Biochemical Studies on Bacterial β-Amylase and Its Secretion

南森, 隆司

(Degree)
博士（学術）

(Date of Degree)
1984-03-31

(Date of Publication)
2007-10-11

(Resource Type)
doctoral thesis

(Report Number)
甲0459

(URL)
https://hdl.handle.net/20.500.14094/D1000459

DOCTORAL DISSERTATION

BIOCHEMICAL STUDIES ON BACTERIAL β-AMYLASE AND ITS SECRETION

(細菌β-アミラーゼとその分泌に関する生化学的研究)

TAKASHI NANMORI

THE GRADUATE SCHOOL OF SCIENCE AND TECHNOLOGY

KOBE UNIVERSITY

DECEMBER 1983

Contents

Introduction

β-Amylase (α-1,4-D-glucanmaltohydrolase EC 3.2.1.2), which was first named by Kuhn,[1] attacks α-1,4-glucosidic linkages and liberates β-maltose successively from the non-reducing ends of starch, glycogen and maltooligosaccharides. Ball et al.[2] first crystallized sweet potato β-amylase. Then, barley,[3)-5)] wheat,[6),7)] and soybean[8] β-amylases were crystallized or purified and their physicochemical properties were studied. Each isoelectric point of those β-amylases was observed in acidic side and their molecular weights were estimated to be 197,000 (sweet potato, tetramer),[9),10)] 57,200 (barley),[5)] 64,200 (wheat),[11),12)] and 61,700 (soybean),[8)] respectively. The amino acid compositions were also determined. Five sulfhydryl residues were found in a molecule of most plant β-amylases,[5),8),13)-16)] some of which were thought to be essential for catalytic activity because of its inhibition by sulfhydryl reagents like pCMB.

Thoma et al.[13] pointed out that the SH groups which were modified by iodoacetamide, were located on a surface of the molecule of sweet potato β-amylase, and weren't related essentially to catalytic activity. Some SH groups of soybean β-amylase reacted with DTNB under the conventional conditions, but the others could not react without the addition of KCl at high

ionic strength or denaturing the enzyme by guanidine hydrochloride.[14] The latter sluggish SH groups were seemed to play an essential role to exhibit enzyme activity.

On the other hand, we have very few knowledge on the properties of bacterial β-amylases. Robyt et al.[17] reported that the amylase from B.polymyxa selected by Tilden et al.[18] liberated mainly β-maltose but that the catalytic mechanism of the amylase seemed to be different from those of not only plant β-amylases but also α-amylases. Recently Okada et al.[19),20] identified an extracellular enzyme from B.megaterium strain No 72 as β-amylase by measuring the change of optical rotation and using the oligosaccharide map method.[21] Independently, Fogarty et al.[22),23] separated β-amylase from the culture filtrates of B.polymyxa containing a debranching enzyme, and found the same mechanism of action to substrates as those of plant β-amylases.

Shinke et al.[24] also isolated the β-amylase producing bacterium (B.cereus BQ 10). Then, Marshal[25] pointed out that the amylase from B.polymyxa acted on the substrates as equally as β-amylases from higher plants. Consequently, it was accepted in general that some kinds of bacteria, such as B.polymyxa, B.megaterium and B.cereus could produce β-amylases.

However, the amounts of β-amylases produced by these bacteria were much less than those from soybean or other plant sources, and such a poor productivity was thought to be unable to meet industrial demands.

On the other hand, the physico-chemical properties of bacterial β-amylases have not been clear. Their molecular weights were reported differently even in the *Bacillus* groups, for example, 3.5×10^4-3.8×10^4(*B.megaterium*),[19),26)] $3.5\times10^4\pm5{,}000$ (*B.cereus*),[27),28)] 4.4×10^4(*B.polymyxa* No72);[29)] 5.9×10^4(*B.polymyxa*).[30)]

Shinke *et al.*[31)] attempted the experiments for increasing the extracellular amounts of bacterial β-amylase, and obtained by UV-irradiation the mutant which could produce about 25 times as much β-amylase as that of *B.cereus* BQ10 and named it *B.cereus* BQ10-S1. Their attempt to examine the relation between spore formation and the β-amylase productivity resulted in selection of rifampin-resistant, asporogenous mutants by NTG (N-methyl-N-nitro-N-nitrosoguanidine) treatment.[32)] Among them, a mutant which could produce about 7 times as much β-amylase as that of *B.cereus* BQ10-S1 was found, and named *B.cereus* BQ10-S1 SpoI.[27)] But this mutant was not enough for industrial use because the β-amylase activity was decreased rapidly after reaching the maximum activity during the stationary phase.

