaction cannot be reconstructed *in vitro*, the each of reaction is feasible because it was assigned to ribozyme or enzyme. In biotechnology field, we take out the enzyme or use the whole cell for specific reaction.

Biotechnology is defined as the industrial exploitation of biological systems or processes and it is largely based upon the expertise of biological systems in catalysis inside tha cell. This is reflected in the ability to recognize other biological systems and specific chemical structure and an extraordinary ability of enzymes to catalyze a vast range of specific chemical reactions under moderate conditions. Even today, the catalytic chemist cannot complete with the efficiency and specificity of biological catalysis, and our understanding of

enzymatic catalysis is quite limited. In spite of our achievements in chemistry, on which most of our industry is based, we are still native in our understanding of catalysis in organisms.

For thousands of years, human being has exploited biotechnology in such activities as brewing, wine-making, bread-making, food preservation and modification by fermentation, the manufacture of soap from fats, primitive medications and waste treatment. Recently, the discovery of genetic engineering techniques via recombinant DNA technology was discovered and improved. While the study of microbial genetics has made fundamental contributions to our understanding of gene structure and function, it has also opened the door to one of most exciting areas of biological sciences today, *genetic engineering*. The development of genetic engineering is a consequence of the large amount of support given to a dramatic research into molecular biology which are most likely to lead to important applications, and a further impact on engineering. All-embracing term, biotechnology was encompassed by terms such as applied biochemistry, enzyme technology, bioengineering, biochemical engineering, biomimetics and bioelectronics.

As the biotechnology developed, the need in many promising areas for close international co-operation between the technologists and scientists involved. The co-operation has already been exemplified most obviously by the highly multinational nature of several of the larger biotechnology companies established over the last years. To realize the industrial production from the biomaterial produced by organisms, such as microorganisms, animals and plants, we have to improve all of steps in combination with various knowledge and techniques. After the development of molecular biology, it has become essential to understand the engineering factors that significantly affect the overall economics of manufacture by evaluating a process with a view to improving its profitability by integration and optimization.

As far as biological processes are concerned, two important orientations exist, *i. e.* the approach based on biochemical engineering and the approach based on physiology. However, there is a sole goal in both of approaches. Sometimes, it is difficult to classify them. I will try to continue learning molecular biology, biochemical engineering, bioengineering and bioelectronics, and understand both of basic and applied fields.

This study is organized into two topics, i) gene structure and enzyme (Chapter 1-2), ii) fermentation engineering (Chapter 3-4). The contents in all of them involved molecular biology, enzyme engineering and biochemical engineering.]

One gene two enzyme structure

Genetic principles are universal. The study of the inheritance and variability of the characteristics of microorganisms has contributed greatly to what we now know about the genetics of all organisms. Gergor Mendel discovered the rule of inheritance in 1859 by doing carefully controlled breeding experiments with the garden pea plant, *Pisum sativum*.. Through this century, the study of mutations has been driving force in genetics. It led to find out the pathway of the biosynthesis of niacin 'vitamin B3' in the pink bread mold, *Neurospora crassa*. Pioneering work in the concept that one gene controls the production of one enzyme was carried out by George Beadle and Edward Tatum, who eventually shared the Nobel Prize for their work. Not only did they put forth the one-gene-one enzyme hypothesis but they used mutants to work out the details of biochemical passway. The one-gene-one-enzyme hypothesis has been in essence refined to a more accurate one-cistron-one-polypeptide concept. To date, many types of overlapping gene such as polycistronic gene structure were discovered. Among them, this finding discribed in the chapter 1 was a first example of one-gene-two-enzymes.

After the protein (polypeptide) was synthesized through the central dogma (DNA-RNA-protein), the protein was processed by some modification or digestion by protease. The bifuctional enzyme or the precursor protein were obligatorily subjected to proteolytic processing.

The structure and catalytic mechanism of enzyme

In industrial enzymes were often supplied from bacteria, not but animal or plant because of a diversity of bacterial enzymes. Concerning amylase, α-amylases were derived from thermophilic bacteria enzyme, while β-amylase used was plant-derived enzyme. To improve the properties of β-amylase, the bacterial β-amylase should be tested and developed.

The basic features of most of the mechanisms are known by three main techniques, chemical modification, X-ray diffraction methods and site-directed mutagenesis. The information from these data provides the positions of the catalytic groups on the enzyme. It is important that we begin on a note of caution. All experiments including those from x-ray crystallography, are subject to interpretation: one crystallographer may interpret a particular feature in the electron density map as being significant, whereas another may consider it an artifact of statistical noise. In addition, the three-dimensional structure of enzyme interpreted does not represent the native structure. Likewise, the chemical modification and site-directed mutagenesis involve an artifact. It was said that the cysteine residues in β-amylase played an essential role on β-amylase catalysis. In the chapter 2, it is proven that the whole cysteine residues in β-amylase do not participate in its catalytic reaction.

The crucial problem in the structural work is the determination of the enzyme-substrate complex. Without this, it is not possible to obtain the fine details of the reaction, such as whether there is distortion of the enzyme or substrate, and precisely where the substrate is located relative to the catalytic groups. In a few case-serineprotease such as trypsin, it is possible to solve the

structure and catalytic reaction. In order to narrow down the candidate catalytic center residues in β-amylase, genetic engineering in combination with oligonucleotide dependent site-directed mutagenesis was applied.

Biochemical engineering

The information that has been accumulated on gene cloning, transformation, protein structure and so forth has made it possible in microorganisms various peptides and proteins. In this respect, species barriers between microorganisms, plants and animals have been, in principle, eliminated. From the macroscopic viewpoint, there is increasing demand for the development of microorganisms as sources for food protein, medicinal substrates, energy and the natural environment. The contribution of biochemical engineering is not simply a set of useful techniques but, perhaps more importantly, a more of thought in describing and designing ways through which we can achieve an objective. Biochemical engineering was a example of important introduction to industrial work on antibiotics fermentation. The concept and technique were based on the development of more efficient culture operation and stirred tank reactors with blade turbine impellers designed for sterile operations. The fermentation process always requires effective culture and proper control for culture condition. It is essential to establish the conditions that will enable the cell to express its character in high yield, to grow vigorously in large vessels, to have high stability. The fundamental works are described in chapters 3 and 4.

Transgenic plant cultivation

These two areas of research have in recent years become associated with the general field of biotechnology. This is generally taken to be the use of living organisms (e. g. bacteria, yeast) or their component parts (e. g. enzyme) in the processing of materials to provide consumables or biochemicals converted. Since microorganism, animal cell, plant cell include a series of enzymes, raw

material are converted into complicated products by the whole cells. The living cells are classified as procaryotic (bacteria and their close relatives) or eucaryotic. Eucaryotic cells are larger and more complex than procaryotic cells and contain more DNA, together with components that allow this DNA to be handled in elaborate ways. Animal and plant are composed of eucaryotic multi-cells. Multicellularity enables a plant to become physically large; to have roots in the ground, where one set of cells can take up water and nutrients; and to have leaves in the air, where another set of cells can efficiently capture the radiant energy from the sun. In the trunk of the whole plant are specialized cells that form channels for transporting water and nutrients between the roots and the leaves. Tissue specific behavior and production are organized. When the plant culture is carried out in the bioreactor, the specific tissue or organ (e. g. root) is suitable for production of the useful chemicals.

The development of *Agrobaterium* plasmids as vector systems for plants is a natural progression of the organisms: firstly, on the plant disease associated with it, and secondly in respect of the similarity of the disease symptoms to certain cancers.

Agrobaterium tumefaciens and *Agrobacterium rhizogenes* respectively are responsible for crown gall and hairy root induction in infected sensitive plants. In both cases, the phenomenon is due to the transfer, integration, and expression in the plant cell genome of DNA (T-DNA) originating form large plasmid called Ti (tumor inducing) and Ri (root inducing) plasmids. The hairy root is similar as originated root in shape and its property. By phytophormone supplementation, root is induced from the originated plant tissue. Hairy root, transgenic plant does not, however, require the phtophormone for growth. The induced hairy root has some advantage properties, such as the higher content of target secondary metabolites and growth rate increment. Hairy root culture is a promising application for biochemical production. The last chapters 3 and

4 are concerned with the application of hairy roots to the efficient culture in combination with biochemical engineering technique.

The objectives of this thesis are to apply gene engineering to the analysis as well as application of enzyme, its gene and procaryotic (bacterial) and eucaryotic (plant) cell. The thesis described some of topics on the overall process involved in bioengineering. The study in combination with the techniques outlined above will offer much wider scope for basic and applied engineering in ways that are of value to the human consumer.

Chapter 1 A Single Gene Directs the Synthesis of a Precursor Protein with β- and α-Amylase Activities in *Bacillus polymyxa* - One Gene Two Enzymes

Introduction

β– and α–amylases hydrolyze the α–1, 4-glucosidic linkages in exo- and endo- fashions from the nonreducing ends of starch-type substrates, respectively. β–Amylase (EC 3.2.1.2) produces maltose, and is present in certain bacteria such as *Bacillus polymyxa*[19, 22], *Bacillus cereus*[11, 39] and *Clostridium thermosulfurogenes*[7], as well as in plants[2, 11]. α–Amylase (EC 3.2.1.1.) produces a series of maltooligosaccharides, and is widely distributed in microorganisms as well as in plants and animals[2]. Many microbial and eucaryotic α–amylase genes have been cloned and well characterized[4, 16, 24, 26, 29, 33, 44, 45].

It was found that *Bacilllus polymyxa* 72 produces multiform β–amylases with approximate molecular weights of 70 kDa, 56kDa, 48kDa and 42 kDa. A large precursor protein with an approximate molecular weight of 130 kDa was detected transiently in the culture broth at an early phase of enzyme production and also when *B. polymyxa* was grown in the presence of protease inhibitors. To elucidate the structures of the *B. polymyxa* β–amylases and the regulation of their formation and secretion, the *B. polymyxa* β–amylase gene was cloned and its partial nucleotide sequence of the gene has been determined. In addition the properties of amylases has been characterized. These results showed that the *Bacillus polymyxa* amylase is synthesized as a precursor protein having β– and α–amylase activities, which gives rise to β– and α–amylases. The finding has been the first demonstration on 'one gene two enzyme' production system.

Materials and Methods

Materials All restriction enzymes, T4 DNA ligase and a dideoxy sequencing kit were purchased from Takara Shuzo Co., Ltd. The universal translation terminator, 5'GCTTAATTAATTAAGC3', was from Pharmacia Fine Chemicals. [α–P]dCTP (400 mCi/mmol) was from Amersham International Ltd. Biodyne A membranes were from PALL Ultrafine Filtration Corp. Achromobacter lysylendopeptidase (EC 3. 4. 21. 50) was from Wako Pure Chemical Industries Ltd. *B. licheniformis* α–amylase (Termamyl) was a gift from Novo Industri Japan Ltd. Amylose was from Sigma. α– And β–cyclodextrins and oligosaccharide mixtures were gifts from Hayashibara Biochemical Laboratories Inc. S-AI, an oligosaccharide produced by *S. diastaticus* which inhibits α–amylases of animal, plant and microbial origins[21)], was a gift from Drs. S. Murao and M. Arai of the University of Osaka Prefecture. α–EPG, 2, 3-epoxypropyl α–D-glucopyranoside, which inhibits β–amylases of plant and microbial origins[9)], was a gift from Dr. Y. Nitta of the University of Osaka Prefecture. All other reagents were of reagent grade.

Bacterial Strains, Plasmids, Media and Transformation The bacterial strains used were *B. polymyxa* 72, which was supplied by S. Murao[22)], *E. coli* HB101, JM83, JM103 and DH20[18)], *B. subtilis* 1A289 (amyE sacA321 aroI906 metB5; Bacillus Genetic Stock Center, Ohio State Univesity, Columbus) and *B. brevis* HPD31. *B. polymyxa* 72 was grown in medium containing 10 g each of soluble starch, polypeptone, meat extract, 5 g of monosodium L-glutamate and 0.1 g of $MgCl_2$ $6H_2O$ per liter (pH 7.0) at 30 ℃. *E. coli* and *B. subtilis* were grown in LS medium containing L broth (10 g of tryptone, 5 g of yeast extract, and 5 g of NaCl per liter [pH 7.0]) and 0.5 % soluble starch, or in antibiotic medium 3 (Difco). *B. brevis* HPD31 was grown at 37 ℃ in T medium [43)]. When required, ampicillin, kanamycin and erythromycin were added at concentrations of 50, 10 and 10 μg/ml, respectively. Transformation of *E. coli* , *B. subtilis* and *B. brevis*

was performed by the methods of Lederberg and Cohen[15], Chang and Cohen and Takahashi et al.[38], respectively. Amylase positive clones were detected by staining the plates with a 1.7 mM I_2-KI solution[41]. pRU100 was constructed as follows: a 55 base pairs *Hind*III-*Eco*RI fragment, the multi-cloning sequence, on pUC19 was inserted into pHW1[6] after the *Pvu*II and *Hind*III sites on pHW1 had been converted to *Hind*III and *Eco*RI sites, respectively.

Isolation and Analysis of DNAs *B. polymyxa* chromosomal DNA was isolated as described by Saito and Miura[30]. Plasmid DNAs were isolated from *E. coli* and *B. brevis* as described by Birnboim[24]. Treatment of DNAs with restriction enzymes and ligation were carried out under the conditions specified by the supplier, and DNA fragments were analyzed by electrophoresis in 0.7% agarose gels and 5% polyacrylamide gels[18]. *B. polymyxa* DNA was partially digested with the restriction enzyme *Hind*III and then ligated to pBR322 DNA, which had been cleaved completely with *Hind*III and treated with bacterial alkaline phosphatase. The ligated DNA was used to transform *E. coli* HB101 to ampicillin resistance. Transformants were selected on LS plates containing ampicillin. Southern blot analysis of the *B. polymyxa* chromosomal DNA was performed using Biodyne A membranes as described by Southern[36]. The β(1149 bp *Dra*I-*Dra*I), J(1521 bp *Hinc*II-*Cla*I) and α(1180 bp *Bcl*I-*Pvu*II) fragments were isolated from pYN49, labeled by nick translation with [α–P]dCTP and then used as hybridization probes (Fig. 1). DNA sequencing was carried out by the dideoxy chain-termination method of Sanger *et al.*[32] for most of DNA sequencing and the chemical technique of Maxam and Gilbert for restricted regions of the seqence after subcloning of appropriate restriction fragments into derivatives of bacteriophage M13.

Purification of β–amylases produced by *B. polymyxa* and *B. subtilis* 1A289 (pYN501) *B. subtilis* 1A289 (pYN501), *B. polymyxa* were grown at 37 °C for 24. Proteins in the *B. polymyxa* or *B. subtilis* 1A289 (pYN501) supernatant were concentrated by addition of ammonium sulfate to 80% saturation. The

precipitate was dialyzed against 5 mM phosphate buffer (pH 7.0) and then subjected to chromatography on hydroxyapatite as described above. β–Amylase in the eluate with 75 to 125 mM phosphate buffer was precipitated with ammonium sulfate (80 % saturation), dissolved in 20 mM borate buffer (pH 7.2), dialyzed against the same buffer, and then applied to a column of Bio-Gel P-100 (Bio-Rad Laboratories, Richmond, Calif.). The enzyme fractions were pooled. The purified β–amylases with different molecular masses were pooled and used for subsequent experiments. The 70-and 42-kDa enzymes produced by *B. polymyxa* and the 42-kDa enzyme produced by *B. subtilis* 1A289 (pYN501) appeared to be homogeneous, whereas the 56-kDa enzyme produced by *B. polymyxa* could not be separated from the 42-kDa enzyme. For determination of the NH_2-terminal amino aid sequences of the 56-kDa enzyme produced by *B. polymyxa,* the enzymes were further purified by electrophoresis on preparative sodium dodecyl sulfate (SDS)-polyacrylamide gel (PAGE). The final protein samples extracted from the gels were dialyzed extensively against distilled water and then used for determination of the NH_2-terminal amino acid sequences. The specific activities of these β–amylases were above 120.

Immunoblot anylysis of the products of clones carrying the β–amylase gene. Rabbits were inoculated via their hind footpads with 2 mg of the purified *B. polymyxa* 70-kDa β–amylase (1 ml) previously dialyzed against 0.9 % NaCl and emulsified with an equal volume of Freund complete adjuvant. After 3 weeks, each rabbit was given a subcutaneous booster injection of 2 mg of the β–amylase suspended in Freund complete adjuvant. At 3 weeks after the booster injection, the rabbits were bled. After the blood samples had been allowd to clot overnight at 4 ℃, the serum was obtained by low-speed centrifugation. Samples fro immunoblot analysis were prepared as follows. The culture conditions were the same as those used for the purification of the β–amylases described above. Cells of *E. coli* were collected from a 3-ml culture and then lysed by incubation at 37 ℃ for 30 min in 0.1 ml of a solution containing 50 mM

glucose, 10 mM EDTA (pH 8.0), 25 mM Tris hydrochloride (pH 8.0), and 2 ng of lysozyme per ml. The supernatant obrained on centrifugation was directly subjected to gel electophoresis. After electrophoresis on sodium dodecyl sulfate-10 % polyacrylamide slab gels, the separated proteins were transferred electrophoretically to nitrocellulose sheets (Bio-Rad). To visualize proteins cross-reactive with the antibody to the β–amylase, protein A conjugated with horseradish peroxidase and the chromophore 4-chloro-1-naphthol were used.

Purification of 48 kDa Amylase *B. polymyxa* 72 was grown at 30 °C for 40 h in the medium described above. The following steps were performed at 4 °C. The amylase was precipitated by adding ammonium sulfate to 80% saturation to the culture fluid. The precipitate was dissolved in a small volume of 10 mM Tris-HCl buffer (pH 8.0) and then dialyzed extensively against the same buffer. After the removal of the precipitate by centrifugation (10,000 x g, 10 min), the dialyzed sample was applied to a column of DEAE-cellulose (2.9x15cm) that was equilibrated and eluted at a flow rate of 18 ml/h, with 10 mM Tris-HCl buffer (pH 8.0). Both the 48 kDa amylase and β–amylases were recovered in the flow-through fraction, precipitated by the addition of ammonium sulfate to 80% saturation, and then dissolved in and dialyzed against 50 mM Tris-HCl buffer (pH 7.5). The dialyzed sample was subjected to gel filtration on a column of Sephadex G-75 (2.5x40cm) with the same buffer at a flow rate of 18 ml/h. The fractions containing the 48 kDa amylase were visualized in situ in the sodium dodecyl sulfate-polyacrylamide gels after electrophoresis as described below and then pooled. The enzyme was precipitated with ammonium sulfate (80% saturation), dissolved in and dialyzed against 10 mM acetate buffer (pH 6.0). The dialyzed sample was applied to a column of carboxymethyl-cellulose (2x10cm) previously equilibrated with the same buffer. The enzyme was eluted at a flow rate of 18 ml/h from the column with a linear gradient of 0-0.3M NaCl in the same buffer. The 48 kDa amylase waseluted at 0.15 M NaCl.

Production and Isolation of 130 kDa Amylase To produce the 130 kDa amylase, a plasmid, pYN520, was constructed as described below using *B. brevis* HPD31 as a host. First, the *Acc*I site situated in the 5' region of β–amylase gene (Fig. 1) was converted to a *Bam*HI site, followed by digestion with *Pvu*II. The resultant *Bam*HI-*Pvu*II fragment (3.6 kb) was isolated. Second, the *Pvu*II-*Hpa*II fragment (0.45 kb) containing the 3' region of the gene (Fig. 1) was also isolated. Third, the *Bam*HI-*Pvu*II and *Pvu*II-*Hpa*II fragments were ligated to pRU100, which had been cleaved with *Bam*HI and *Acc*I, and then used to transform *B. brevis* HPD31 to erythromycin resistance. The transformants were assayed for amylase production on T2 plates supplemented with 0.5% soluble starch, 10 mg/ml erythromycin and 1.5% agar, as described above. One of the amylase positive clones contained pYN520 (Fig. 1). *B. brevis* HPD31carrying pYN520 was grown at 30 °C for 24 h in T medium supplemented with 0.5% soluble starch and 10 mg/ml erythromycin. The following steps were performed at 4 °C. The amylase was precipitated with ammonium sulfate (80% saturation). The precipitate was dissolved in a small volume of 10 mM Tris-HCl buffer (pH 8) containing 1 mM EDTA and then dialyzed against the same buffer. After the removal of the precipitate by centrifugation (10,000xg, 10 min), the dialyzed sample was applied to a column of DEAE-cellulose (2.9x8cm) equilibrated with the same buffer. The enzyme was eluted at a flow rate of 18 ml/h from the column with a linear gradient of 0-1M NaCl in the same buffer. The fractions containing the 130 kDa amylase were visualized *in situ* in the SDS-PAGE gels as described below and pooled. The enzyme was precipitated with ammonium sulfate (80% saturation), dissolved in and dialyzed against 100 mM Tris-HCl buffer (pH 7.5), and then subjected to gel filtration on a column of Toyopearl HW-55 (2.2 x 65cm) with the same buffer containing 0.5 M NaCl, at a flow rate of 18 ml/h. The fractions containing the 130 kDa amylase were visualized, pooled, concentrated with ammonium

sulfate (80% saturation) and then dialyzed against 50 mM Tris-HCl buffer (pH 7.5).