The author *et al.* succeeded in selection of a mutant (*B.cereus* BQ10-S1 SpoII) which could secret higher amounts of β-amylase (4 times those of *B.cereus* BQ10-S1) and keep its activity even in the later stationary phase,[33)] which will be described in Chapter I.

Then, the author tried to purify the β-amylases from B.cereus BQ10-S1 and its mutants up to a homogeneous state on disc electrophoresis and analytical ultracentrifugation, which will be described in Chapter II. The molecular weights of the β-amylases were calculated by various methods. The amino acid compositions and the number of cysteine residue in the molecule were determined.[34)]

In Chapter II and III enzymological properties of the β-amylase will be compared with those of plant or other bacterial β-amylases already reported. Moreover, it is known that Taka-amylase or α-amylases from some bacteria contain some amounts of sugars in the molecule,[35)-38)] there is no information on β-amylases. So the detection of sugar in bacterial β-amylases was attempted, which will be described in Chapter III.

On the other hand, the author prepared a rabbit anti-bacterial β-amylase serum, which will be written in detail in Chapter IV.

The β-amylase from B.cereus BQ10-S1 SpoII will be also compared with others immunologically by using its antiserum.

The mutant capable of secreting large amount of β-amylase (B.cereus BQ10-S1 SpoII) seemed to be a good bacterium for researching a mechanism of protein secretion in Bacillus groups. In the case of α-amylase from B.subtilis, its membrane bound form seemed to be its precursor.[39),40)] But its properties

or differences of molecular weight between extracellular α-amylase and its precursor are not clear.

Furthermore, an attempt to detect a precursor protein in the cell-free protein synthesis system from E.coli was not successful.[41),42)]

Though it was not on bacterial amylase, Okita et al.[43)] succeeded in detection of an α-amylase precursor produced by wheat aleurone cells which seemed to have a signal peptide.

Lampen et al.[44),45)] studied the processing of penicillinase from B.licheniformis 794/C and determined the amino acid compositions of its signal peptide, which sustained the signal hypothesis by Blobel.[46),47)] Thereafter, many papers on the processing of bacterial protein were reported. However, there is no information on bacterial β-amylase.

The author tried to detect an intracellular precursor of exo-β-amylase using the Western-Blotting method. Furthermore RNA fraction extracted from B.cereus BQ10-S1 SpoII was translated by incubation with polysome solutions prepared from E.coli K-12. Incorporation of radioactivity to the immuno-precipitates of cell-free synthesized proteins with anti-β-amylase serum was measured and immuno-reactive proteins was identified on SDS-electrophoresis. The mechanism of β-amylase secretion in B.cereus BQ10-S1 SpoII is discussed in Chapter V.

Chapter I. Selection of a Mutant Capable of Producing Larger Amounts of β-Amylase.

In this Chapter, the author deals with selection of mutants capable of secreting larger amounts of β-amylase and its ability of protein secretion.

Materials and Methods.

Microorganisms and culture methods B.cereus EQ10-S1 was used for the experiments for improving β-amylase productivity. The microorganism was cultured in a Polypepton medium consisting of 3% Polypepton (Daigo Nutritive Chemical, Ltd.), 0.5% KCl and 0.5% NaCl (pH 7.2) at 30°C for 22 hr in a 500ml shaking flask with shaking at 140 reciprocation per min. The cell growth was measured by turbidity at 660nm.