Amino Acid Sequence Analysis 500 mg of the 48 kDa amylase in 10 mM acetate buffer (pH 6.0) was extensively dialyzed at 4°C against 50 mM triethylamine acetate buffer (pH 9.0), digested at 37°C for 6 h with 0.1units of lysylendopeptidase and then lyophilized. The lysylendopeptidase digest was dissolved in formic acid and then subjected to HPLC with a Shimadzu LC-6A system equipped with a reverse phase Toso ODS-120T column (0.46x25cm). Elution was performed with a linear gradient of acetonitrile in 0.1% trifluoroacetic acid, from 5 to 60%, in 90 min, at a flow rate of 0.5 ml/min. The peptides were monitored by measuring the absorbance at 220 nm. The amino acid sequences were determined with a JEOL JAS-47K sequence analyzer. The NH_2-terminal amino acid sequence of the 130 kDa amylase was determined with an ABI 477A-120A protein sequencer.

Assaying of Amylase Activities - The β– and α–amylase activities were determined at 37 °C and 45 °C with soluble starch as a substrate by the methods of Murao et al.[22] and Saito[31], respectively. One unit of β– and α–amylase was defined as the activity causing the formation of 1μ mole maltose from soluble starch in 1 min and the hydrolysis of 0.1 mg of soluble starch in 10 min, respectively. The hydrolysis products from soluble starch and cyclodextrin were analyzed by paper chromatography with n-propanol-H_2O (7:3, v/v) or n-butanol-isopropanol-H_2O (10: 5: 4) as the solvent. Three ascents of approximately 20 cm each were completed on each chromatogram at room temperature. After drying, the chromatograms were developed by the silver nitrate dip method of Robyt and French[27]. Blue-value-reducing-value curves were determined at 37°C, with 0.5% amylose as a substrate, by the method of Robyt and French[28]. Amylase activity bands were detected *in situ* after electrophoresis as described by Lacks and Springhorn[13]. Amylases were heated at 100 °C for 5 min in the presence of 2% sodium dodecyl sulfate and 5%

2-mercaptoethanol, and then electrophoresed on SDS-polyacrylamide gels(10%) as described by Laemmli[14]. The gels were briefly rinsed with water and washed twice at room temperature by shaking gently in 200 ml of 40 mM Tris-HCl buffer (pH 7.5) for 1 h each, followed by incubation for 1h with 100 ml of 0.5% soluble starch in 100 mM Tris-HCl buffer (pH 7.5). Finally, the gels were stained with a 1.7 mM I_2-KI solution.

Other Methods - Protein was determined by the method of Lowry et al. with bovine serum albumin as a standard[17].

Results

Cloning of the *B. polymyxa* β–amylase gene into *E. coli* and deletion derivative subacloning.

A library of *B. polymyxa* 72 DNA was constructed in *E. coli* HB101, and about 7,500 transformants were screened for amylase activity. Five amylase-positive cloned were obtained in the initial screening. Only one clone, designated as YN49, stably produced amylase, even after several transfers onto LS-ampicillin plates. When the plasmid isolated from pYN49 was used to transform *E. coli* HB101, all the transformants produced amylase.

The restriction map of the 9.3-kilobase (kb) insert on pYN49 is shown in Fig. 1. To reduce the size of the insert, pYN 49 DNA was digested with *Eco*RI and *Cla*I, ligated with pBR322 cleaved by *Eco*RI and *Cla*I, and then used to transform *E. coli* HB101. Amylase-positive clone, designated as YN4901, which contained a 4.8-kb insert on the plasmid was obtained by the amylase assay on the plate.

The properties of the amylase produced by *E. coli* HB101(pYN4901) were compared with those of the 70-kDa enzyme purified from the donor strain, *B. polymyxa*. Both enzymes degraded starch to form maltose exclusively (data not

shown). They hydrolyzed neither α–nor β–cyclodextrin, and similar mutarotation of the product was seen when they acted on dextrin, indicating that the maltose formed was of the β–form (data not shown). Furthermore, the enzymes were sensitive to 1 mM *p*-chloromercuribenzoate, as are various β–amylases isolate from other sourced[20, 26]. None of the α–amylases studied so far is sensitive to this sulfhydryl reagent. These results confirmed that the product of *E. coli* HB101 (pYN4901) was β–amylase.

To further localize the gene conferring the β–amylase activity, a series of derivatives was generated by deleting various segments of pYN4901 with various restriction enzymes. The religated deletion derivatives were transformed into both *E. coli* HB101 and *B. subtilis* 1A289, and then the production of β–amylase activity was tested. It became apparent that all the clones containing the 1.8-kb *Acc*I-*Acc*I fragment were able to produce β-amylase, whereas the clones lacking some of this region (pYN4906 and pYN4907) produced no amylase at all. Since β–amylase production increased when a promoter was placed to the left of the inset on pYN4904, pYN4917, or pYN501, the direction of transcription appeared to be toward the *Cla*I site. Therefore, the 1.8-kb *Acc*I-*Acc*I region of the DNA seemed to be essential for β–amylase synthesis.

Characterization of the β–amylases produced in different hosts.

When the proteins were electophoresed on a sodium dodecyl sulfate-polyacrylamide gel, multiple-activity bands were unexpectedly obseved by the *in situ* detection method for amylase not only in the *B. polymyxa* culture supernatant but also in the extract of *E. coli* cells containing various lengths of cloned DNA (Fig. 2A). The possibility that one enzyme protein gave the multiple activity bands was ruled out by the observation the purified β–amylases showed not only a single band in gels stained with a dye but also a single activity band at the respective position. The enzyme proteins were also

detected immunologically at approximately the same positions as those at which the activities were detected (Fig. 2B). Production of multiple amylases by *B. subtilis* 1A289(pYN503) was also demonstrated by immunoblot analysis but not by in situ detection of the activity (Fig. 2). Table 1 summarizes the relationship between the size of the inserted DNA in various plasmids and the molecular weight of the β–amylase synthesized.

By using the purified amylases, the properties of the multiple amylases were compared. The activity profiles with respect to temperature and pH of the amylases with different molecular weights purified from different hosts appeared to be essentially the same. The specific activities of the 70-kDa β–amylase purified from *B. polymyxa* and the 42-kDa enzyme from *B. subtilis* 1A289(pYN501) were found to be 180 and 171, respectively. The β–amylases with various molecular weight that were purified from the culture broths of *B. polymyxa* and *B. subtilis* 1A289(pYN501) were used to determine the NH_2-terminal amino acid sequences. The 14-residue NH_2-terminal amino acid sequences of these β–amylases were exactly the same (Table 1). These results strongly suggest that the NH_2-terminal regions of all the β–amylases are the same, regardless of the molecular weights or the host bacteria in which the β–amylases are synthesized.

Nucleotide Sequence and Characterization of the Amylase gene.

The amylase gene comprises 3,588 nucleotides and appears to be divided into two portions by a large direct repeat (nearly 300 base pairs long) at about the middle of the sequenced DNA (Fig. 3). The predicted amino acid sequence comprises a putative 35 amino acid signal sequence and a 1,161 amino acid amylase sequence. The mature amylase should thus be synthesized as a protein with a molecular weight of 127,134. The amino acid sequence from amino acid 36 (Ala, +1) to 50 (Ala, +15) coincides with the NH_2-terminal amino acid sequences of all the β–amylases mentioned above. There are a possible

RNA polymerase binding and recognition sites (-35 and -10) and a possible ribosome-binding site (Shine-Dalgarno) in the upstream portion of signal peptide sequence. The amino acid compositions calculated from the deduced amino acid sequence approximately matched those determined chemically by using the purified amylases of *B. polymyxa* and *B. subtilis* 1A289 (pYN501) (data not shown). These results confirmed the reading frame of the gene.

To determine the similarity of the deduced amino acid sequence of the *B. polymyxa* amylase with those of amylases of various origins, the amino acid sequences of the latter were optimally aligned with the *B. polymyxa* sequence. The *B. polymyxa* amylase contained sequences homologous with ones in other β– and α–amylases in the NH_2- and C-terminal portions, respectively. The NH_2- and C-terminal portions of the *B. polymyxa* amylase did not show any significant similarity with those of the other α– and β–amylases, respectively. Interestingly, these two portions were separated by the direct repeat sequence described above.

In the NH_2-terminal portion upstream of the direct repeat sequence, three highly conserved sequences were recognized at similar intervals among the β–amylases derived from *B. polymyxa*, *C. thermosulfurogenes*[10], and barley[11] (Ile-77 to Pro-94, Gly-159 to Pro-171 and Leu-319 to Ser-329 of the *B. polymyxa* β–amylase), which were suggested to comprise the active site[10]. In the C-terminal portion downstream of the direct repeat sequence, the *B. polymyxa* amylase contained four sequences homologous at similar intervals with ones in Taka-amylase A[40]. X-ray crystallographic analysis of Taka-amylase A has shown that His-210 and Asp-297 are located in the active center cleft, and His-122, His-296 and Asp-297 participate in substrate binding[20]. Three out of the four homologous sequences observed in the *B. polymyxa* amylase should thus function as active centers of α–amylase. Further comparison with other prokaryotic (*B. licheniformis* and *B. stearothermophilus*) and eucaryotic (*Aspergillus oryzae* and human) amylases showed almost the same homologous

sequences in the same regions as demonstrated previously[8, 23]. This suggests that the *B. polymyxa* amylase gene encodes a single translation product, which possesses two enzymatic activities.

I next examined, described below, whether or not the *B. polymyxa* genome contains a DNA fragment identical in size with the plasmid segment, since it is conceivable that the insert on pNY49 resulted from artificial DNA rearrangement during propagation of the plasmid in *E. coli*. Three DNA probes were prepared from the insert on pYN49: the β–probe (1,149 bp *Dra*I-*Dra*I fragment within the β–amylase coding region), J-probe (1,521 bp *Hinc*II-*Cla*I fragment at the junction of the β– and α–amylase coding sequences) and α–probe (1,180 bp *Bcl*I-*Pvu*II fragment within the α–amylase coding region). Both the *B. polymyxa* chromosomal and plasmid DNAs were cleaved with restriction enzymes, *Eco*RI and *Pvu*II, and probed with the ^{32}P-labeled β, J and α DNA fragments. As shown in Fig. 4, all three probes hybridized to DNA fragments of the same sizes generated from the chromosomal and plasmid DNAs with different restriction enzymes. The *B. polymyxa* genome thus appears to contain an amylase coding fragment of the same size as plasmid pYN49, and thus presumably contains in-phase β– and α–amylase coding sequences.

Characterization and Amino Acid Sequence Analysis of 48 kDa Amylase.

The 48 kDa amylase and 70 kDa β–amylase were purified to homogeneity from the culture broth of *B. polymyxa*. To determine what type the 48 kDa amylase is, the action pattern of the 48 kDa amylase on soluble starch was compared with those of the 70 kDa β–amylase and *B. licheniformis* α–amylase (Fig. 6A, lane 1 and 2). The 48 kDa amylase produced a series of maltooligosaccharides ranging from maltose to maltopentaose, maltose being the most prominent species. The *B. licheniformis* α–amylase also produced a series of maltooligosaccharides, with glucose as the main product. The 70 kDa β–amylase, on the other hand, yielded only maltose. The 48 kDa amylase was

able to hydrolyze β–cyclodextrin, but showed no detectable activity toward α–cyclodextrin. The blue-value-reducing-value curve of the 48 kDa amylase was similar to that of the *B. licheniformis* α–amylase, but quite different from that of the 70 kDa β–amylase. Furthermore, an α–amylase specific inhibitor, S-AI, inhibited the 48 kDa amylase activity but not the 70 kDa β–amylase activity. On the basis of the data described above, the 48 kDa amylase is regarded as having alpha-type amylase activity andto act in an endo-fashion.

To determine the amino acid sequence of the 48 kDa amylase, the enzyme was digested with lysylendopeptidase and the resulting peptides were isolated by high performance liquid chromatography, since the NH_2-terminal of the enzyme is blocked. The amino acid sequences of ten representative peptides determined by Edman degradation were determined. The agreement found between the sequences of the 10 representative peptides and some nucleotide sequences in the 3' part of the gene suggests that the rest of the predicted amino acid sequence of the protein is in agreement with the nucleotide sequence. This clearly indicates that the 3' region of the amylase gene codes for the 48 kDa α–amylase.

Characterization and cleavage of the 130-kDa amylase secreted.

Since the nucleotide sequence indicated that the gene codes for a protein of 130 kDa, a search for a large enzyme in the culture supernatants was made by growing *B. polymyxa* in the presence of various protease inhibitors such as chymostatin and antipain after various incubation periods. β–Amylases of larger molecular weight were indeed found in the *B. polymyxa* culture by using a large amount of chymostatin (Fig. 5). Furthermore, these two high-molecular-mass amylases were cross-reacted with anti-70-kDa β–amylase serum when analyzed by Western blotting. Such large enzymes were also found at an early phase of enzyme production, but they disappeard rapidly as the incubation proceede. When the culture broth containing the large enzymes

was incubated with culture broth prepared at a later phase of enzyme production, the large enzymes were rapidly degraded. However, degradation of the large enzymes was prevented by the presence of protease inhibitors such as chymostatin and diisopropyl fluorophoshate. This suggests that a serine protease(s) produced simultaneously is involved in the degradation of β–amylase.

As the isolation of 130 kDa amylase from *B. polymyxa* was not successful because of its rapid degradation, *B. brevis* HPD31 was used as a host to produce the 130 kDa amylase, since *B. brevis* HPD31 has a large capacity to produce extracellular protein (20 g per liter) and produces extracellularly protease(s) at almost undetectable levels, when determined with casein or bovine serum albumin as a substrate. The 130 kDa amylase was thus isolated from *B. brevis* HPD31 carrying pYN520. The 130 kDa amylase had the same NH_2-terminal amino acid sequence as the major β–amylases produced by *B. polymyxa* (Fig. 3). What type the 130 kDa amylase is was determined as follows. The 130 kDa amylase produced maltose, maltotriose and maltotetraose, maltose being the main product from soluble starch, and hydrolyzed β–cyclodextrin (Fig. 6A, lane 3, and Fig. 6B, lane 3). Although the action pattern of the 130 kDa amylase on soluble starch was in general similar to that of the 48 kDa amylase, the former appeared not to be able to degrade β–cyclodextrin into small oligosaccharides (Fig. 6B, lane 3). Amylase inhibitors, S-AI and α–EPG, were used to separately determine the β– and α–amylase activities of the 130 kDa amylase. Under the conditions where the α–amylase inhibitor, S-AI, inhibited the 48 kDa amylase, the 130 kDa amylase produced only maltose from soluble starch (Fig. 6B, lane 2) and showed no hydrolysis activity toward β–cyclodextrin (Fig. 6B, lane 1), while the 70 kDa β–amylase was not affected. Under the conditions where the β–amylase inhibitor, α–EPG, inhibited the 70 kDa β–amylase (Fig. 6C, lane 3), the 130 kDa amylase produced a series of maltooligosaccharides from soluble starch (Fig. 6C, lane 1). All these data

indicate that the 130 kDa amylase is a bifunctional enzyme, possessing both β– and α–amylase activities.

Discussion

The cloned *B. polymyxa* amylase construction gene appeared to have an amylase coding fragment of the same size as the *B. polymyxa* genome. A large enzyme protein with an approximate molecular weight of 130 kDa (127,314) was transiently detected in the culture broth of *B. polymyxa*, which disappeared upon prolonged culture generating multiple β–amylases of 70, 56 and 42 kDa as the main enzymes. All three β–amylases had exactly the same NH_2-terminal amino acid sequence, which coincided with the deduced amino acid sequence. This ruled out the possibility of overlapping reading of the gene. Therefore, we previously proposed that the 130 kDa enzyme might be proteolytically cleaved to produce multiform β–amylases after secretion. This further suggests that the NH_2-terminal portion of the 130 kDa protein is responsible for the β–amylase activity. To minimize the size of the β–amylase at the NH_2-terminal side, a universal translation terminator, 5'GCTTAATTAATTAAGC3', was inserted into various restriction enzyme sites in the 5' region of the insert on pNY49. One of the clones, containing the terminator at the *Hinc*II site (nucleotide 1620; Val-393) exhibited β–amylase activity. Furthermore, the 5' sequence up to the *Hinc*II site contained three sequences homologous with ones in other β–amylases described above, which might comprise the active sites of the enzyme. This clearly indicates that only the approximately one-third of the gene in the 5' region is required for synthesis of the functional β–amylase.

In the 3' region downstream of the direct repeat sequence, four sequences were found to be homologous with ones in α–amylases of various origins[8,

[23]. This prompted us to search for enzymes showing α–amylase activity in the culture broth of *B. polymyxa*. The 48 kDa amylase was proved to have α–amylase activity and to act in an endo-fashion. Although the NH_2-terminal amino acid sequence of the 48 kDa α–amylase was not established, the amino acid sequences of peptides generated on lysylendopeptidase digestion showed complete agreement with the nucleotide sequence in the 3' region. The 48 kDa α–amylase is thus concluded to be encoded by the amylase gene. This raises the possibility that the *B. polymyxa* amylase gene directs the synthesis of a bifunctional protein possessing both β–and α–amylase activities. The 130 kDa enzyme isolated from *B. brevis* carrying the gene on a plasmid had the same NH_2-terminal amino acid sequence as three major β–amylases produced by *B. polymyxa*, and exhibited both β– and α–amylase activities. All the results presented here indicate that the *B. polymyxa* amylase gene contains in-phase β– and α–amylase coding sequences in the 5' and 3' regions, respectively, and directs the synthesis of a bifunctional enzyme, which gives rise to β– and α–amylases.

To our knowledge, this is the first demonstration of a single precursor protein for two enzymes in procaryotes. More than one polypeptide can be produced from a single gene as a consequence of either a unique DNA arrangement, such as in the case of overlapping genes, or the processing of precursor polyproteins. Overlapping genes are relativelycommon in viruses and mitochondria, as well as in bacteria[25]. The processing of precursor polyproteins is very rare, the main examples being viral polyproteins and polypeptide hormones in eukaryotes[37]. A single precursor protein for two mitochondrial enzymes, acetylglutamate kinase and acetylglutamyl-phosphate reductase in *Neurospora crassa*[42] and another for yolk proteins in the nematode, *Canorhabditis elegans*[34] were reported.

After my report on this finding, two cases in bacteria have been reported in which one single gene directs the synthesis of two different enzymes or

constituent subunits of an enzyme. (i) The Penicilin G acylase precursor is first translocated to the periplasm, thereby losing its NH_2-terminal signal peptide. An unidentified endoprotease then cleaves off the distal β subunit from the a subunit at a specific site on an exposed endopeptide[46]. (ii) The second example is the *B. japonicum fbcH* gene encoding the cytochromes b and c_1[47]. The production of a bifunctional amylase appears to be advantageous for *B. polymyxa* in at least two respects: more efficient utilization of starch type substrates and reduction of so-called "shipping costs"[12] for secretion.

To characterize enzymes involved in the proteolytic processing of the amylase precursor into β- and α-amylases, two proteases genes were cloned from *B. polymyxa* and sequenced in the subsequent study: the extracellular neutral protease gene (npr) and the intracellular serine protease gene (isp). Both proteases produced by *Escherihia coli* were shown to process the amylase precursor into β- and α-amylases[48] , indicating that theindividual amylases from the primary product, a precursor, was derived by posttranslational proteolytic processing (Fig. 7).

It will be of interest to determine whether the *B. polymyxa* amylase gene is an ancestral gene for β– and α–amylases or generated as a consequence of DNA rearrangement. It is tempting to suggest that the direct repeat sequence might mediate the in-phase fusion of the two genes, since the gene appears to be divided into two functional units by the sequence. Although such sequences are reminiscent of transposable elements, these sequences were found only in the amylase gene of *B. polymyxa,* and not in genomes prepared from other Bacilli, when determined by Southern blot analysis with the direct repeat sequence (194 base pairs, nucleotide 2100 to 2293) as a probe. The existence of similar repeat sequences has been demonstrated in two cellulase genes cloned from *Clostridium thermocellum*[1] and alkalophilic *Bacillus* sp. strain N-4[5]. These sequences, however, showed no similarity with that of the *B. polymyxa* amylase

gene. At present the role of such a direct repeat sequence within a structural gene is not known.

Table 1 NH_2-terminal amino acid sequence analysis of β-amylases produced by *B. polymyxa* 72 and *B. subtilis* 1A289(pYN501)

Degradation step	Identified residue	Amount (nmol) of residue from:			
		B. polymyxa proteins			*B. subtilis*
		70 kDa	56 kDa	42 kDa	42 kDa
1	Ala	3.9	1.14	3.7	1.65
2	Val	2.8	1.07	3.2	1.40
3	Ala	3.4	0.77	2.6	0.70
4	Asp	5.7	1.96	4.0	1.20
5	Asp	6.3	1.81	3.6	0.71
6	Phe	3.3	1.47	1.0	1.04
7	Gln	2.3	0.69	1.9	0.37
8	Ala	3.2	0.63	1.2	1.62
9	Ser				
10	Val	2.3	0.53	1.4	1.00
11	Met		0.41	0.7	0.83
12	Gly	2.4	0.68	2.3	0.70
13	Pro	0.5	0.18	0.3	0.73
14	Leu	2.1	0.40	0.7	0.47
15	Ala			1.8	0.45

Serine was not identified.

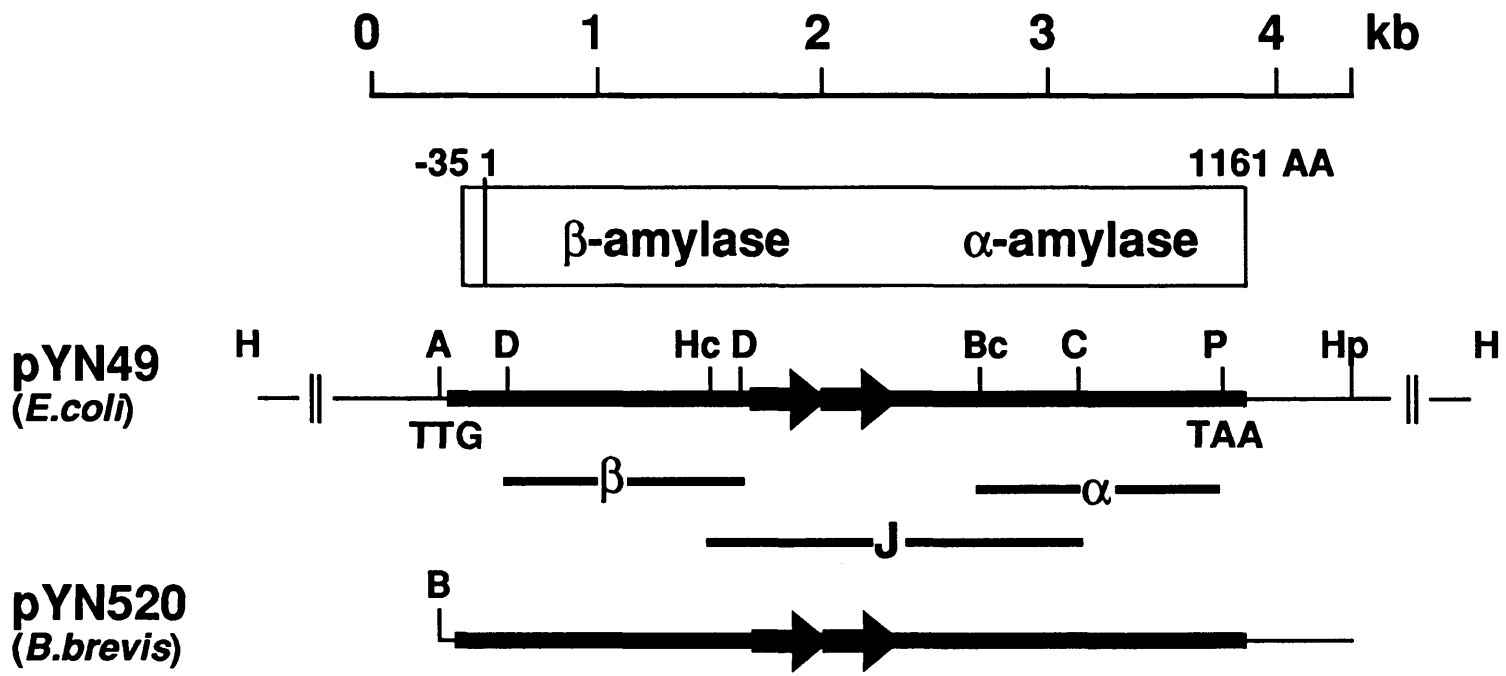

Fig. 1 Diagramatic representation of the amylase gene. At the top is a schematic diagram of the β- and α-amylase coding regions on pYN49. Under this are shown the positions of the restriction enzyme sites used to construct pYN520 and to prepare DNA fragments. The origins of the three DNA probes used in this study, β, J and α, are indicated below the restriction map. The direct repeat sequences are indicated by thick horizontal arrows. The start codon (TTG) and stop codon (TAA) are also indicated. At the bottom is the DNA fragment subcloned onto pRU100. The abbreviations used for restriction enzyme sites are as follows: A, *Acc*I; B, *Bam*HI; Bc, *Bcl*I; C, *Cla*I; D, *Dra*I; H, *Hind*III; Hc, *Hinc*II; Hp, *Hpa*II; P, *Pvu*II.

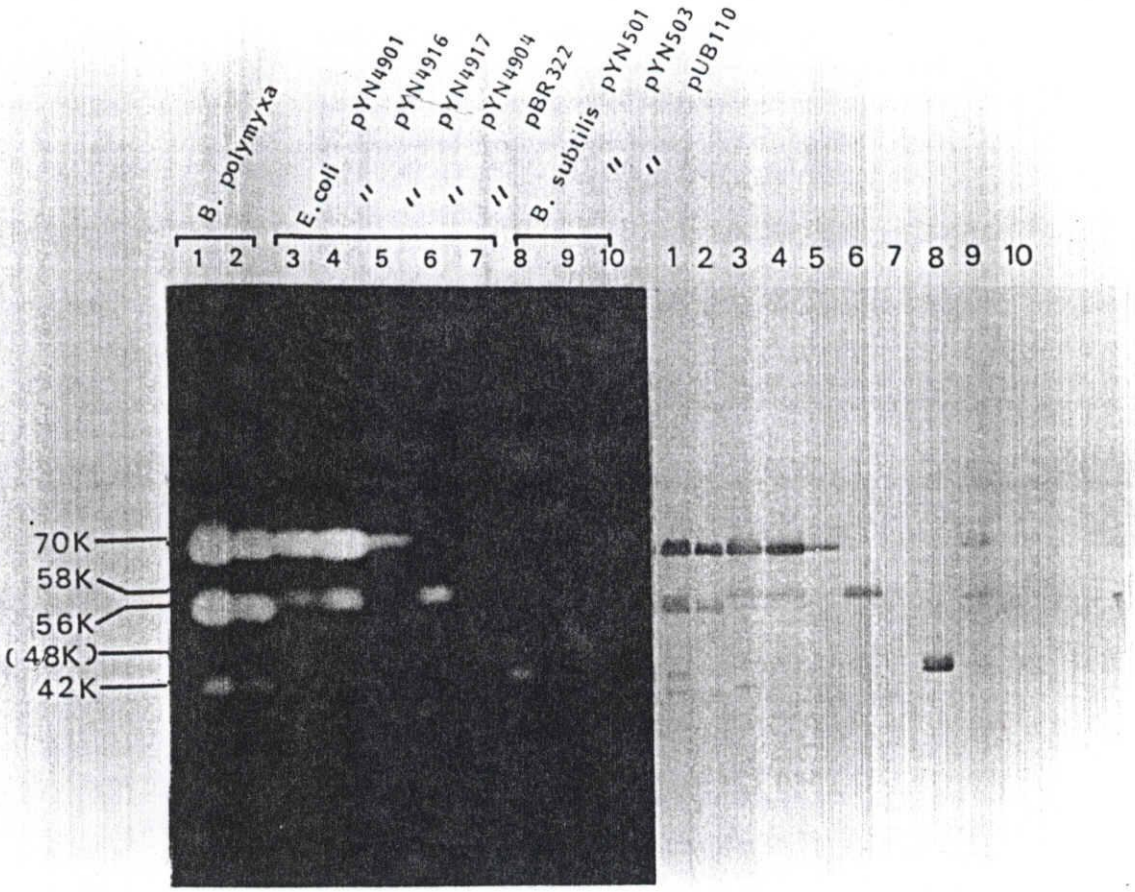

Fig. 2 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of β–amylases produced by various hosts. (A) *In situ* detection of β–amylase activities; (B) Wetern blot anylasis of proteins cross-reacting with anti-β–amylase serum. Culture supernatants of *B. polymyxa* (lane 1, 16 mg [panel A] and 0.8 mg [panel B] of protein; lane 2, 8 mg [panel A] and 0.4 mg [panel B] of protein), *B. subtilis* 1A289(pYN501) (lane 8, 4.7 mg of protein), *B. subtilis* 1A289(pYN503) (lane 9,40 mg of protein), and *B. subtilis* 1A289(pUB110)(lane 10, 40 g of protein), and cell extracts of *E. coli* HB101(pYN4901) (lane 3, 37 mg [panel A] and 15 mg [panel B] of protein), *E. coli* HB101 (pYN4916) (lane 4, 37 mg [panel A] and 15 mg [panel B] of protein), *E. coli* HB101 (pYN4917) (lane 5, 74 mg [panel A] and 15 mg [panel B] of protein), *E. coli* HB101 (pYN4904)(lane 6, 37 mg [panel A] and 15 mg [panel B] of protein), and *E. coli* HB101(pBR322) (lane 7, 74 mg of protein) were electrophoresed together with molecular weight marker proteins. A 48-kDa enzyme detected only in the culture supernatant of *B. polymyxa* showed a clearer band than other activity bands and did not cross-react with anti-β-amylase serum.

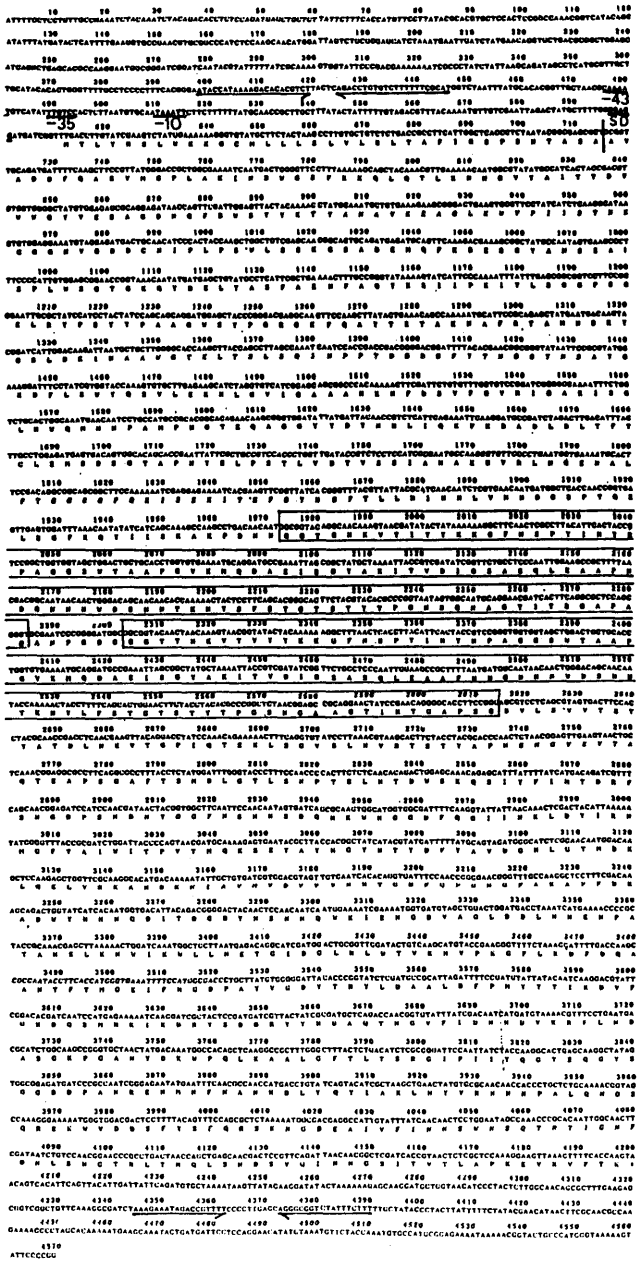

Fig. 3 Nucleotide and amino acid sequences of the Bacillus polymyxa amylase. The underlined sequences marked -35 and -10 refer to the possible RNA polymerase binding and recognition sites, respectively. The cleavage site of the signal sequence is indicated by a vertical bar. The direct repeat sequences are schematically shown as boxes. Three sequences showing homology with ones in other β-amylases are localizedat the 5' region upstream of the direct-repeat sequence. The 3' region downstream of the direct-repeat sequence contains the amino acid sequences of the ten peptides prepared from the 48 kDa amylase. The stop codon is indicated by an asterisk. Palindromic sequences in the 5' and 3' flanking regions are indicated by arrows. The amino acids are numbered from 1from the NH_2-terminus of the mature amylase. The last digit is aligned with the corresponding amino acid or nucleotide.

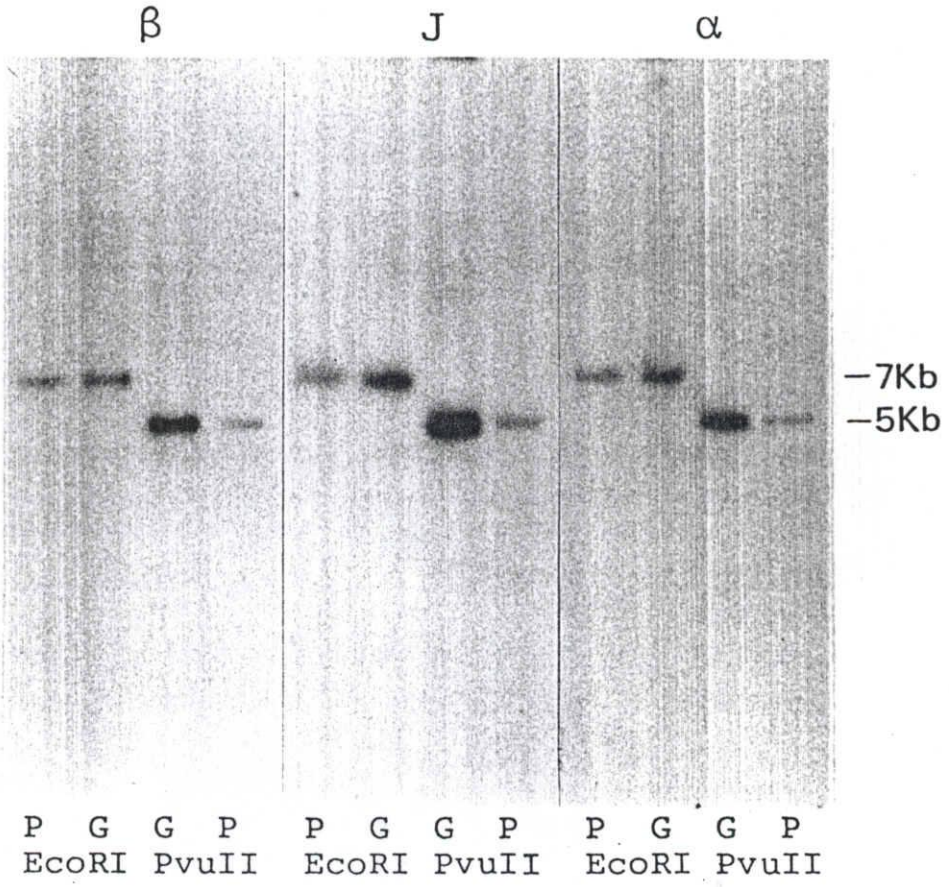

Fig. 4 Comparison of the amylase coding region in the genome and plasmid pYN49. G and P represent genomic and plasmid DNAs, respectively. Three μg of DNA from *B. polymyxa* and 0.1 μg of plasmid DNA were completely digested with *Pvu*II or *Eco*RI, and then processed for Southern blot hybridization with the β, J and α probes as described under Materials and Methods.

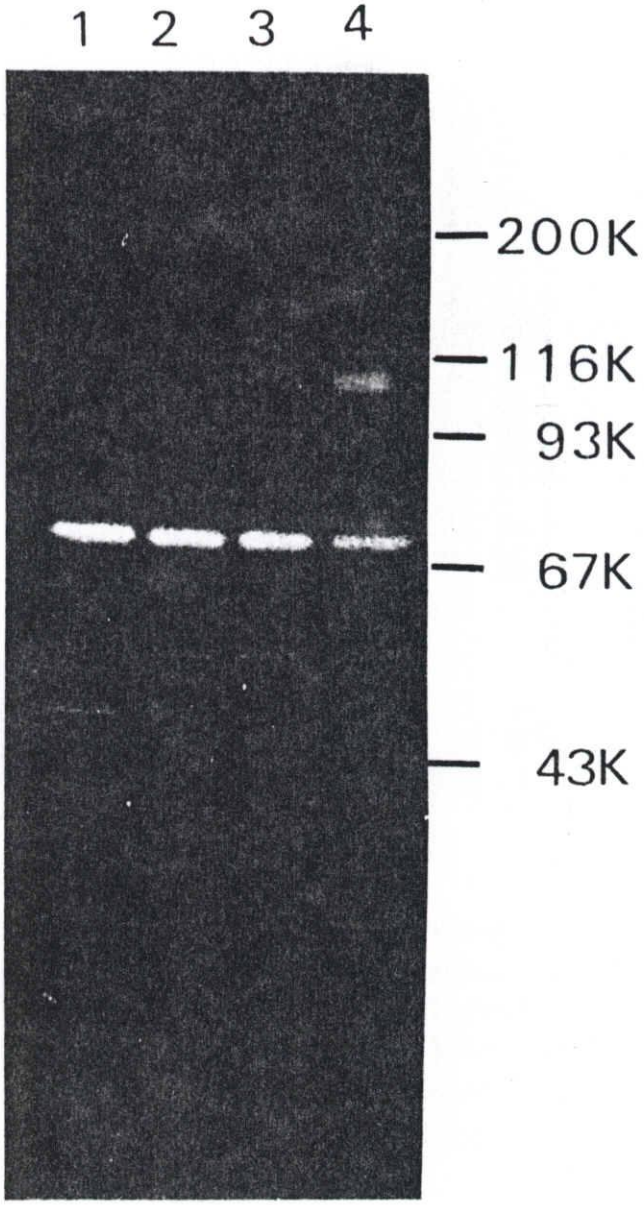

Fig. 5 Profiles of multiform β–amylases in the presence and absence of chymostatin. The supernatants (50 ml) of *B. polymyxa* culture grown at 30 °C for 24 h in the absence (lane 1) and presence of 100 mg (lane 2), 200 mg (lane 3), and 1,000 mg (lane 4) of chymostatin per ml were electrophoresed and then subjected to *in situ* detection of activities.

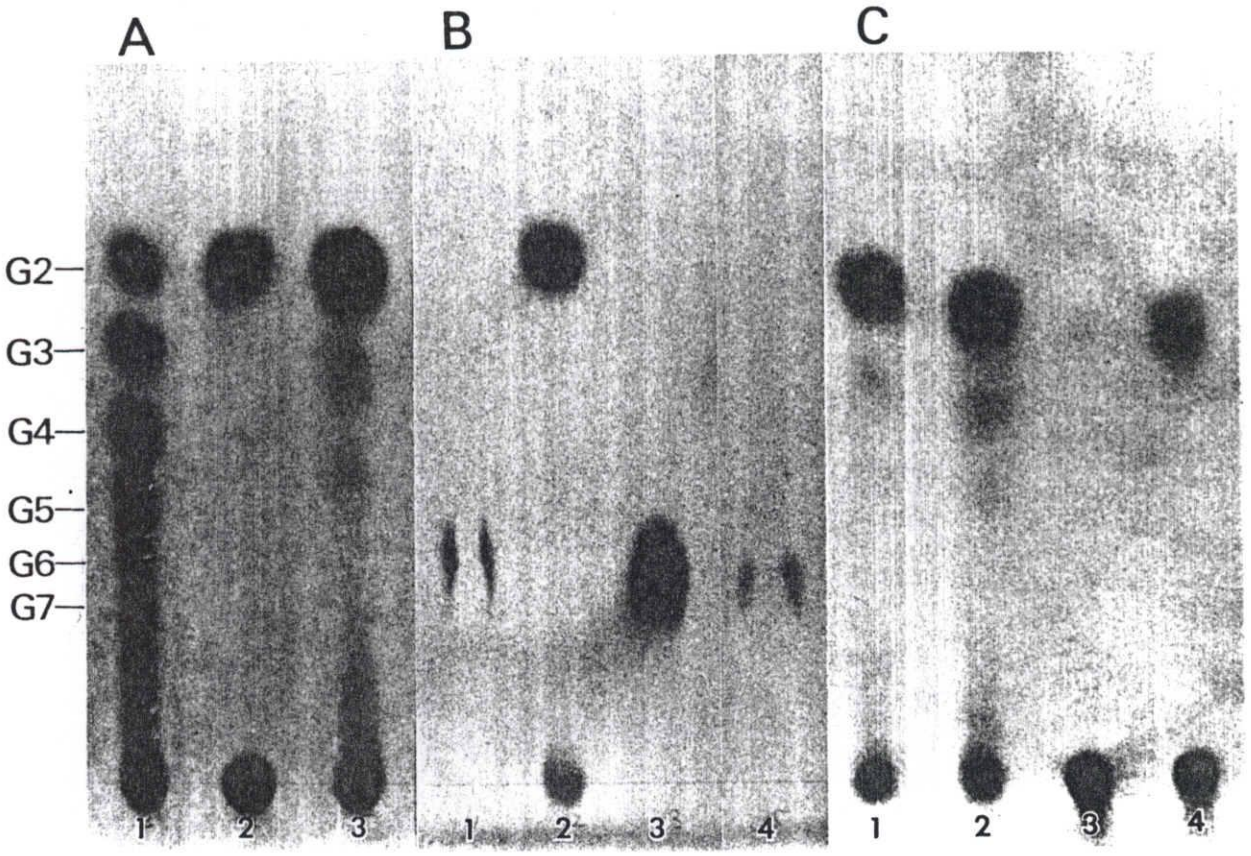

Fig. 6 Paper-chromatographic analysis of the products on the reaction of the 130 kDa amylase with soluble starch and β-cyclodextrin. The enzyme reaction and processing were carried out asdescribed in the legend to Fig. 5. A. Soluble starch was used as a substrate. Products after reaction with the 48 kDa amylase (lane 1), 70 kDa β-amylase (lane2) and 130 kDa amylase (lane 3). B. Products after reaction of the 130 kDa amylase with β-cyclodextrin (lanes 1 and 3) and soluble starch (lane 2). S-AI was included at 200 μg/ml in the reaction mixture (lanes 1 and 2). β-Cyclodextrin used as a substrate (lane 4). C. Soluble starch was used as a substrate. Products after reaction with the 130 kDa amylase in the presence (lane 1) and absence (lane 2) of α-EPG (1 mg/ml). Products after reaction with the 70 kDa β-amylase in the presence (lane 3) and absence (lane 4) of α-EPG (1 mg/ml).