Isolation of rifampin-resistant, asporogenous mutants

B.cereus BQ10-S1 was cultured in Schaeffer's medium and the spores formed were collected by the method of Sacks and Alderton[48] and stirred at 4°C in the suspension of 3.5×10^9 spores per ml. The spore suspension (0.1ml) was plated on a Polypepton agar plate containing 5μg/ml of rifampin (Calbiochem-Behring Corp.), to which UV light was irradiated with "National" germicidal lamp (10 watt) at a distance of 50cm for 60 to 80 sec. The UV-irradiated plates were incubated at 30°C overnight. Each isolate from the colonies (5 to 10 colonies per plate)

was again plated on the Polypepton agar plate and incubated at 30°C overnight. From rifampin-resistant colonies appearing on the plates, rather thin and transparent colonies were picked up as probable asporogenous mutants. Asporogenicity of the isolated strains was confirmed by microscopic observation and sporulation tests in Schaeffer's medium.

Enzyme assays i) β-Amylase activity. β-Amylase activity was measured by Bernfeld method[49] with 3,5-dinitrosalicylic acid reagent and expressed as mg maltose produced by one ml of culture broth at 40°C for 60 min. ii) Protease activity. Protease activity in the culture broth was measured with casein as substrate and expressed as μg of tyrosine N formed per ml of culture broth at 35°C for one min.[50]

Measurement of protein content Protein content was measured as follow; each 10ml of culture broth during incubation was taken at regular intervals, to which 10ml of 10% trichloroacetic acid (TCA) was added. The precipitates formed were collected by centrifugation at 20,000xg for 20 min and subjected to protein measurement by Lowry's method.[51]

Results

Isolation of rifampin-resistant, asporogenous mutants

From the rifampin-resistant colonies, 100 strains were isolated and tentatively designated as R-1 to R-100. The germination rate of the spores was 45.4% and, based on eight colonies per Polypepton agar plate in average, the rate of mutation was calculated to be 5.03×10^{-6}%. The examination of β-amylase productivity, as seen in Table I, showed that only the R-59 strain was capable of producing about four times as much β-amylase as the parent strain under the same culture conditions, and that the other strains produced less β-amylase than the parent strain (BQ10-S1), including the R-62 strain with no β-amylase productivity. Furthermore, the β-amylase produced by the R-59 strain was found to be more stable in the culture broth than that by BQ10-S1 SpoI. Therefore, the R-59 strain was selected as a promising mutant for fermentative β-amylase production and named Bacillus cereus BQ10-S1 SpoII.

β-Amylase production by BQ10-S1 SpoII

The time course of β-amylase production by BQ10-S1 SpoII was pursued with the Polypepton medium. As shown in Fig.1, the β-amylase activity reached the maximum value (1100 units/ml) on 21 hr incubation in this condition and didn't show such a rapid decrease as in the case of BQ10-S1 SpoI.[27)]

The extracellular protease activity (13μg tyrosine N/ml) on 21 hr incubation was also higher than that (4μg tyrosine N/ml) of its parent (BQ10-S1). (Fig. 2.)

As also shown in Fig.1, the increase in TCA-insoluble protein contents corresponded to that in β-amylase in the culture.

β-Amylase production by feeding the Polypepton medium

As another attempt to increase β-amylase production, each 5ml of the Polypepton medium was added at regular intervals after taking out each 5ml of the culture broth in the logarithmic growth held constant up to 35 hr incubation and reached the maximum value (33.6), which was about two times as high as that with no feeding, leveling off after 40 hr incubation. After the longer period of β-amylase production the activity reached the maximum value (3240 units/ml) on 30 hr incubation which, thereafter, did not decrease for more than 10 hr (Fig.3).

Discussion

An attempt to isolate more industrially promising β-amylase producers resulted in selection of BQ10-S1 SpoII.[33] This mutant could produce not only about 4 times as much β-amylase as its parent (BQ10-S1), but also more amounts of protease.

To examine the effect of protease activity on extracellular β-amylase from B.cereus BQ10-S1 SpoII, the enzyme was incubated in the phosphate buffer (pH 7.0) and in the Polypepton medium with or without neutral protease from B.subtilis. The β-amylase was remarkably inactivated only in the presence of the protease.

However, the β-amylase was not inactivated when it was incubated in the same conditions with the crude protease from B.cereus BQ10-S1 SpoII, suggesting that the β-amylase in the culture broth seemed to be protected from the attack of the protease.