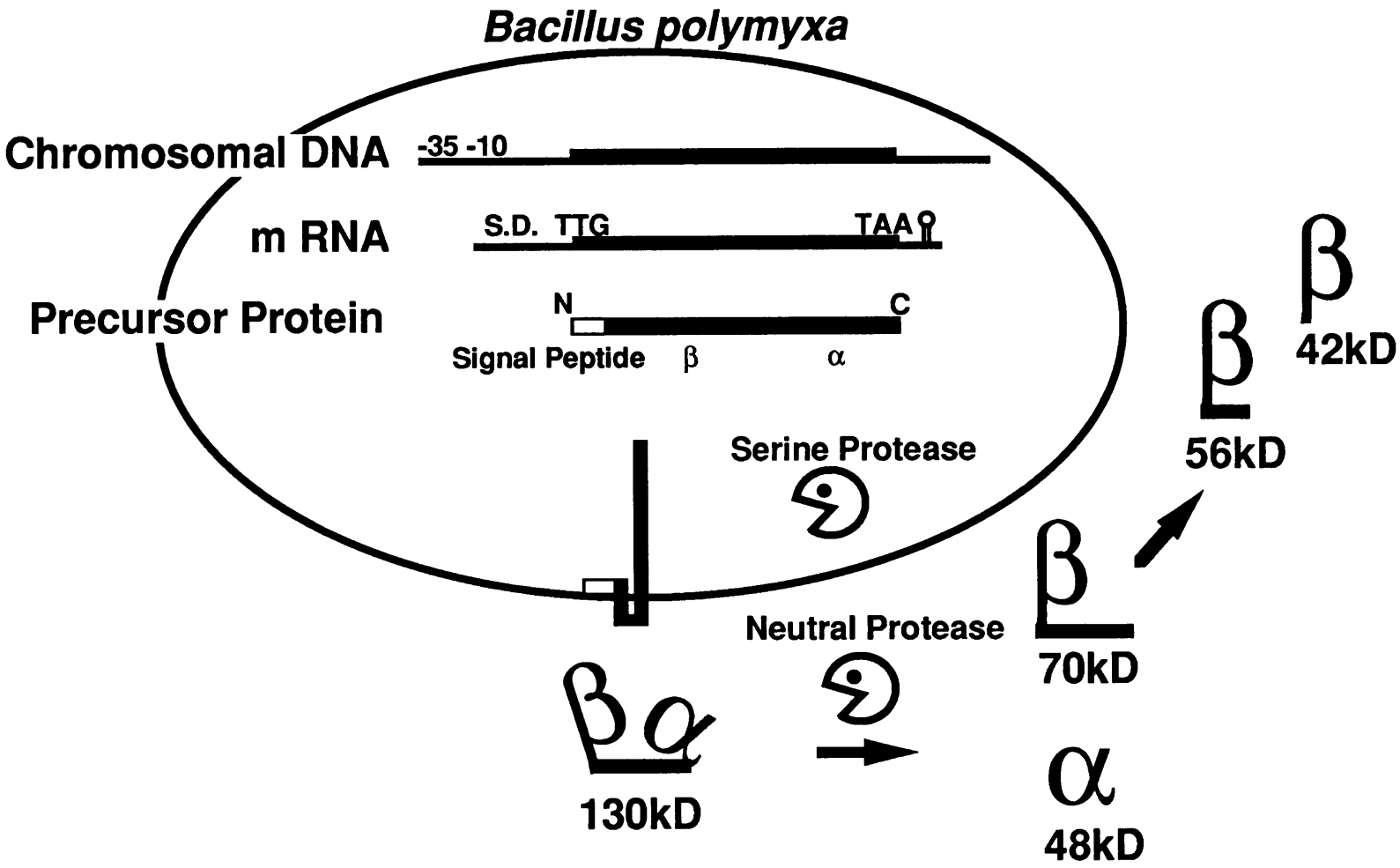

Fig. 7 Production of β- and α-amylases in *Bacillus polymyxa*

Summary

The *Bacillus polymyxa* amylase gene comprises 3,588 nucleotides. The mature amylase comprises 1,161 amino acids with a molecular weight of 127,314. The gene appeared to be divided into two portions by the direct repeat sequence located at almost the middle of the gene. The 5' region upstream of the direct repeat sequence was shown to be responsible for the synthesis of β–amylase since NH_2-terminal amino axid sequence of the major three β–amylases of 70, 56 and 42 kd matched completely the amino adid sequence deduced from 5' region DNA of the amylase construction gene. The 3' region downstream of the direct repeat sequence contains four sequences homologous with ones in other α–amylases such as Taka-amylase A. The 48 kDa amylase isolated from *B. polymyxa* was proved to have α–amylase activity. The amino acid sequences of the peptides generated from the 48 kDa amylase showed complete agreement with the predicted amino acid sequence of the C-terminal portion. The *B. polymyxa* amylase gene was thus concluded to contain in-phase β– and α–amylase coding sequences in the 5' and 3' region, respectively. A precursor protein, a 130 kDa amylase, directed by a plasmid, pYN520, carrying the entire amylase gene had both β– and α–amylase activities. This represents the first report of a single protein precursor in prokaryotes which gives rise to two enzymes, so called 'one gene two enzyme' production system .

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Chapter 2 Structural and Functional Roles of Cysteine Residues of *Bacillus polymyxa* β-Amylase

Introduction

β-amylase (α-1,4-glucan maltohydrolase, EC3.2.1.2) catalyses the liberation of β-anomeric maltose from the non-reducing ends of α-1, 4-glucan and is present in certain bacteria[1-5] as well as in higher plants[6, 7]. Five genes encoding β-amylase have been cloned and sequenced from both prokaryotes and eukaryotes[8-13]. Three highly conserved sequences are recognized among them, and suggested to comprise the active site[13]. β-amylases characterized to date are sensitive to various sulfhydryl-modifying reagents and are considered to contain an SH group essential for the activity[2, 14-18]. The exact role of cysteinyl residues in the enzymatic action, however, remains uncertain, since he derivatization of the SH groups with alkylating reagents might inhibit the catalytic action only by steric hindrance at the active site[19].

Bacillus polymyxa produces multiform β-amylases with approximate molecular masses of 70, 56 and 42 kilodaltons (KDa) and a 48 KDa α-amylase. The amylase gene contains in-phase β- and α-amylase-coding sequences in the 5' and 3' regions, respectively. A precursor protein, a 130-KDa amylase, has both β- and α-amylase activities and is proteolytically cleaved to produce multiform β-amylases and a 48-KDa α-amylase after secretion. The three major β-amylases contain three cysteine residues and have essentially the same enzymatic properties.

To determine whether the *B. polymyxa* β-amylase contains an essential active cysteine residue, we employed site-directed mutagenesis to replace three cysteine residues by serine or valine residues utilizing the DNA fragment encoding the 42-KDa β-amylase. The mutant enzymes were purified and

characterized. The *B. polymyxa* β-amylase contained a disulfide linkage. None of the cysteine residues appear to be essential for catalytic activity.

Materals and Methods

Bacterial Strains, Media, and Transformation L-broth and antibiotic medium 3 (Difco) were used to grow *Escherichia coli* JM103 and *Bacillus subtilis* 1A289 (amyE sacA321 aroI906 metB5; Bacillus Genetic stock Center, Ohio State University, Columbus), respectively. When required, ampicillin and kanamycin were added at 50 and 10 μg/ml, respectively. The transformation of*E.coli* and *B.subtilis* was carried out as described previously. β-Amylase-producing transformants were identified by staining the plates with a 1.7 mM I_2-KI solution.

Plasmids pYN520 containing in-phase β-and α-amylase coding sequences of the *B.polymyxa* amylase gene was described previously. pYN4941 was constructed as follows: a 2.4-Kb *Bam*HI-*Kpn*I fragment containing the β-amylase coding region was isolated from pYN520 and inserted between the *Bam*HI and *Kpn*I sites on pUC118 (Fig. 1). A universal translation terminator, 5'-GCTTAATTAATTAAGC-3' (Pharmacia Fine Chemicals, Piscataway, N.J.) was inserted into the *Eco*RV site after removal of the 0.3-Kb EcoRV fragment on the resultant plasmid. pYN541 was constructed as follows: a 2.1-Kb *Bam*HI-*Eco*RI fragment was isolated from pYN4941 utilizing the *Eco*RI site located 12-bp downstream of the 3' end of the insert and inserted between the *Bam*HI and *Eco*RI sites on pUB110, followed by the transformation of *B.subtilis* 1A289 to kanamycin resistance.

Site-directed Mutagenesis of the β-Amylase Gene The 0.6-kb *Eco*47III-*Eco*T22I and 0.4-Kb *Kpn*I-*Hinc*II fragments on pYN4941 containing the region I and III conserved among β-amylases respectively, were inserted between the

corresponding sites on M13mp19. Single strand M13mp19 DNA containing the insert was subjected to mutagenesis with the aid of the oligonucleotide-directed in vitro mutagenesis system (Amersham Corp.). To confirm that only the desired mutations occurred during the manipulations, the gene from each was sequenced for its entirety by the dideoxy chain termination method. The *Eco*47III-*Eco*T22I or *Kpn*I-*Hinc*II fragment with the confirmed mutations was inserted between the corresponding sites on pYN4941 and used for the transformation of *E.coli* JM103 to ampicillin resistance. To analyze the gene products, the *Bam*HI-*Eco*RI fragment containing the mutated gene was subcloned onto pUB110 as described above and expressed in *B. subtilis.* Mutagenic deoxyoligonucleotides, 5'-CAACGCATAAGAGTGGAGG-3', 5'-GTAGGAGATGACGTCAACATCCC-3', 5'-GACATTTACTAGCCTGGAG-3', 5'- CATCTCAACGCTTAAGTGT-3' and 5'-GTCCTTCCGGGCAATTGCG-3' were synthesized at the Center for Gene Research of Nagoya University and were used to obtain pYN541-C83S, pYN541-C91V, pYN541-C323S, pYN541-H81L and pYN541-E163Q, respectively. pYN541-C-free was constructed as follows: the *Eco* 47III-*Eco*T22I fragment on pYN541-C83S containing the region I was further mutated at codon 91 to produce double mutations, C83S-C91V, followed by insertion in the corresponding region on pYN541-C323S.

Purification of β-Amylases *B. subtilis* 1A289 cells with plasmids containing wild-type and mutant β-amylase genes were grown for 40 h at 28 ℃ in antibiotic medium 3 supplemented with 0.5% soluble starch and 10 μ/ml kanamycin. β-Amylases were purified as described previously with some modifications. The non-binding fractions containing β-amylase on a DEAE-cellulose column were pooled, dialyzed against 10mM acetate buffer (pH 6.0) and applied to a column of CM-cellulose equilibrated with the same buffer. The non-binding fractions containing the enzyme activity were pooled and dialyzed extensively against 100 mM phosphate buffer (pH 7.0) containing 25% ammonium sulfate, followed by fractionation on a phenyl-Sepharose CL-4B

column previously equilibrated with the same buffer. The enzyme was eluted at a flow rate of 18 ml/h from the column with 10 mM phosphate buffer (pH 7.0). The β-amylase-containing fractions were pooled and dialyzed against 50mM phosphate buffer (pH 7.0).

Protein Derivatization The general conditions for protein labeling and isolation of resultant derivatives were as described previously[20, 21]. Wild-type and mutant C91V β-amylases (400 μg each) were labeled at 25 ℃ for 2 h with 4.5 mM N-Iodoacetyl N-(8-sulfo-1-naphthyl) ethylenediamine (IAEDANS) in 75 mM Tris-HCl buffer (pH 8.0) containing 6M guanidine hydrochloride and 4.5 mM EDTA. The derivatized proteins were protected from light to avoid photodecomposition of the fluorophor. This also applied to the analytical procedures described below. The reactions were stopped by adjusting the pH of the reaction mixture to 5 with acetic acid, followed by extensive dialysis against 100 mM Tris-HClbuffer (pH 8.0) and then dialysis against the same buffer containing 1 mM $CaCl_2$.

Protein Cleavage and Peptide Separation Protein derivatives were digested by adding aliquots of 1% (W/V) trypsin at a ratio of β-amylase to trypsin by weight of 50 to 1. After incubation at 37 ℃ for 16 h, digestion was terminated by freezing the reaction mixture and subsequent lyophilization. The resultant peptides were dissolved in 0.1% CF_3COOH in H_2O and separated by HPLC (JASCO, Tokyo, Japan) on a reverse-phase column (Biofine RPC-PO, 4.6x150 mm, JASCO, Tokyo, Japan). Chromatography was performedat a flow rate of 1 ml/min. using a linear gradient of increasing percentages of CH_3CN relative to H_2O while maintaining a constant concentration of 0.1% (V/V) CF3COOH. Programs for rates and extents of increase in CH_3CN were optimized for each particular separation. Absorbance of the eluates were monitored at 220 nm in addition to fluorescence emission at 540 nm due to excitation at 340 mn. Peaks of fluorescence were collected manually and lyophilized. If necessary, samples

were digested further with trypsin and peptides bearing fluorescence were isolated as described above.

Other Analytical Procedures The NH_2-terminal amino acid sequences were determined with a gas-phase sequence analyzer (ABI 477A-120A protein sequencer). The circular dichroism spectrum of each protein was measured at room temperaturewith a JASCO, J-500C spectropolarimeter. The β-amylase activity was determined at 25 ℃ with soluble starch as a substrate. Protein was determined with bovine serum albumin as the standard[22]. The purity of protein was determined by sodium dodecylsulfate-polyacrylamide gel electrophoresis. α-EPG (2, 3-epoxypropyl a-D-glucopyranoside) was gifted from Dr. Y Nitta of the unversity of Osaka Prefuctre.

Results

Construction and Expression of the Mutant β-Amylase Genes

The amino acid sequences deduced from various β-amylase genes were optimally aligned to the *B. polymyxa* sequence. This alignment revealed three highly conserved regions (Fig. 2). The *B. polymyxa* β-amylase contained one additional cysteine residue in region I, which was replaced with a valine residue in the other enzymes. Taking into consideration the sequence similarity, we constructed four mutant genes by means of oligonucleotide-directed mutagenesis. The cysteine codons at 83 and 323 were replaced by a serine codon to construct C83S and C323S, respectively. C91V, cysteine codon at 91 replaced by a valine codon, contained the sequence most homologous to the other enzymes. In C-free, three cysteine codons were replaced by serine codons at codons 83 and 323 and by a valine codon at codon 91. The mutant enzymes were expressed in *B. subtilis* and purified by the same procedure used for the wild-type (Wt) enzyme. The proteins obtained were homogeneous as

judged by SDS- polyacrylamide gel electrophoresis and had approximate molecular masses of 42 KDa.

Identification of Disulfide Bond

To assess whether two cysteine residues at 83 and 91 in the *B. polymyxa* β-amylase form a disulfide bond, both Wt and C91V enzymes were derivatized with a fluorescent label, IAEDANS, under denaturating conditions. Tryptic digestion of fluorescent derivatives followed by high-performance liquid chromatography yielded two and three major peptides bearing the fluorescent label from Wt and C91V proteins, respectively (Fig. 3). Among those five peptides, peptide, C91V-3, was unique and should be generated as the consequence of mutation. Wt-1 and -2 were eluted from a Biofine RPC-PO column at exactly the same retention time as that of C91V-1 and -2, respectively. Upon further digestion of C91V-2 with trypsin, a major peptide, C91V-2', bearing the fluorescent label was eluted at nearly the same position as those of Wt-1 and C91V-1. All the major fluorescent peptides were subjected to amino acid sequencing. The NH_2-terminal amino acid sequences of those peptides are shown along with the deduced amino acid sequence (Fig. 4). Four peptides, Wt-1, Wt-2, C91V-1 and C91V-2' included Cys323, although the NH_2-terminal amino sequences varied because of partial enzymatic cleavage. Peptide C91V-3 had Cys83 at the NH_2-terminus, since the NH_2-terminal amino acid sequence from 2 to 13 was exactly the same as the deduced sequence, although the NH_2-terminus amino acid was not assigned as the consequence of derivatization. Furthermore, the amino acid sequence chemically determined confirmed that Cys91 of the wild type enzyme was replaced by avaline residue in the C91V mutant. Therefore, the differences in the number and the position of modified cysteine residues between Wt and C91V proteins clearly show the presence of a disulfide bond between Cys83 and Cys91 in the wild type enzyme. This further indicates that Cys323 contains a free SH group.

Secondary structures of wild-type and Mutant Enzymes

The circular dichroism (CD) spectrum of each mutant varied depending on amino acid substitutions (Fig. 5), although each protein behaved identically during purification. C91V and C323S revealed essentially the same CD spectrum as that of Wt, whereas the CD spectra of C83S and C-free differed distinctly from that of Wt. The main cause for the gross alteration of the protein structure observed with the latter mutants appeared to be due to the replacement of Cys83 by a serine residue rather than the disruption of the disulfide bond, since C91V retained the structural integrity essentially the same as Wt.

Kinetic Parameters of Wild-type and Mutant Enzymes

Kinetic constants, Km and kcat, were determined from initial rate measurements for hydrolysis of soluble starch (Table 1). The mutants, C91V and C323S, had Km values for this substrate comparable with that of Wt, while the other mutants, C83S and C-free exhibited a marked increase in the Km, approximately 7 and 8 -fold increases, respectively. For all the mutants, the kcat/Km ratio, which reflects the specific activity for soluble starch of the enzyme, was reduced drastically compared to that for Wt; approximately 5-fold for C323S, 20-fold for C91V and 60-fold for both C83S and C-free.

Inactivation of Wild-type and Mutant Enzymes by SH Reagents

To determine whether substitution of cysteine residues has any effect on inactivation, the mutant enzymes as well as the Wt enzyme were subjected to modification by SH reagents such as pCMB, NEM and DTNB and then assayed for enzyme activity (Table 2). Reaction of pCMB with enzymes resulted in the complete inactivation of Wt, C83S and C91V. Both C323S and C-free retained full activity under the same condition. Of the mutant enzymes, only C91V was

inactivated by all three SH reagents, although DNTB modification caused only a partial inactivation of its activity. Assuming that a loss of the enzyme activity is due to the chemical modification of cysteine residues, all these data clearly indicate that alkylation of Cys323 by pCMB causes a loss of the activity as a consequence of introduction of steric bulk. Therefore, none of the cysteine residues participate in the catalytic reaction.

His residues were not active site in β–amylase.

To norrow down the catalytic site of β–amylase, the terminal translation linker were inserted at the various restriction site on β–amylase. The recombinat which produced trancated proteins consisted of 248 amino acids represented the halo when the assay with KI-I_2 was carried out. Within these amino acids, there is only one histidine residue, which is located at 81 on the basis of NH_2-terminal amino acid. By using site direcded mutagenesis, His 81 was replaced by leuicine. The resultant β–amylase, H81L, possesssed a detectable β–amylase activity, more than 1/100 compared with that of Wt (Table 3). This indicates that histidine residues do not participate the β–amylase catalytic reaction.

Glu 163 involves the β–amylase catalytic reaction.

According to affinity labering experiment data by Nitta et al., one of glutaimic acid residues involved the catalytic reaction. The mutant E163Q, in which Glu163 was replaced with Gln163 was constructed and induced into B. sutilis. The purified E163Q exhibited barely detectable activity for soluble starch, approximately 10^{-4} of the specific activity of the wild-type enzyme (Table 3). This result confirmed that the Glu163 was essential for the β–amylase catalytic reaction.

Discussion

β–Amylase from diverse origins have been shown to be sensitive to inactivation by sulfhydryl reagents[2, 14-18]. To assess the role of cysteine residues in the catalytic activity, we constructed four mutants of the *B. polymyxa* β–amylase and analyzed effects of the mutations on protein structure and on enzymatic properties in combination with protein-modifying reagents. The *B. polymyxa* β–amylase contains three cysteine residues, two of which, Cys83 and Cys91, were shown unambiguously to form a disulfide bond between Cys83 and Cys91. This, in turn, indicates that Cys323 contains a free SH group. No disulfide bonds have been identified in β–amylases from other origins. Mutations to prevent disulfide formation are expectedto induce some perturbations in tertiary structure. The mutation of Cys83 to Ser83 resulted in a gross structural alteration as judged by the CD spectrum, while the mutation of Cys91 to Val91 had no apparent effect on the structure. Val91, a well conserved residue among β–amylases might be able to restore, to some extent, the structural perturbations induced by disruption of a disulfide bond.

None of the cysteine residues were shown to participate directly in the catalytic reaction based on the enzymatic properties of mutants. This is also the case with soybean β–amylase as described below. The mutant C323S, however, had a rather low specific activity, approximately 20 % of that of the wild-type enzyme, suggesting a structural function for Cys323. The thioalcohol hydrogen of Cys323 might participate in formation of a hydrogen bond that, if absent, subtly alters the enzyme structure and lowers the specific activity of C323S.

The soybean β–amylase has been well characterized and shown to contain two types of SH groups: highly reactive SH groups (Cys82, Cys208 and Cys343) and essential SH groups (Cys95, Cys288 and Cys448)[23, 24]. None of the cysteine residues participate either in catalysis or in substrate binding[19]. Cys95 has, however, been suggested to be located close to the substrate binding site in

the active site region, since modification of Cys95 results in the loss of the activity[24]. Interestingly, these three residues, Cys95, Glu186 and Cys343 in the soybean β–amylase, are included in the highly conserved regions among β–amylases.

A few examples were revealed completely that catalytic reaction mechanism of enzyme . In particular, it is well known the catalytic triard (Ser-His-Glu or Asp) of serine protease[25]. Histidine redidue possessed higher reactivity among L-amino acids. His 81 is completely conserved in β-amylase sequence derived from various spieces, and located within three conservative regions (Fig. 6). I predicted that His81 would be an important residue in hydrolysis of starch. However, H81L was at a detectable level in β-amylase activity. Even if β-amylase is classfied to hydrolase, such as serine protease, histidine residue do not involve the catalytic reaction.

The Glu163 residue might participate in the catalytic reaction, since α-EPG (2,3-epoxypropyl α-D-glucopyranoside) caused the inactivation of the enzyme activity as a consequence of esterification of Glu 186 in soybean β-amylase[8]. The *B. polymyxa* β–amylase contains Glu at position 163, equivalent to Glu186 in the soybean enzyme. This result demonstrated that the Glu 163 in the consereved sequence was essential residue for catalytic reaction. Further works on β-amylase catalytic mechanism has been progressed by Y. Nitta, his coworkers and us.

Table 1 Kinetic parmeters of various β–amylases with soluble starch as a substate

	k_m (mg/mL)	k_{cat} (/sec)	k_{cat}/k_m(mL/mg s)
Wt	0.071 ± 0.014	128 ± 2	1820
C83S	0.460 ± 0.007	14.5 ± 0.1	31.5
C91V	0.099 ± 0.021	9.5 ± 0.1	96.6
C323S	0.118 ± 0.012	40.5 ± 4.0	343
C-free	0.549 ± 0.005	16.2 ± 0.2	29.5

Kinetic parameters of various β–amylases with soluble starch as a substrate. Initial reaction rates were determined at 25 ℃ as described in Materials and Methods. Kinetic data were fitted to the Michaelis-Menten relationship to evaluate k_m and V_{max}. Turnover number (k_{cat}) was calculated based on the amount of enzyme used.

Table 2 Effects of various sulfhydryl group reagents on the activity of β–amylases

	pCMB	NEM		DTNB	
	40mM	2 mM	10 mM	1 mM	5 mM
Wt	< 1	112	95	94	95
C83S	< 1	90	98	103	100
C91V	< 1	25	< 1	46	53
C323S	115	86	97	100	100
C-free	103	92	97	102	99

Effects of various sulfhydryl group reagents on the activity of β–amylases. Enzyme (200nM) was incubated at 4 ℃ for 60 min. in 50mM phosphate buffer (pH 7.0) with various sulfhydyl group reagents at concentrations indicated, followed by measurement of the activity at 25 ℃ with soluble starch as a substrate.

Table 3 The contribution of His 81 and Glu 163 to β-amylase catalysis

	Specific Activity (U/mg)	Activity Ratio
Wt	142	1
H81L	1.79	1.26×10^{-2}
E163Q	0.0151	1.06×10^{-4}

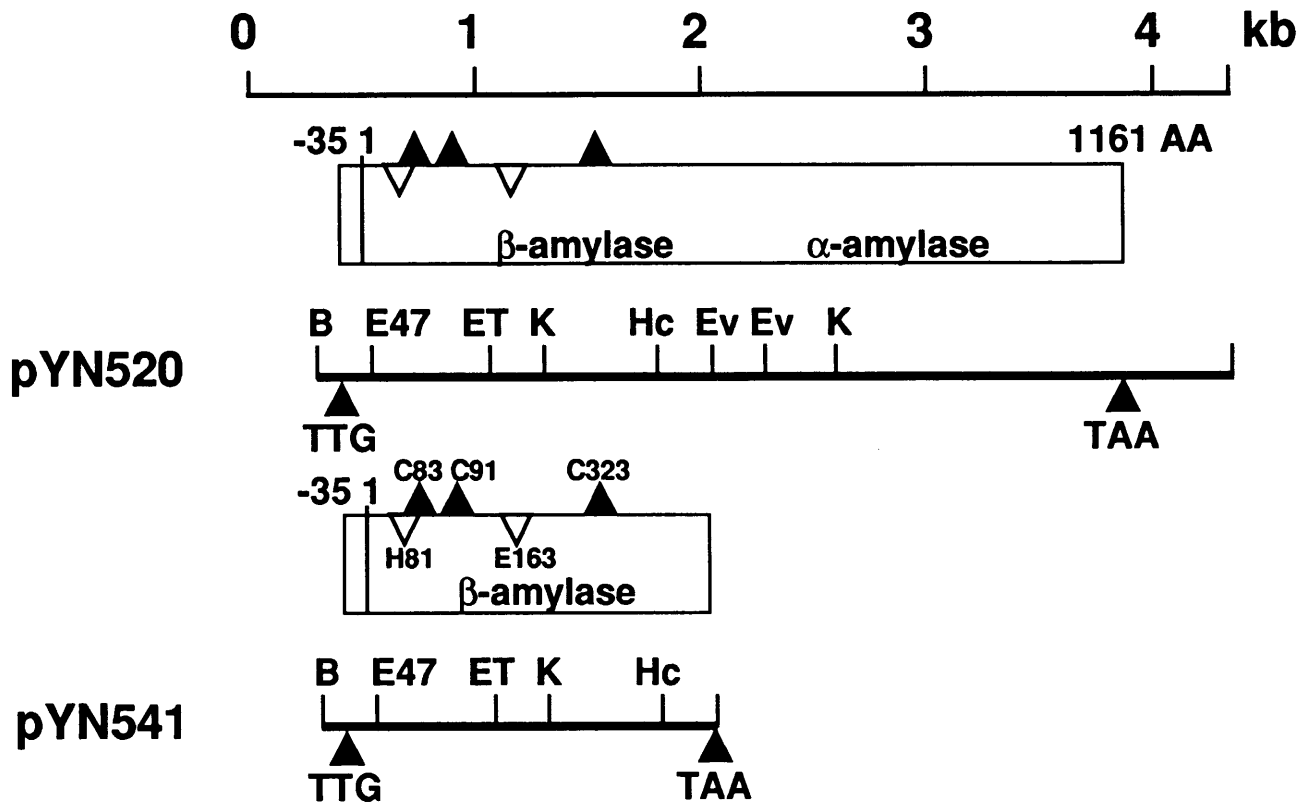

Fig. 1. Diagramatic presentation of the β-amylase gene from *B. polymyxa*. The upper half represents the correlation of the β- and α–amylase coding regions on plasmid pYN520, while the lower half shows the amylase gene on plasmid pYN541 used. The start codon (TTG) and stop codon (TAA) are also indicated. Abbreviations for restriction enzyme sites; B, *Bam*HI ; E47, *Eco*47III ; ET, *Eco*T221 ; EV, *Eco*RV ; Hc, *Hinc*II ; K, *Kpn*I.

```
B. polymyxa          77 IISTHKCGGNVGDDCNIPLPSW 98  159 GPSGELRYPSYYP 172 319 LTFTCLEMSDS 329
                        * *** *******  *** ***          *********** *          ******* **
C.thermosulfurogenes 77 IMSTHACGGNVGDTVNIPIPSW 98  159 GPSGELRYPSYNP 172 320 MTFTCLEMDDS 330
                        *** * ******* ****** *          ** ******** *           ****** **
Soybean              89 IMSFHQCGGNVGDIVNIPLPQW 110 182 GPAGELRYPSY-P 193 339 LNFTCLEMRDS 349
                        ************* ********          ***** ***** *          **** ****
Barley               87 IMSTHQCGGNVGDDCNIPLPSW 108 180 GPAGEMRYPSY-P 191 337 ITFTCAEMSDL 347
                        *********** *** ******          * *** ***** *          **** ****
Sweet Potato         90 IMSTHQCGGNVADDCFIPLPSW 111 184 GAAGELRYPSY-P 195 341 LTFTCLEMSDS 351

Consreved               I-S-H-CGGNV-D---IP-P-W          G--GE-RYPSY-P
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Fig. 2. Conserved amino acid sequences in three homologous regions (I, II, and III) of β-amylases. Identical residues are denoted by asterisks between the sequences. The amino acids are numbered from the NH2-terminus of each mature enzyme. Closed triangles indicate positions of cysteine residues of the *B. polymyxa* β-amylase. Amino acid sequences of the following amylases were aligned to maximize the homologies; *B. polymyxa*, *C. thermosulfurogenes*, Soybean, Barley, Sweet Potato. Conserved amino acids among five β-amylases are shown at the bottom.

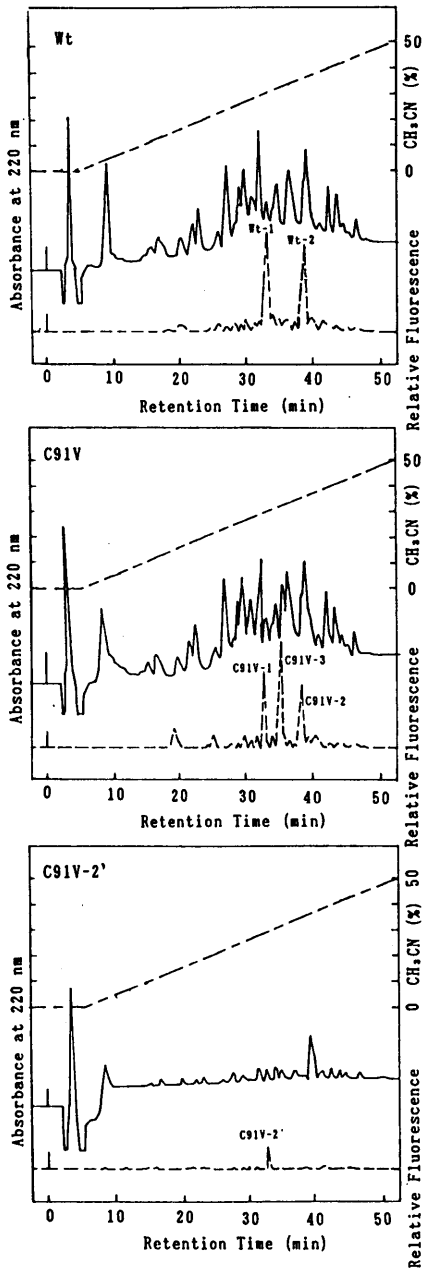

Fig. 3. Separation of fluorescent peptides from the tryptic digests of Wt and C91V derivatized with IAEDANS. The tryptic digests were fractionated on a reverse phase HPLC column.

```
1                        25                        50                        75
AVADDFQASVMGPLAKINDWGSFKKQLQTLKNNGVYAITTDVWWGYVESAGDNQFDWSYYKTYANAVKEAGLKWV
      83      91      100                       125                       150
PIISTHKCGGNVGDDCNIPLPSWLSSKGSADEMQFKDESGYANSEALSPLWSGTGKQYDELYASFAENFAGYKSI
       *************
       C91V-3
                       175                       200                       225
IPKIYLSGGPSGELRYPSYYPAAGWSYPGRGKFQAYTETAKNAFRTAMNDKYGSLDKINAAWGTKLTSLSQINPP
                       250                       275                       300
TDGDGFYTNGGYNSAYGKDFLSWYQSVLEKHLGVIGAAAHKNFDSVFGVRIGAKISGLHWQMNNPAMPHGTEQAG
                   ***********             **********   *********************
                   Wt-2                    C91V-2'      Wt-1
                                                        ********************
                                                        C91V-1
                     323                       350                       375
GYYDYNRLIQKFKDADLDLTFTCLEMSDSGTAPNYSLPSTLVDTVSSIANAKGVRLNGENALPTGGSGFQKIEEK
****
                       400                       425
ITKFGYHGFTLLRLINNLVNNDGSPTGELSGFKQYIISKAKPDNNGGTGNKVTIYYKKGFNVYYIHY···
```

Fig. 4. Alignments of amino acid sequences of tryptic peptides bearing fluorescence and the deduced sequence of β-amylase. Wt-1, Wt-2, C91V-1, C91V-2' and C91V-3 were isolated as fluorescent peptides from the tryptic digests (Fig. 4). Numbers indicate amino acid positions from the amino terminus. Asterisks denote amino acids identical to the deduced amino acid. X indicates amino acid not assigned.

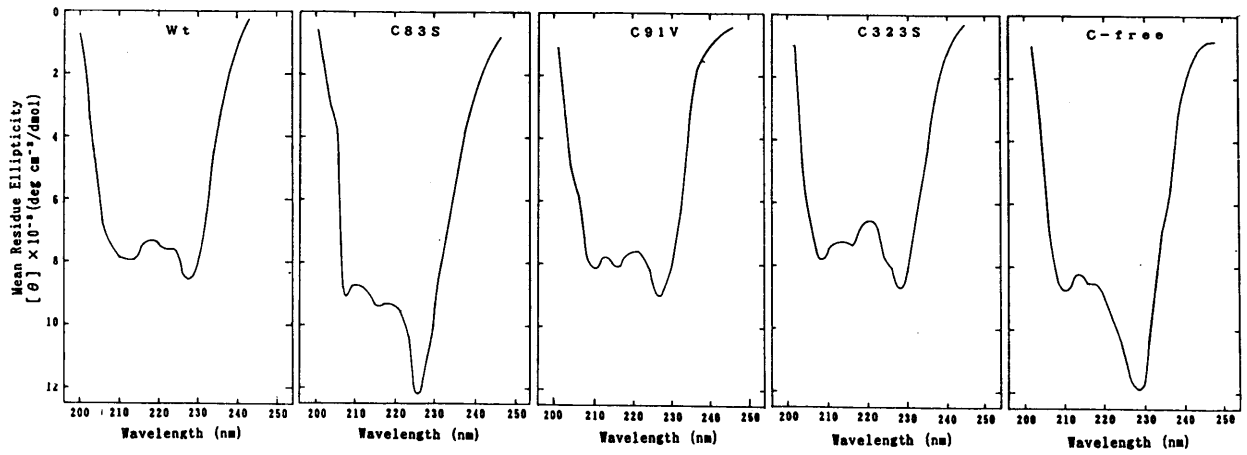

Fig. 5 Comparison of far ultraviolet CD spectra among β-amylases. The CD spectrum of each protein was measured in 50 mM phosphate buffer (pH 7.0) using 200 mg of protein per ml.

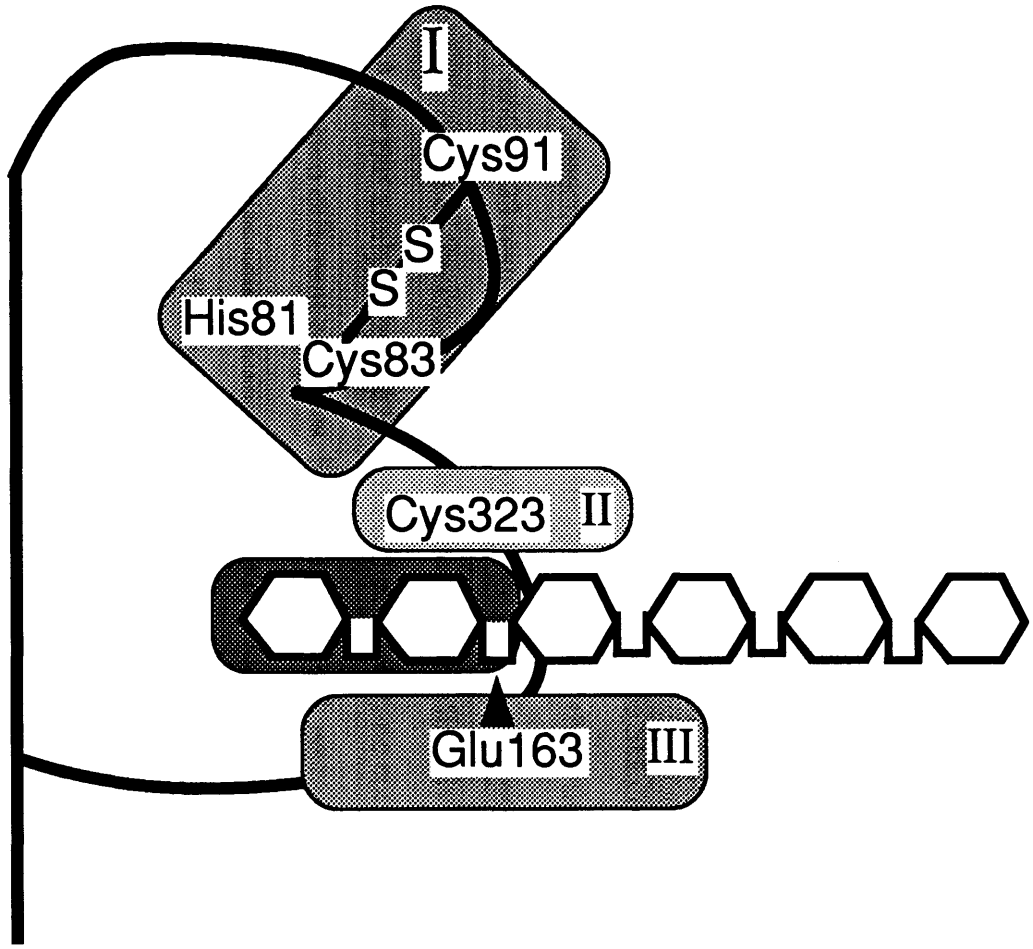

Fig. 6 The position of amino acid residues in *Bacillus polymyxa* β-amylase

Summary

Bacillus polymyxa β–amylase contains three cysteine residues at positions 83, 91 and 323, which can react with sulfhydryl reagents. To determine the role of cysteine residues in the catalytic reaction, cysteine residues were mutated to construct four mutant enzymes, C83S, C91V, C323S and C-free. Wild type as well as mutant forms of the enzyme were expressed in, and purified to homogeniety from *B. subtilis*. A disulfide bond between Cys83-Cys91 was identified by isolating tryptic peptides bearing a fluoresent label, IAEDANS, from wild type and C91V enzymes, followed by amino acid sequencing. Therefore, only Cys323 contains a free SH group. Replacement of cysteine residues with serine or valine residues resulted in a significant decrease in the kcat/Km value of the enzyme. C323S containing no free SH group, however, retained a high specific activity, approximately 20 % of the wild type enzyme. Alkylation of Cys323 resulted in a loss of the activity as a consequence of steric interference. None of the cysteine residues participate directly in the catalytic reaction. After the analysis by the trancated β-amylse gene construction, I predicted that two different residues, His81 and Glu163, were candidate residues for β-amylase catalytic center. The relative β-amylase activity of H81L and E163L revealed histidine residues were not essential for the catalytic reaction, but Glu163 was one of active center

Nomenclature

The abbreviations used are: pCMB, p-chloromercuribenzoate; DTNB, 5, 5'-dithiobis (2-nitrobenzoate); α-EPG, 2, 3-epoxypropyl α-D-glucopyranoside; IAEDANS, N-iodoacetyl N-(8-sulfo-1-naphthyl) ethylenediamine NEM, N-ethylmaleimide.

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Chapter 3 Enhancement of Peroxidase Production and Excretion from Horseradish Hairy Roots by Light, NaCl and Peroxidase-Adsorption *in Situ*

Introduction

Plant cell produces useful complicated chemicals (*e.g.*, medicinals, flavors, cosmetics, pigments, fragrances, and pesticides). The chemicals are often localized at specific tissues, and some types of them are produced and stored in roots. Hairy root, transgenic plant generated by the integration of T-region on Ri plasmid in *Agrobacterium rhizogenes* represents root in morphology. The adventage of the hairy root propeties is that the hairy roots which have a higher contents of chemicals than that of original root can be induced. Hence, the improvement of hairy roots culture will shed light on biochemials production.

To develop an effective production system, we have studied alternative means of stimulating production and excretion of a useful enzyme from horseradish (*Armoracia rusticana*). Peroxidase (EC 1.11.1.7.) is present in higher plants, and widely used in the colorimetric analysis of biological materials. Horseradish peroxidase consists of more than 30 multiforms determined by isoelectric points[1,2]. Shannon *et al.*[3] isolated seven peroxidases among many isozymes from horseradish and characterized their properties. A study of peroxidase localization in the onion root indicated that this enzyme seemed to be present in the cell wall, in the plasmalemma, in the the Golgi apparatus cisternae and vesicles, in the endoplasmic reticulum and so on[4]. Despite many investigations about peroxidase, the localization of the peroxidase isozymes is not completely assigned.

A previous study reported that small amounts of extracellular peroxidase from the hairy root are found in medium and that low levels of peroxidase are excreted from the cells when the medium is supplemented with polypeptone[5].

This chapter shows whether illumination with fluorescent white light, addition of NaCl and product adsorption *in situ* would lead to significant increase in extracellular peroxidase production, and that the feasibility of using the technique to facilitate peroxidase recovery from horseradish hairy roots was examined.

Materials and Methods

Plant cell culture and culture medium Horseradish hairy root cells induced by the leaf-disc method[6] were used, and maintained by regular subculture in the dark, every 3 weeks, on hormone-free Murashige and Skoog (MS) medium supplemented with 2 % (w/v) sucrose. For the culture experiments, the roots were grown for 14 days at 25°C and then were transferred aseptically into 100-ml Erlenmeyer flask containing 40 ml aliquot of the required medium (2g/l fresh weight of roots). Temperature during the culture was kept at 25°C. In the light condition, the period of illumination with fluorescent white light (ca. 3500 lux) was 14 hours a day. On-line estimation of cell concentration during the culture was based on conductometry as reported previously[7]. Dry cell mass was gravimetrically measured after drying the roots at 60°C for 24 hours.

Peroxidase assay. Peroxidase activity was determined at 25°C with *o*-aminophenol as a substrate[8]. For extraction of intracellular peroxidase, the hairy root cells were disrupted in 10 mM phosphate buffer (pH 6.0) using a mortar with a pestle on ice. The supernatant was obtained by centrifugation (17000 x g, 10 min) at 4°C and used as a crude enzyme solution.

Configuration of adsorption system The experimental reactor configuration is illustrated in Fig. 1. Cultures were performed at 25°C in a flask with a working volume of 40 ml of MS medium containing 50 mM NaCl. These cultures were kept on a gyratory shaker (100 rpm) in the light condition with 14 hours light / 10 hours dark cycle. Aeration was continuously supplied at flow rate of 3 l/h. The hydrophobic adsorbent resin (DAIYAION HP-20, Mitsubishi Kasei Co., Ltd.) was packed in a column with a bed volume of 20 ml, and a working volume of 7 ml. The columns were equilibrated with 7 ml of MS medium supplemented with 2 % sucrose prior to use. Adsorption operations were carried out by passing the reactor medium through the column, using a peristaltic pump. The initial 7 ml, composed mostly of the fresh medium, was drained out from this circuit and 40 ml volume of culture broth was recycled into the flask to reuse as medium successively. At the last circulation, the liquid in the column was flushed and substituted with fresh MS medium supplemented with 2 % sucrose derived from the reservoir.

Scanning electron microscopy For scanning electron microscopy, the cells were fixed with 2 % glutaraldehyde in 10 mM phosphate buffer (pH 6.0) for 2 h. After this, they were dehydrated with an increasing acetone series and then soaked in isoamyl acetate for 2 h. After critical-point drying and sputtering, micrographs were taken using a Hitachi S-570 scanning electron microscope.

Results

Effects of light and NaCl on cell growth and peroxidase production

Figure 2 provides growth data from horseradish hairy root in the dark and light conditions and also the effect of various concentrations of NaCl on cell mass and peroxidase production in the hairy root cultures. Illumination with the light enhanced final cell mass in the medium with low concentration of

NaCl, while at higher NaCl concentrations (50 -500 mM), the light effect on cell growth was not significant for the final cell mass. Light-grown culture increased approximately 2-fold peroxidase production compared with that from dark-grown culture.

Maximum cell mass decreased with addition of NaCl to the medium. The cell mass was almost the same in the presence of low concentration of NaCl (20mM) and without NaCl. On the other hand, the cell mass decreased when the NaCl concentration was above 50 mM and a severe growth inhibition was observed at higher NaCl concentration (500 mM). NaCl concentration from 0 to 100 mM did not have much effects on enhancement of peroxidase productivity. However, addition of have NaCl to the dark- and light-culture enhanced peroxidase excretion into the medium. In the light condition, peroxidase activity of the culture containing NaCl increased drastically compared with culture without NaCl; approximately 2.4-fold for the culture medium containing 50 mM NaCl and 3.4-fold for the culture medium containing 100 mM NaCl at day 34.

Morphological analysis of hairy root

Surface of the hairy root cells at the late logarithmic growth phase was examined by scanning electron microscope. Figure 3A shows hairy root in the dark condition. In this case, the surface appeared to be rather smooth. In the light condition, the root surface looked wavy and wrinkled (Fig. 3B). Diameter of the light-grown hairy root was approximately 3 times as wide as that of the dark-grown root. However, the light-grown and the dark-grown cell mass were comparable. Therefore light effect was likely to expand cells or the interspace between the cells. Fig. 3C shows the morphology of the hairy root grown in the light condition with 50 mM NaCl. Needle-like tissues were detached from the root surface. These detachments seem to contribute to extensive peroxidase excretion. Microscopic examination of the samples from

different parts of the hairy root showed different microscopic morphologies. The detachments were not observed at the apical meristem on NaCl-treated roots (Fig. 3D). The older cells apart from the apical meristem tended to be more susceptible to NaCl stress.

Cultivation in combination with adsorption

Preliminary studies have shown that repeated batch cultivation improved growth rate and peroxidase productivity compared with batch cultivation (data not shown). To prevent peroxidase degradation during the culture, excreted peroxidase should be recovered from the medium periodically. Recovering of peroxidase from the medium will also enhance the enzyme excretion by maintaining its concentration in the medium at a low level. As a consequence, we tested the cultivation in combination with the resin adsorption. The adsorption operations were carried out at each several days. The results are shown in Fig. 4. The culture was performed with MS medium supplemented with 2 % sucrose and 50 mM NaCl in light condition. When peroxidase activity in the medium was kept below the maximum level of the batch culture, the productivity after 20 days retained above 2.8 U/ml-day. Thus, the stepwise adsorption gave rise to a significant increase in the total amount of peroxidase produced. In addition, the adsorption caused slightly higher final cell mass. Final total peroxidase activity reached approximately 70 U/ml level.

Discussion

A major limitation in continuous production of proteins and secondary metabolites from plant cells is that desirable products are stored intracellularly. Several methods have been tested to overcome this limitation and to excrete products in the medium. These methods include temperature shift[9], use of

agents for permeablizing[10,11]. Even if such strategies succeeded in excreting products, they are usually detrimental to cell viability and overall productivity. Other strategies are required to improve overall yield productivity. Kim *et al.*[12] has reported that light plays an important role in product formation, but unfortunately light suppressed the secretion of secondary metabolites from plant cells.

The results in the present paper clearly show that the combined use of illumination with light, NaCl addition and the adsorption of excreted peroxidase leads to peroxidase overproduction. To release peroxidase in the medium, it is necessary to alter cell membrane and cell wall properties. Taya *et al.* observed that polypeptone enhanced intracellular peroxidase activity, but unfortunately was not effective for excretion. Taking the polypeptone content into consideration, the enhanced production was attributed to NaCl present in the polypeptone. In this study, NaCl was found to induce peroxidase excretion into the medium, rather than to activate peroxidase synthesis by expression of peroxidase gene (Fig. 2). In contrast, light enhanced peroxidase content in the cell, but not peroxidase excretion.

Excretion of desirable products is sometimes difficult to achieve without damaging cells. Microscopic observations on the light-grown cultures supplemented with NaCl revealed that the hairy root surface in the late stage of the cultivation was altered (Fig. 3). This alteration, the tissue detachment on cell surface, seemed to be correlated with decrease of cell viability, which results in leakage of intracellular peroxidase into the medium.

Some of the peroxidases are known to be associated with the cell wall[13]. Only specific proteins were excreted as the cells grew, which was measured by sodium dodecylsulfate-polyacrylamide gel electrophoresis (data not shown). Assuming that there was no extensive leakage of intracellular protein after the late growth phase, the detachments would make a large contribution to the cell-

wall associated peroxidase excretion, since the amount of excreted peroxidase depended on cell surface area in contact with culture medium.

On the other hand, the morphology at the apical meristem of the roots looked normal, as shown in Fig. 3D, which indicates that the roots possessed good viability. It is unclear whether the cause of increased peroxidase activity in the medium was due to ionic strength or specific ions. Further work is now in progress to clarify the excretion mechanism.

Enhanced enzyme production was observed during cultivation in combination with the resin adsorption of peroxidase (Fig. 4). The adsorption seems to be useful to retain peroxidase production and to prevent peroxidase degradation. Metabolic by-products which inhibit growth of root cells may also be adsorbed, since the final cell mass with the adsorption operations was 1.3-fold higher than that in the batch culture (Fig. 4). Nevertheless, the substrates required for the cell growth were not removed by the adsorption operation, which was checked by another experiment without the root cells. Regulation of peroxidase concentration at a low level in the medium by the adsorption led to the stimulation of enzyme excretion, thereby providing a large concentration gradient of peroxidase across the cellular membrane.

I have attempted to determine favorable conditions to elute the peroxidase from the resin. The best product recovery was 85 % when we used 80 % cold acetone as elution agent (data not shown). For effective production of peroxidase from plant cell culture, a more efficient elution agent should be developed.

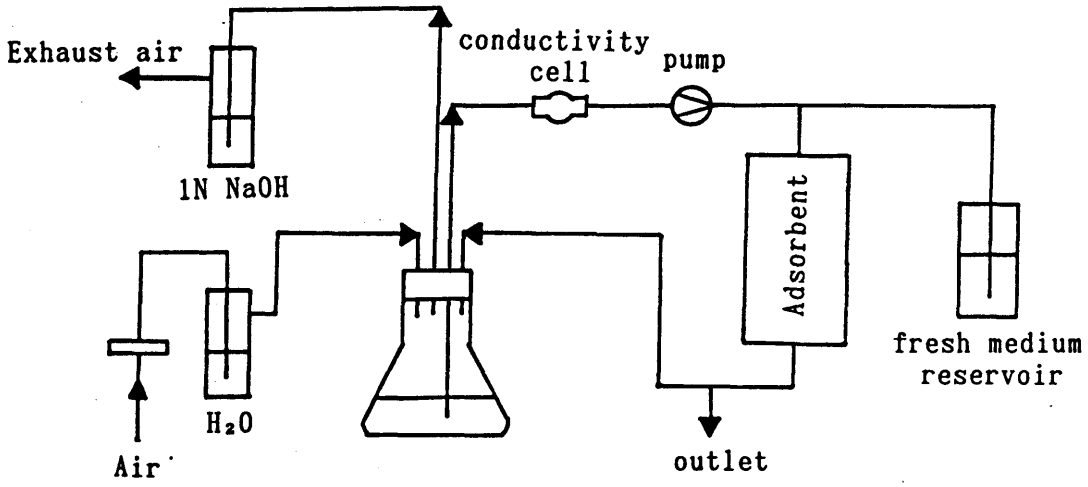

Fig. 1 Experimental set-up for operation of peroxidase adsorption.

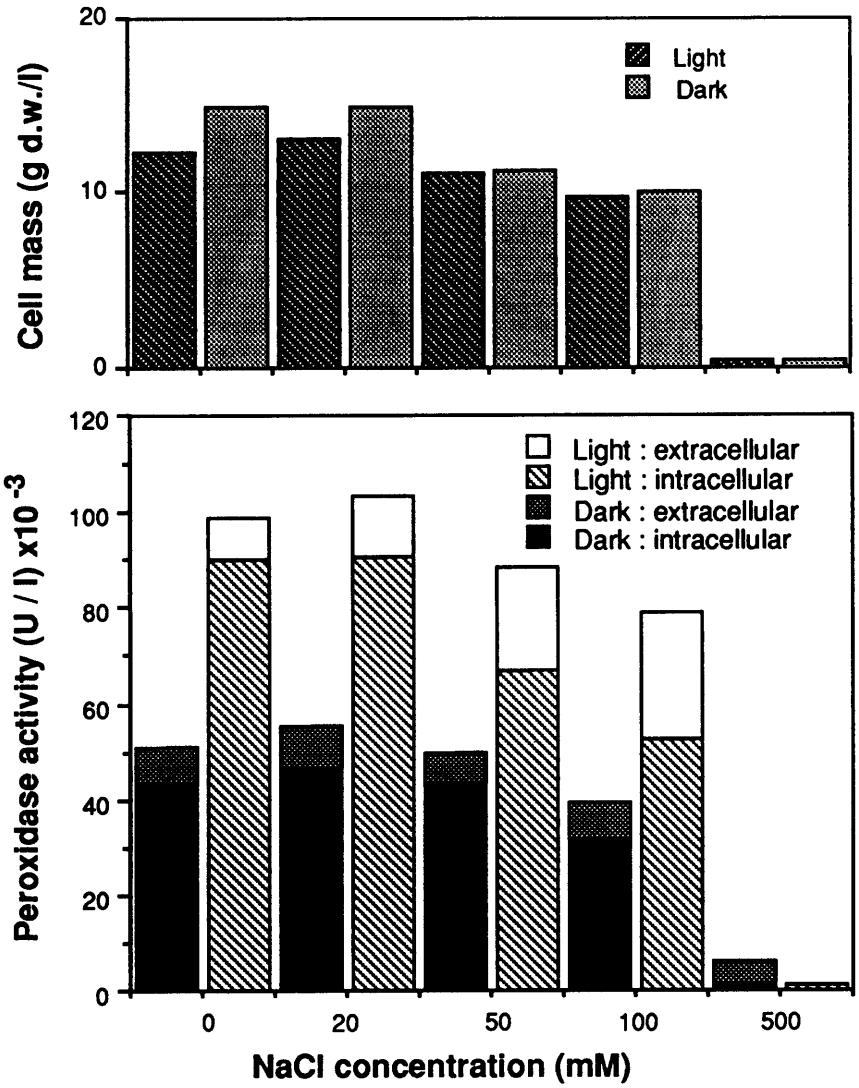

Fig. 2. Effect of NaCl and light on cell mass, peroxidase production and excretion from horseradish hairy roots at the 34th day. The upper half represents the cell mass grown in culture with various NaCl concentrations. The lower half shows peroxidase activity of horseradish hairy roots.

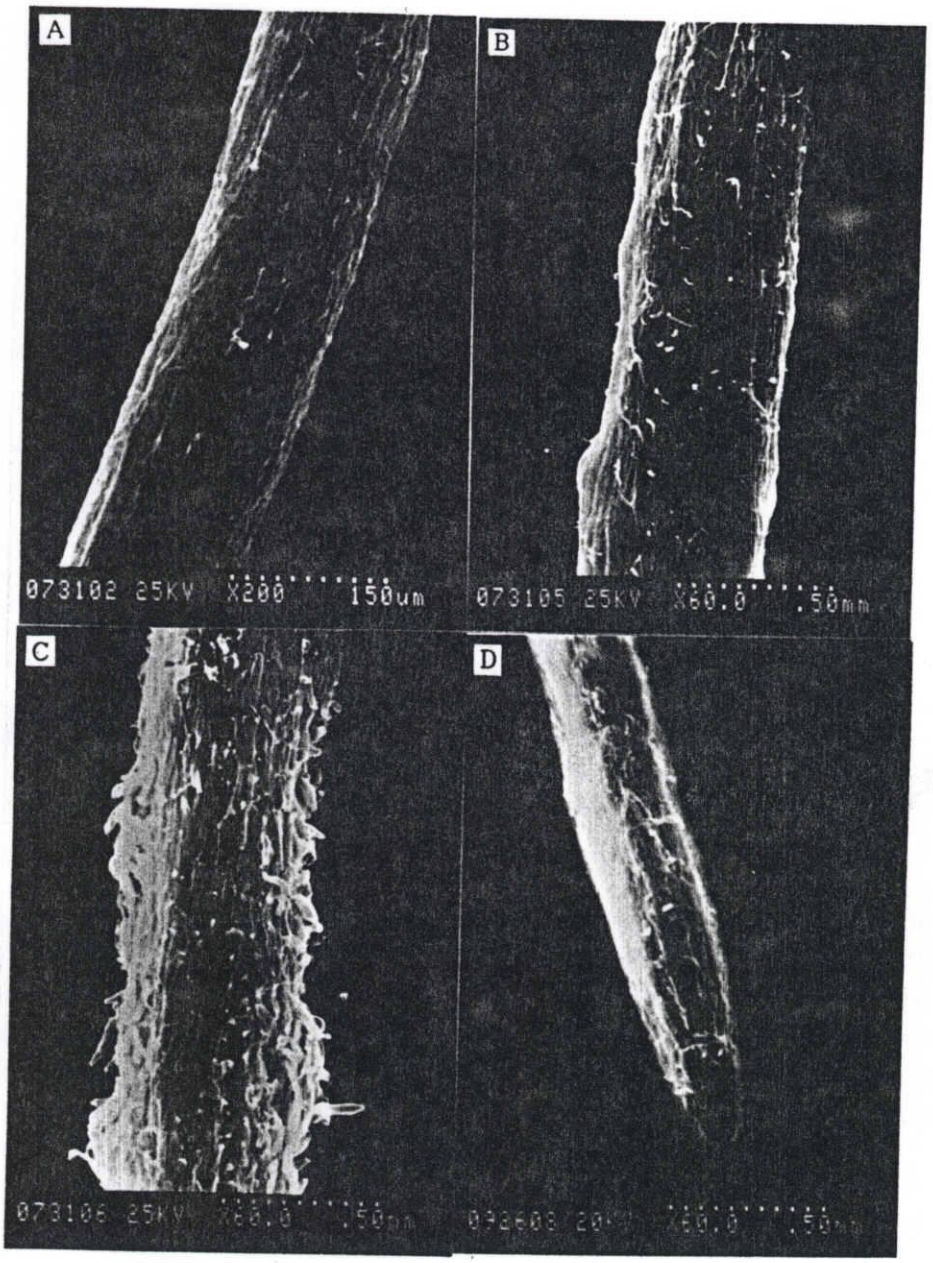

Fig. 3. Scanning electron micrographs of surface of the horseradish hairy roots grown for 30 days in various conditions. A, Dark-grown control hairy root; B, Light-grown hairy root; C and D, Light-grown hairy root treated with 50 mM NaCl.

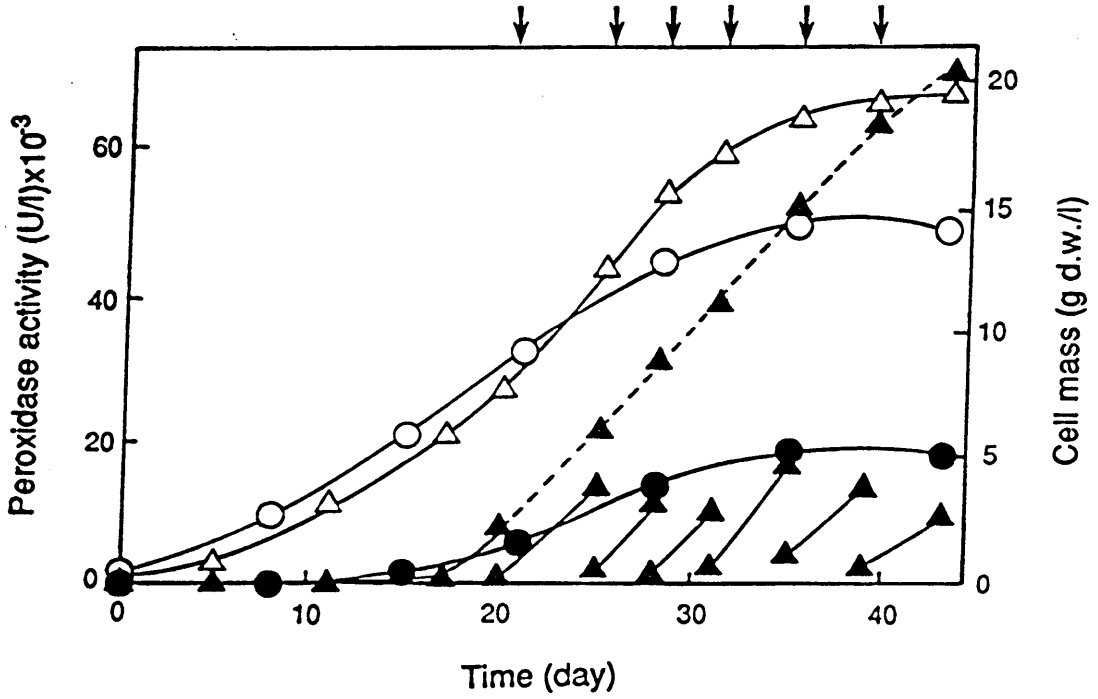

Fig. 4. Enhancement of extracellular peroxidase production on hairy root culture in combination with the resin adsorption. Peroxidase activities in the medium supplemented with 50 mM NaCl and without NaCl addition are indicated by closed triangles and circles, respectively. The total peroxidase activity estimated from each adsorption recovery is shown by a dotted line. Cell mass are denoted by open triangles (addition of 50 mM NaCl) and open circles (control). The adsorption operations are indicated by arrows.

Summary

Effects of light, NaCl and adsorption *in situ* on production and recovery of peroxidase from horseradish hairy root cell are reported in this chapter. Illumination with fluorescent white light increased peroxidase content in the cell 2-fold. The treatment of NaCl stimulated excretion of peroxidase from the cell, and also it was subject to alteration in morphology without serious influence on proliferation. Adsorption of peroxidase from the extracellular medium with hydrophobic resin greatly enhanced release of peroxidase. For a 43-day culture period, 70 U/ml of peroxidase was produced compared to 5 U/ml without special treatments. These results suggest that production and product recovery from plant cells can be greatly improved by application of the combined treatments.

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Chapter 4 Plant Hairy Root Growth and Metabolism and High Cell Density in Fed-batch Culture on Monosaccharide Medium

Introduction

For efficient production on the industrial scale, high cell density culture is required. The optimization of the fermentor-scale plant cell culture requires knowledge of substrate requirements and utilization rates. This chapter shows the fed-batch culture for a large amount of plant cells using monosaccharide and the estimation on cell yield and maintenance enegy.

In general, sucrose has been used as a carbon source for plant cell culture[1]. Extracellular invertase hydrolyzes sucrose to monosaccharides, *i.e.* glucose and fructose. Invertase is excreted into the medium during plant cell growth and the monosaccharide concentrations change with cultivation time. Therefore, control of monosaccharide concentrations is very difficult. Taking this problem into account, as far as plant cells could consume the monosaccharides, these saccharides should be a more interesting carbon source and the control of carbon source would become easier.

To obtain high density culture of plant cells, the culture condition should be maintained at the optimum level. From the view point of process development, I am interested in characterizing carbon utilization of hairy root in liquid culture to evaluate metabolic energy costs[2]. Since the proliferation of hairy roots occurred only at the apical meristem and was expressed by a linear growth, the balance equation for hairy growth should be used to determine the values of maintenance coefficient and cell yield under carbon-limited culture. The aim of our study is to develop a monosaccharide medium on which carrot hairy root cells would grow to a high cell density. I determined the suitable medium composition for fed-batch culture, in particular its dependency on

monosaccharides for cell growth prior to bioreactor culture. Then, I examined the possibility of reaching a high cell density culture of carrot hairy roots with mono- and di-saccharide carbon sources in a turbine-blade reactor. In this chapter, after the preferential monosaccharide on *Ajuga* hairy root growth was determined, fed-batch culture was carried out with the monosaccharide. On the basis of the data, a relationship between cell growth using monosaccharide, instead of mixed saccharides derived from sucrose, and its consumption was evaluated.

Materials and Methods

Plant materials and cultivation Hairy root induced by infection with *Agrobacterium rhizogenes* A4 from carrot was used[3]. *Ajuga* hairy root cell was a gift from Dr. N. Tanaka (Daicel Chemical Industries Co., Hyogo)[4]. The hairy root was maintained by regular subculture under dark conditions, using hormone-free Murashige-Skoog (MS) medium[5] containing 30 g/*l* of sucrose and 10 g/*l* of agar, at 25°C in a 100 ml Erlenmeyer flask as described previously[6, 7]. About 2 g (fresh weight) of carrot hairy roots were inoculated into 1 *l* liquid MS medium containing 2% of sucrose in a turbine-blade reactor (TBR-2, Sakura Seiki Co., Tokyo). The volume of the blade rotation space where the root could not grow was 150 ml. The aeration rate was increased with the proliferation of the root in order to maintain dissolved oxygen concentration above the critical value. When cell density became higher than 10 g-dry weight/*l*, a mixture of air and oxygen was introduced into the medium. The aeration rate was increased with the proliferation of root cells in order that dissolved oxygen concentration in the medium. When cell concentration became high, a mixture of air and oxygen was introduced into the medium. Dry cell mass was gravimetrically measured after drying the

roots at 60°C for 24 h. On-line estimation of cell concentration during the culture was based on the conductometry as described previously[8]. Final dry cell mass was measured gravimetrically after drying the roots at 60°C for 24 hours. For the repeated-batch culture, the medium was drained and supplied through a silicone rubber tubing at the sampling port. When the conductivity decreased by 15 %, five fold concentrated modified MS medium was supplied through a tubing in the fed-batch culture and then the equivalent volume of medium was removed from the reactor.

Analytical methods Glucose concentration was measured by a glucose analyzer, Model 27 (Yellow Spring Instrument Co., Ohio, USA). Fructose concentration was evaluated according to the method described by Blakeney *et al.* and Tawfik *et al.* [9, 10]. Fructose and sucrose concentrations were also assayed by a Tri Rotar-V HPLC (JASCO, Tokyo) equipped with a PNH_2-10/S2504 column (Shimadzu Co., Kyoto) as described previously[3]. Ammonium ion was measured by the Nessler method with ammonium nitrate as the standard. Nitrate and phosphate ion concentrations were measured respectively by the brucine method and molybdenum blue method using KNO_3 and KH_2PO_4 as the standard[11]. Copper ion was assayed by using an atomic absorption spectrophotometer, SAS 760 (Seiko Instruments & Electronics Co., Tokyo). Potassium and other cation concentrations were determined by flame emission spectrophotometry and flame atomic absorption spectrophotometry respectively, using an atomic absorption flame emission spectrophotometer, Model AA-1MK (Nippon Jarrell Ash Co., Kyoto) equipped with a C_2H_2-air flame atomizer. 20-Hydroxyecdysone in *Ajuga* hairy root was isolated and analyzed as described by Matsumoto *et al.* [4].

Results

Cell yields determination for main components

Carrot hairy root was cultivated in a batch system with a turbine-blade reactor (initial sucrose concentration: 20 g/*l*). The final cell mass reached 10 g-dry weight/*l* at 31 d. We assayed the concentrations of the residual key components in the batch culture broth after 31 d. The cell yield for each component was determined. We then evaluated the maximum cell mass of hairy root that can be attained per liter of MS medium. The maximum attainable cell mass (X_{max}) should be obtained theoretically if the other nutrients were sufficiently supplied. As shown in Table 1, the values for the inorganic ion components, except for Ca^{2+} and Cu^{2+} ions, were from 10 to 14 g/*l*, while the values for Ca^{2+} and Cu^{2+} were much larger than 14 g/*l*. This shows that the proliferation of the roots ceased owing to the depletion of not only the carbon source but also of most of the inorganic ion components.

Repeated-batch culture in bioreactor

Substrate depletion in batch culture can be avoided by employing the repeated-batch technique. The purpose of the replacement of used medium with fresh medium is to supplement essential nutrients up to basal level and avoid the accumulation of growth inhibitor. The total volume of the culture broth was replaced every 4 d with fresh medium. Figure 1 shows the time course of the repeated-batch culture using the turbine-blade reactor. The cultivation exhibited a 2-fold increase in cell density, *i.e.* 19 g/*l* at day 40 compared with the batch culture. Generally speaking, repeated-batch culture is not economical even if a high cell density can be attained.

Fed-batch culture using sucrose as a carbon source

A more efficient culture system may be fed-batch culture, which can be readily performed and would be inexpensive compared with the repeated-batch culture system. We determined the composition of a suitable medium for MS nutrients feeding (Table 2). Since components of the feeding medium may accumulate in the culture broth and inhibit cell growth, each nutrient concentration in feeding was determined from the cell yield shown in Table 1.

The conductivity of the culture medium corresponded to the concentration of the total components. The hairy root growth can be estimated from the decrease of the conductivity[3)], and it was also observed that the correlation between two values remained constant through culture. When the conductivity decreased to 15% from the initial level, solutions A and B were fed to keep the conductivity constant. Figure 2 shows the result of time courses of the cell mass and the concentrations of main components during the fed-batch culture. At day 48, 17 g-dry weight/*l* of hairy root was obtained. The concentrations of the main components including sucrose were kept at almost a constant level. However, glucose and fructose accumulated up to 30 g/*l*, which may have adversely influenced cell growth.

Fed-batch culture using monosaccharide

To-date, there is limited information about sucrose uptake directly into plant cells. Kubota *et al.* reported that sucrose was degraded to glucose and fructose by extracellular invertase associated with the cell wall, and the hexoses were subsequently transported across the cell membrane (Kubota. K. and Ashihara H., Abstr. 11the sympo. Plant Cell Culture, Japan, p. 81, 1989).

To obtain information on the monosaccharide utilization for carrot hairy root and carrot hairy root, batch cultures supplemented with various single monosaccharides were carried out in shake flasks (Fig. 3). At 33 d, 6.2 g-dry weight/*l* of the hairy root grown on sucrose was obtained. Preliminary analyses on utilization of various monosaccharides revealed that either glucose

or fructose was a suitable carbon source. In particular, supplementation of medium with fructose instead of sucrose or glucose resulted in a higher final cell mass, 6.8 g-dry weight/*l*.

Figure 3 shows the dependency of cell growth and secondary metabolite, 20-hydroxyecdysone production on various saccharides obtained in shake flasks. The growth rate and final cell mass supplemented with glucose and galactose were comparable with those of sucrose. Fructose was also utilized for growth, whereas neither lactose nor mannitol were consumed by the hairy root. There was no significant difference in 20-hydroxyecdysone content in the hairy root among them. Hence, glucose was used in the subsequent experiments, which is less expensive than sucrose or galactose.

Figure 4 shows time courses of the fed-batch culture using fructose as a sole carbon source. The inorganic ion concentrations in solutions A and B were the same, except that sucrose was substituted by a concentrated fructose. When the medium conductivity decreased to approximately 15% from the initial level, solutions A without sucrose and B, together with a concentrated solution of fructose were added to the bioreactor. During this period, the concentrations of the major nutrients including fructose were kept constant. As a consequence, the final cell mass at day 38 reached 30.1 g-dry weight (approximately 436 g-fresh weight) per liter medium. Since turbine-blade reactor involved a dead space where the blades rotated, the cell density was evaluated to be 35.4 g-dry weight/*l* based on the working volume. This is a marked improvement compared with the previous cultures.

In the case of *Ajuga* hairy root, concentrations of glucose, phosphate, ammonium and nitrate ions were almost kept at a constant levels. The final cell mass increased markedly, 27.2 kg-dry cell/*l* at day 39. *Ajuga* is well-known to produce the insect molting hormone, 20-hydroxyecdysone. This fed-batch culture exhibited approximately 2.0 fold increase in 20-hydroxyecdysone

production, 44 mg/ l of 20-hydroxyecdysone, compared with batch culture in a shake flask with sucrose.

The roots grown by fed-batch operation using fructose in the reactor at day 38 were condensed extensively (Fig. 5). They bound tightly to the stainless mesh cage which provided a support matrix for the roots and invaded into the central space encompassing the mesh and a bottom section of the impeller blades.

The yield coefficient for the hairy root biomass and maintenance energy

It is vital for fed-batch culture to control the concentration of components in MS medium. As the cells uptook the inorganic ions from the medium, there was a consequent gain in the cell mass. The cell mass obtained was accurately proportional to the cell yield coefficient of each inorganic ions.

$$Y_{x/s} = -dX/ds \qquad (1)$$

The $Y_{x/s}$ values for PO_4^{3-}, NH_4^+ and NO_3^- (g-dry cell/g) were 33 , 32 and 4 g, respectively. To keep inorganic ions in the medium constant, the five fold concentrated modified MS medium based on the above values was fed as the hairy root grew.

On the other hand, the carbon source was not supplied by the same manner since the carbon source is utilized to gain biomass and to maintain cell viability. Carbon dioxide released from the reactor during the culture is produced through the cellular respiration and the metabolic pathway to yield the maintenance energy. Thus, the cell yield coefficient for the hairy root biomass and maintenance coefficient should be determined. According to the following balance equation[12, 13, 14], metabolic energy costs can be assessed from carbon utilized for growth (cell yield, $Y_{x/s}$) and carbon used to maintain existing

biomass (maintenance coefficient, m), which are deduced from the following relationship:

$$q = (ds/dt)/X = \mu / Y_{x/s} + m \qquad (2)$$

Specific growth rate (μ) and specific net uptake rate of carbon substrate into cells (q) can be estimated when the cell grows exponentially. The coefficients, $Y_{x/s}$ and m on callus are generally obtained when the callus is proliferated exponentially.

However, the balance equation for the specific growth rate[15, 16] can not be applied to the hairy roots proliferation pattern since hairy roots have a linear growth[17]. In the case of carrot hairy root, the linear growth was also observed from 16 d to 30 d as shown in Fig. 4. Hence, the relationship between cell growth and saccharide consumption should be rewritten in the following balance equation :

$$- ds/dt = (dX/dt)/ Y_{x/s} + mX \qquad (3)$$

Figure 6 shows the results plotted from the experimental data using monosaccharides on the *Ajuga* hairy root and carrot hairy root. Equation (3) was applicable to the linear growth phase (dX/dt = const.). The cell yield and maintenance coefficient were calculated from the intercept and the slope, respectively.

Monosaccharide is more adequate than sucrose for evaluating $Y_{x/s}$ and m for carbon utilization of plant cell because it is difficult to control the saccharide in medium at constant concentration since sucrose is divided into two types of hexose, glucose and fructose during the culture (Table 3). Determination of $Y_{x/s}$ and m must be accomplished under condition where the growth rate is carbon limited. Carbon-limited growth is difficult to perform over a sufficient

duration. The methodology using a fed-batch culture meets this requirement sufficiently.

Discussion

There are some reports on high cell density culture with regard to the suspension culture of plant cells[18, 19]. Piehl *et al.* obtained the concentration of 50 g-dry mass/*l* for *Thalictrum rugosum* callus grown in a membrane-stirred bioreactor[18]. Some papers have been published regarding the growth of hairy roots in fermentation. Hilton and Rhodes reported the kinetics of *Datura stramonium* hairy root grown in a 14-*l* fermentor system and continuously fed fermentation[20]. As far as we know, maximum cell density in hairy root is 11g-dry mass/*l* [7]. The fed-batch culture for hairy roots using monosaccharide exhibited high cell density.

It is interesting to compare the fed-batch culture using fructose and repeated-batch culture using sucrose. The difference in final cell mass between both operations seems to be due to carbohydrate utilization. If plant cell is ready to consume a single preferential carbohydrate, such as the case of bacteria, the medium containing monosaccharide, fructose, may provide to attain the higher cell mass. However, we could not identify the factor to improve the cell density completely.

Air was supplied and the dissolved oxygen level in culture medium could be monitored accurately up to 20 d (Fig. 4). After that period, a mixture of air and oxygen was supplied. When cell density increased above 20 g/*l*, oscillations of dissolved oxygen concentration were observed. In fact, we observed that the bubbles were extensively trapped at the interspace of the root materials in the bioreacter. These interspaces would become anaerobic and the cell growth decreased even if concentration of the main components were kept constant.

The purpose of this research was to evaluate the feasibility of estimating $Y_{x/s}$ and m of plant cells in fed-batch cultures. The results estimated are summarized and compared to the values obtained from the literature in Table 4. The *Ajuga* hairy root and carrot hairy root had $Y_{x/s}$ values for saccharide comparable with those of callus, while the cell yields of *Ajuga* hairy root and carrot hairy root were slightly higher than those of microorganisms. These show that the energy produced from carbon source in plant cells was converted more effectively to biomass despite the low plant proliferation than microorganisms. Although there is no significant difference in the maintenance energy requirement between the hairy roots and various callus, those of microorganisms were 5-fold higher than those of the plant cells. This may be an indication of difference in metabolism as a result of a different cellular construction and structure and a different environment. In general, plant cells don't have mobility and only apical meristem of organized plant retained high elongation activity, the loss of the energy obtained from carbon source in plant might be smaller than those of microorganisms.

In plant culture, evaluation of $Y_{x/s}$ and m is required to assess metabolic energy costs associated with cell age and environmental conditions. Often, there are two phase in plant cell growth, which are the growth phase and the production phase for proteins and secondary metabolites. The information on these coefficients allows us to predict the optimum conditions for plant cell culture and to control automatically the environmental condition for the cell growth by feeding the inorganic components and monosaccharide.

Table 1 Cell yield of carrot hairy root for main components in MS medium

Components	Residual amounts of component (mg/*l*)	Content in MS medium (mg/*l*)	Cell yield (g-dry cell/g)	X_{max} [a] (g-dry cell/*l*)
K^+	134	780	15.4	12
Mg^{2+}	7.0	36	345	12
Ca^{2+}	81	120	256	31
Mn^{2+}	2.2	7.2	1961	14
Cu^{2+}	3.6×10^{-3}	6.4×10^{-3}	3.57×10^{6}	23
Zn^{2+}	0.4	2.0	6250	13
NO_3^-	61	2200	45.5	10
NH_4^+	13	300	41.7	12
PO_4^{3-}	15	120	100	12
Sucrose	0	20000	0.5	10

[a] Maximum cell density estimated from the content in MS medium and the cell yield

Table 2 Composition of medium for fed-batch culture for carrot and *Ajuga* hairy root

Components	Medium used in the fed-betch culture	MS medium
Solution A	(g/*l*)	(g/*l*)
Sucrose	20	20
NH_4NO_3	1.8 (1.1)	1.65
KNO_3	2.0	1.9
$CaCl_2\ 2H_2O$	0.15	0.44
$MgSO_4\ 7H_2O$	0.27	0.37
KH_2PO_4	0.2 (0.3)	0.17
Solution B	(mg/*l*)	(mg/*l*)
KI	0.83	0.83
H_3BO_3	6.2	6.2
$MnSO_4\ 4H_2O$	18	22.3
$ZnSO_4\ 7H_2O$	8.0	8.6
$Na_2MoO_4\ 2H_2O$	0.25	0.25
$CuSO_4\ 5H_2O$	0.01	0.025
$CoCl_2\ 6H_2O$	0.025	0.025
$FeSO_4\ 7H_2O$	27.8	27.8
$Na_2EDTA\ 2H_2O$	37.3	37.3
Myoinositol	100	100
Nicotinic acid	0.5	0.5
Pyridoxin HCl	0.5	0.5
Thiamine HCl	0.1	0.1
Glycine	2.0	2.0

The concentration of compositions for *Ajuga* hairy root is designated in parentheses.

Table 3 Comparison of biomass yield from saccharide and maintenance coefficients for saccharide with published values

Organisms	Substrate	Cell yield, $Y_{x/s}$ [$g \cdot g^{-1}$]	Maintenance coefficient, m [$g \cdot g^{-1} \cdot d^{-1}$]
plant			
Ajuga hairy root	glucose	0.77	0.105
Carrot hairy root	fructose	0.60	0.085
Eschscholtzia californica callus	sucrose	0.71	0.074
Apple callus	sucrose	0.57	0.012
Nicotiana tabacum callus	sucrose	0.60	0.086
Nicotiana tabacum callus	glucose	0.52	0.106
Medicago sativa callus	lactose	0.77	0.113
microorganism			
Saccharomyces cerevisiae	glucose	0.14	0.864
Aerobacter cloacae	glucose	0.40	2.256
Penicillium chrysogenum	glucose	-	0.528
Pseudomonas methanica	methane	0.56	-
Candida utilis	glucose	0.51	-

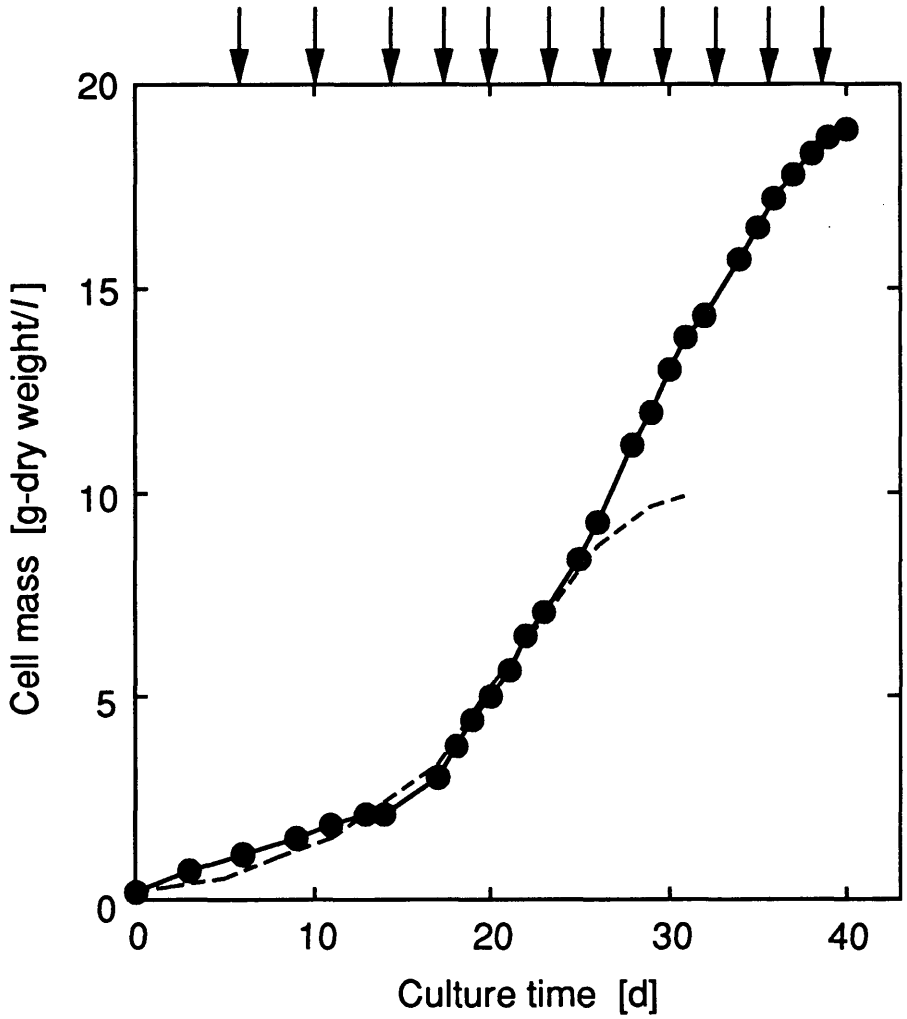

Fig. 1 Batch and repeated-batch culture of carrot hairy root using turbine-blade reactor. Cell mass was estimated from the decrease in conductivity: ● , repeated batch culture ; ···, batch culture. Arrows indicate when fresh medium was used.

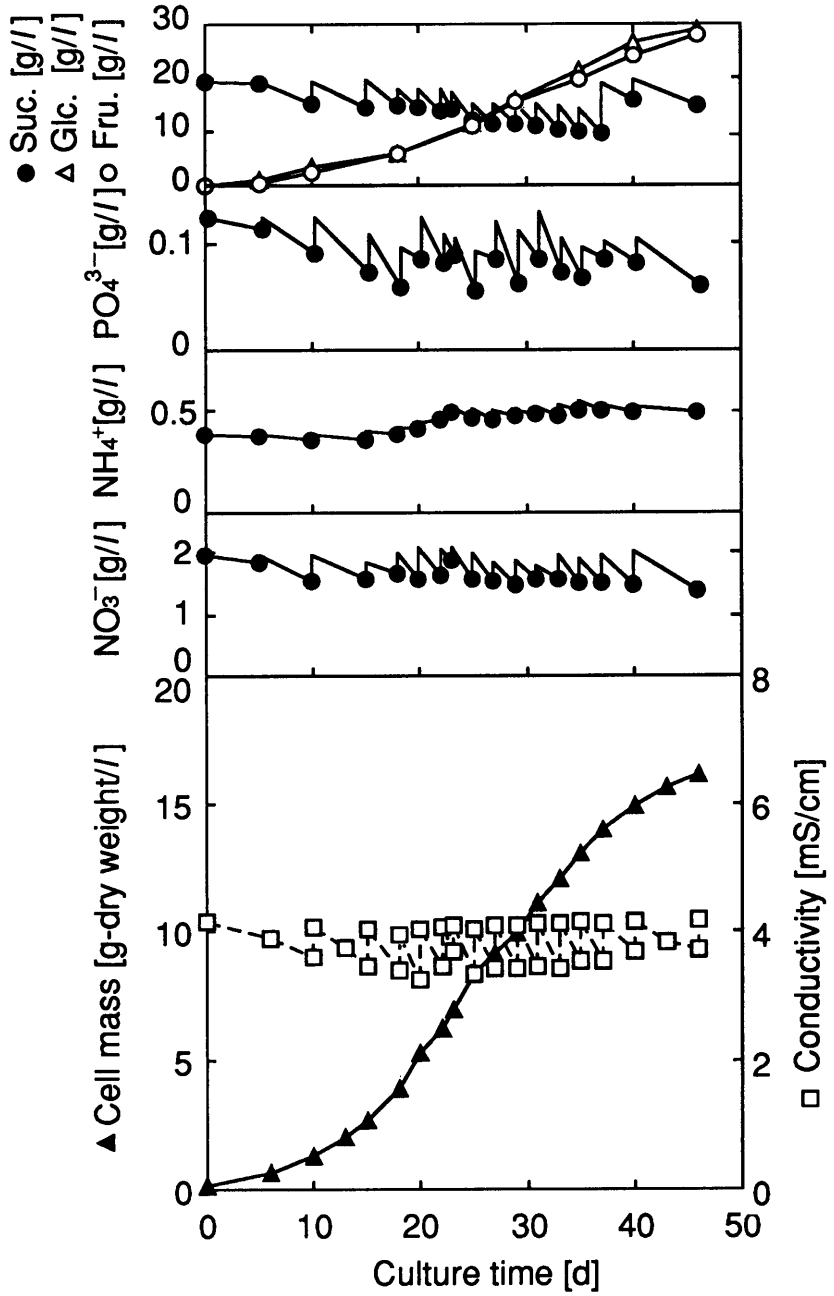

Fig. 2 Fed-batch culture using sucrose as a carbon source of carrot hairy root

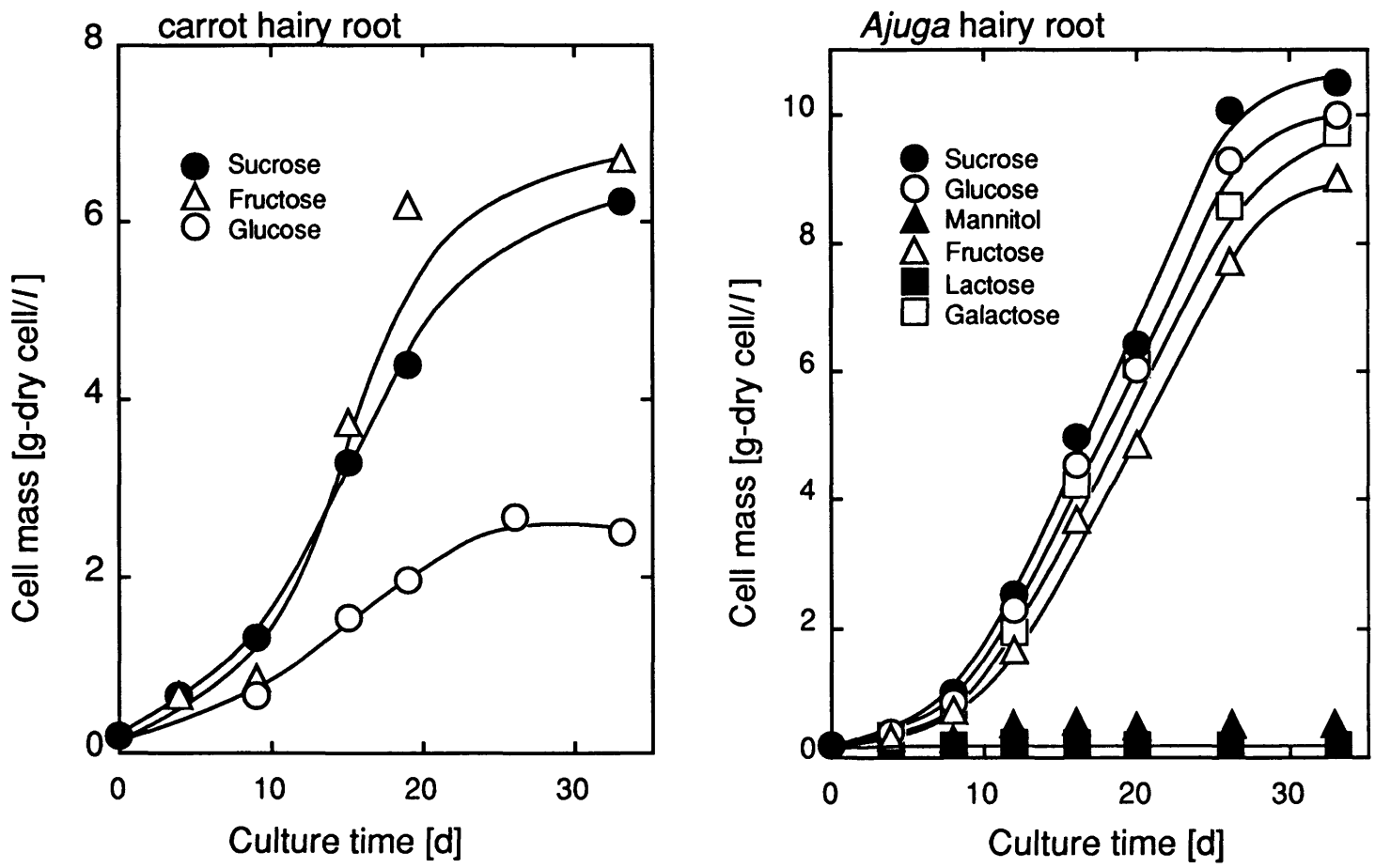

Fig. 3 Effect ofvarious monosaccharides on carrot and *Ajuga* hairy root growth in shake flask.

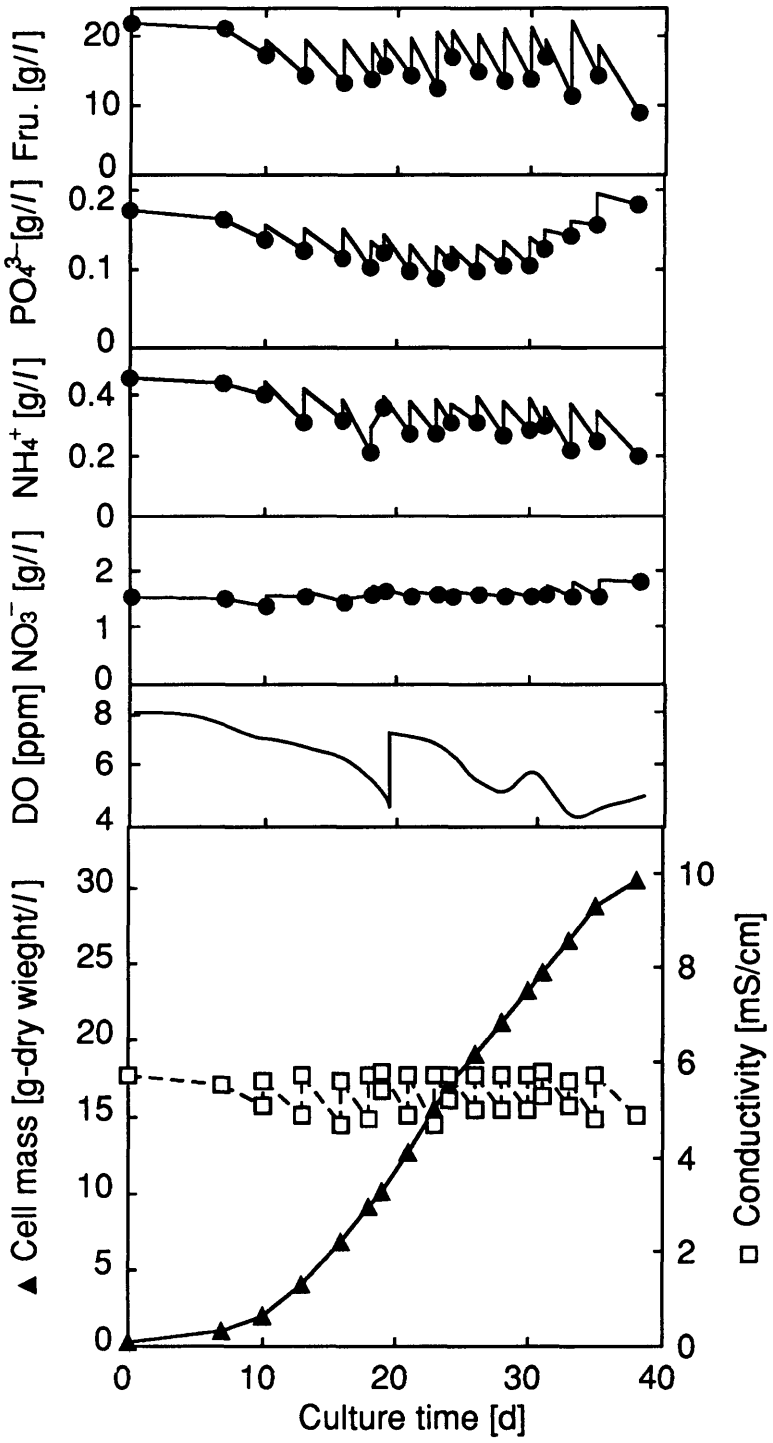

Fig. 4 Fed-batch culture using fructose as a carbon source of carrot hairy root

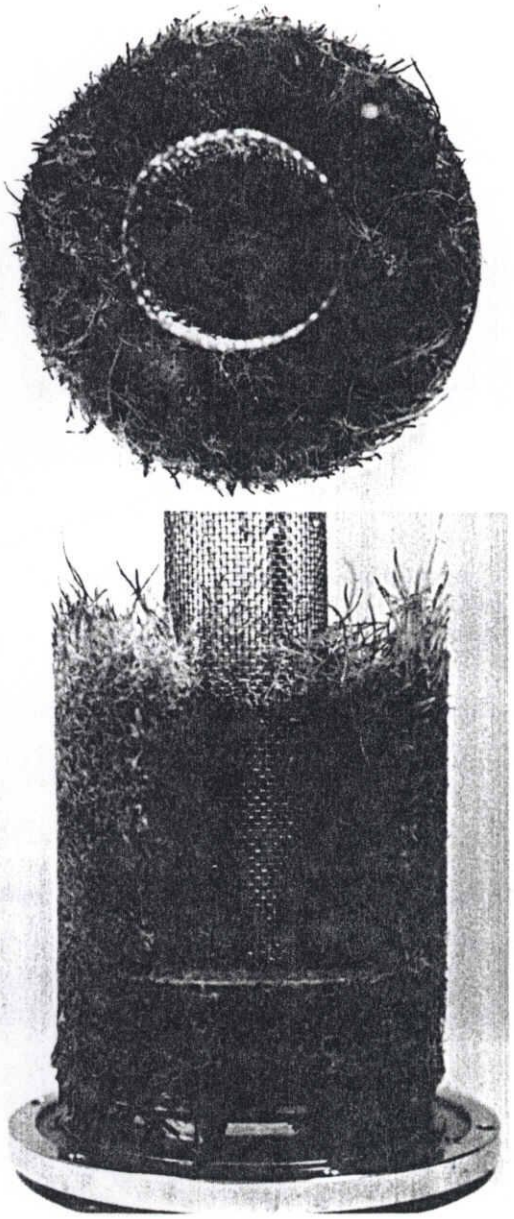

Fig. 5 Growth of carrot hairy root in fed-batch culture using fructose as a carbon source at 38 days. The lower half represents a vertical section view of condensed hairy root materials.

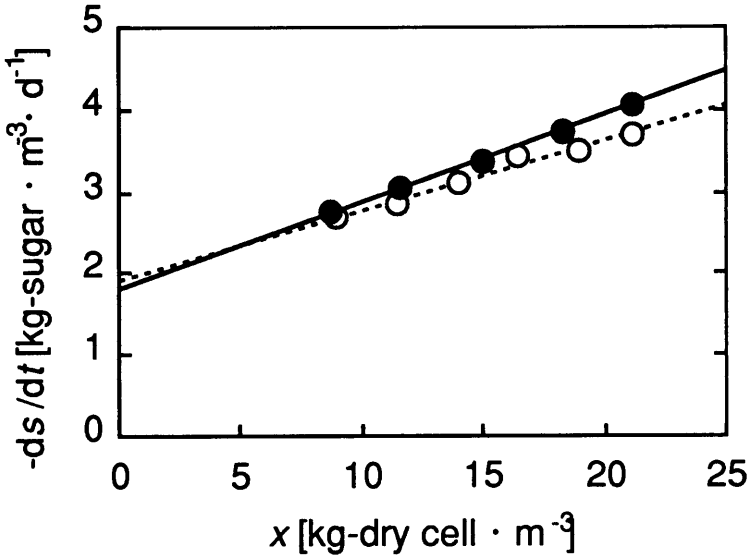

Fig. 6 Comparison of the cell yield coefficient (*Y x/s*) and maintenance coefficient *(m)* on *Ajuga* (●) and carrot (○) hairy root proliferations. The uptake rate of monosaccharide is plotted against the cell concentration.

Summary

From the batch culture experiment, cell yields for main components in the medium were determined and the composition of the medium was modified. When the fed-batch culture was carried out with sucrose, the concentrations of the medium components including sucrose were kept almost constant but fructose and glucose accumulated in the culture medium. The use of monosaccharide instead of sucrose in fed-batch culture avoided the glucose and fructose accumulation, and the drawback of the difficulty in estimating the concentration of saccharides. The plant cell density in the bioreactor can be greatly improved by feeding with a monosaccharide as the carbon source in fed-batch culture. On the basis of the data of the culture in glucose medium, cell yield, 0.60-0.77 g/g , and maintenance energy, 0.085-0.105 g/g•d, for hairy roots were comparable to those of various callus. The cell yield of plant cell was larger than those of microorganisms and the maintenance coefficient was smaller. For industrial scale plant cell culture, their coefficients will be useful to control the feed rate of the concentrated carbon source solution with monitoring the decrease in medium conductivity.

Nomenclature

s = substrate carbon concentration [g- carbon source/l]

X = cell concentration [g-dry cell/l]

t = time [d]

$Y_{x/s}$ = cell yield for hairy root biomass [g - dry cell/g- carbon source]

m = maintenance coefficient [g - carbon source/g-dry cell · d]

q = specific rate of carbon substrate consumption [g - carbon source/g- dry cell · d]

μ = specific growth rate [/d]

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Concluding Remarks

The advence of genetic engineering has made available a vast array of tools to determine the nucleotide sequence of target gene and manipulate the nucleotide sequence of a given gene which facilitates the alteration of a protein structure and its function. The changes that can be affected using recombinant DNA techniques are, in actuality, no different than those that exist in nature, however the ability to intelligently direct these changes is only possible through the use of morden molecular biology. Site-directed mutagenesis permits the precise replacement of any targeted amino acid residue with any other specified residue. The genetic alteration can be objectively analyzed and then its affect on the protein's structure and function determined. Other advantage of genetic engineering is that the foreign gene can be introduced into heterogeneous organisms. The reconbinant microorganisms and transgenic animal or plant acquire the blane new property. The transformation gives us the possibility of recontistitution of the organism.

Based on the above views, gene, enzyme, bacteria and plant which involve the targets in bioengineering field were studied in this thesis. The coclusions obtained in each chapter are summarized as follows:

(1) The amylase gene cloned from *Bacillus polymyxa* genome comprises 3,588 nucleotides. The mature amylase comprises 1,161 amino acids with a molecular weight of 127,314. The gene was organized into two portions, 5' region DNA responsible for the major three β–amylases of 70, 56 and 42 kd and 3' region for the 48 kDa α–amylase. A precursor protein had both β– and α–amylase activities. The *B. polymyxa* amylase gene was thus concluded to contain in-phase β– and α–amylase coding sequences in the 5' and 3' region, respectively. This finding represents the first report of 'one gene two enzyme' production system .

(2) Three cysteine residues at positions 83, 91 and 323, which can react with sulfhydryl reagents in *Bacillus polymyxa* β–amylase were replaced by other amino acids to construct four mutant enzymes, C83S, C91V, C323S and C-free by oligonucleotide dependent site-directed mutagenesis. Wild type as well as mutant forms of the enzyme were expressed in, and purified to homogeniety from, *B. subtilis*. By chemical modification experiments, a disulfide bond between Cys83-Cys91 and only a free SH group of Cys323 was identified. Replacement of cysteine residues with serine or valine residues resulted in a significant decrease in the kcat/Km value of the enzyme. Since C323S containing no free SH group retained a high specific activity, alkylation of Cys323 resulted in a loss of the activity as a consequence of steric interference. None of the cysteine residues participate directly in the catalytic reaction.

(3) To narrow down the essential amino acid in β-amylase catalysis, β-amylse gene was truncated by inserting the stop codon linker in the construction gene. On the basis of the experimental results, two different residues, His81 and Glu163, were candidate residues for β-amylase catalytic center. The relative β-amylase activity of H81L and E163Q constructed revealed histidine residues were not essential for the catalytic reaction, but Glu163 was one of active center.

(4) To develop the effective production from the transgenic plant, horseradish hairy root, the content incremant and excretion of peroxidase were elucidated. Illumination with light increased peroxidase content in the cell 2-fold. The treatment of NaCl stimulated excretion of peroxidase from the cell without serious influence on proliferation. Adsorption of peroxidase from the extracellular medium with hydrophobic resin greatly enhanced release of peroxidase. At the end of the culture, peroxidase produced in the medium from the above treated hairy root exhibited 14-fold, compared without special treatments. The results indicate that production and product recovery from plant cells can be greatly improved by application of the combined treatments.

(5) Cell yields for main components in the medium were determined and the composition of the medium was modified. When the fed-batch culture with monosaccharide was carried out, the final cell concentrations reached higher than that with sucrose. The use of monosaccharide instead of sucrose in fed-batch culture avoided the glucose and fructose accumulationin ths medium, thus the high cell density was achieved. The plant cell density in the bioreactor can be greatly improved by feeding with a monosaccharide as the carbon source in fed-batch culture.

(6) The cell yield and maintenance can be evaluated by fed-batch with monosaccharide which overcame the drawback of the difficulty in estimating the concentration of saccharides. Cell yield and maintenance energy for hairy roots were comparable to those of various callus. The cell yield of plant cell was larger than those of microorganisms and the maintenance coefficient was smaller. The coefficients will be useful for keeping the optimum condition by controling the carbon source concentration.

We convinced that the results could be applied to practical use and have great value on industrial scale.

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