It was reported that tunycamycin resistant-, or D-cycloserine resistant-mutant of B.subtilis could produce more amounts of α-amylase than those of their parent.[52),53)] But the higher β-amylase productivity of rifampin-resistant, asporogenous mutants was first shown by our laboratory.[27),33)] However, the relation between its higher productivity and resistance to rifampin was not clear. The difference of the TCA-insoluble protein contents between BQ10-S1 SpoII and its parent might correspond to the increased amounts of secreted β-amylase.

More details are described in Chapter IV where the determination of extracellular β-amylase was made by using anti-β-amylase serum. This mutant could secret more amounts of not only β-amylase but also other extracellular proteins.

Table I. β-Amylase Productivity of Rifampin-resistant, Asporogenous Mutants from B.cereus BQ10-S1.

Strain isolated	Cell growth (O.D.at 660nm)	β-Amylase activity (unit/ml)
R-8	12.0	220
R-32	10.3	156
R-58	9.4	72
R-59	12.2	1368
R-62	7.3	0
R-77	11.0	232

The strains isolated were cultured in the Polypepton medium at 30°C for 22 hrs and the culture filtrates were subjected to β-amylase measurement. The cell growth and the enzyme activity in the case of B.cereus BQ10-S1 were 11.6 and 318.

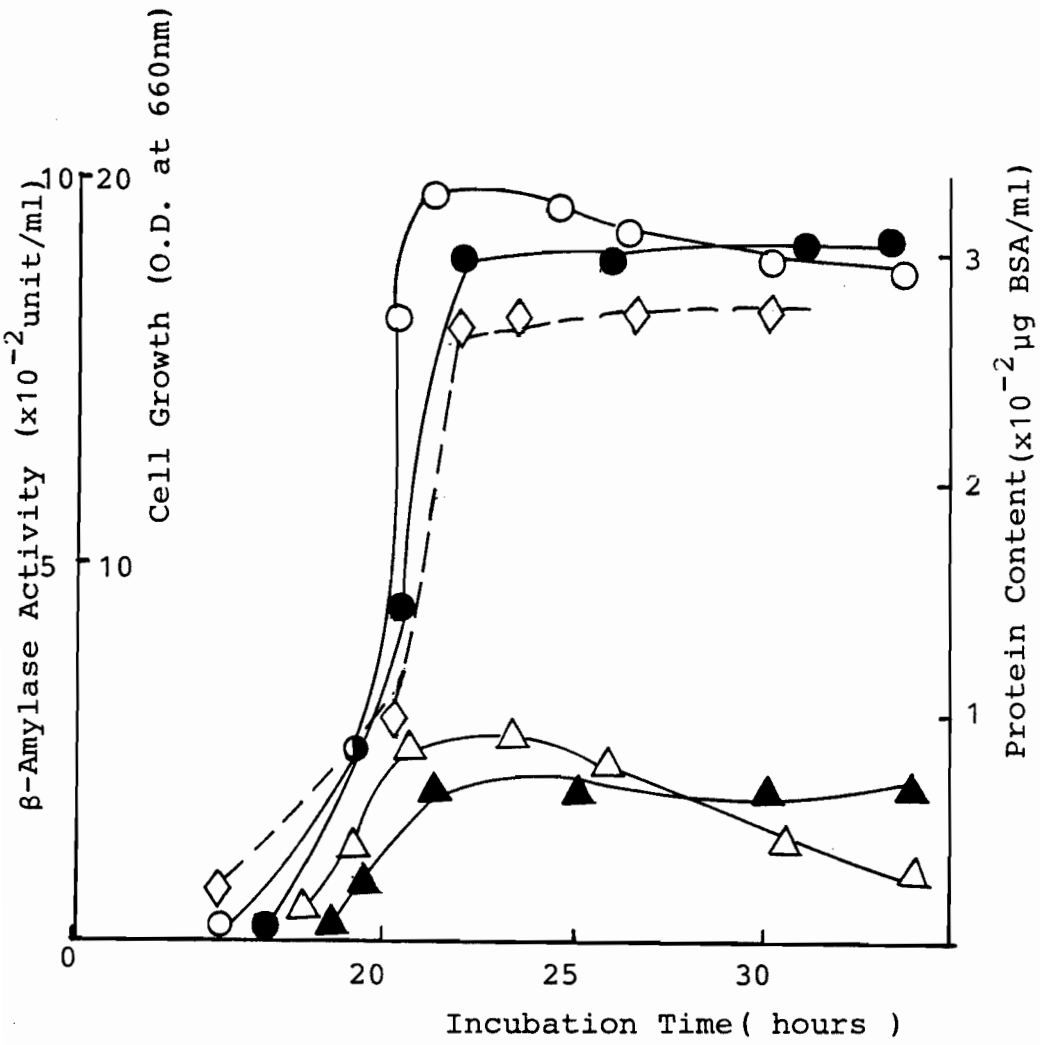


Fig. 1. Time Course of β-Amylase Production by B.cereus BQ10-S1 SpoII and Its Extracellular Protein Content.

The culture was performed with the Polypepton medium at 30°C. Each enzyme activity, cell growth and extracellular protein content were measured as described in Materials and Methods.

◇---◇ cell growth of B.cereus BQ10-S1 SpoII

○—○ β-amylase activity of BQ10-S1 SpoII

●—● protein content of BQ10-S1 SpoII

△—△ β-amylase activity of BQ10-S1

▲—▲ protein content of BQ10-S1

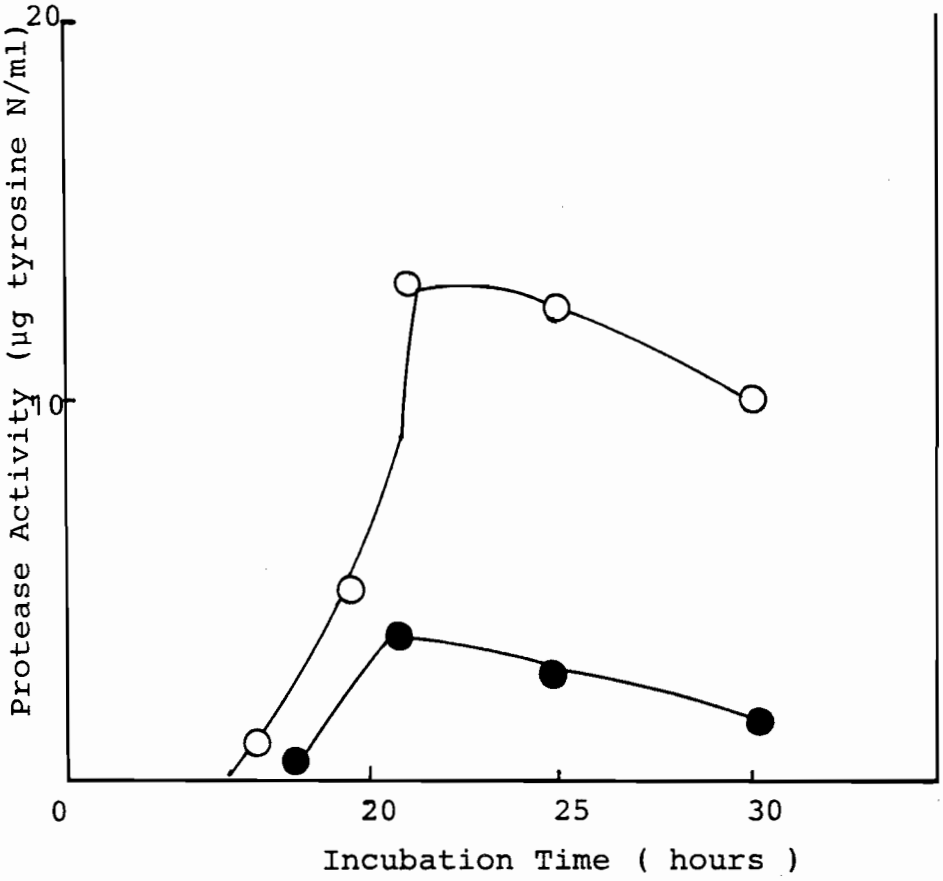


Fig. 2. Time Course of Protease Production by B.cereus BQ10-S1 SpoII and B.cereus BQ10-S1.

The culture was performed with the Polypepton medium at 30°C. Protease activity was measured with casein as substrate and expressed as µg of tyrosine N formed per ml of culture broth.

○—○ protease activity of BQ10-S1 SpoII

●—● protease activity of BQ10-S1

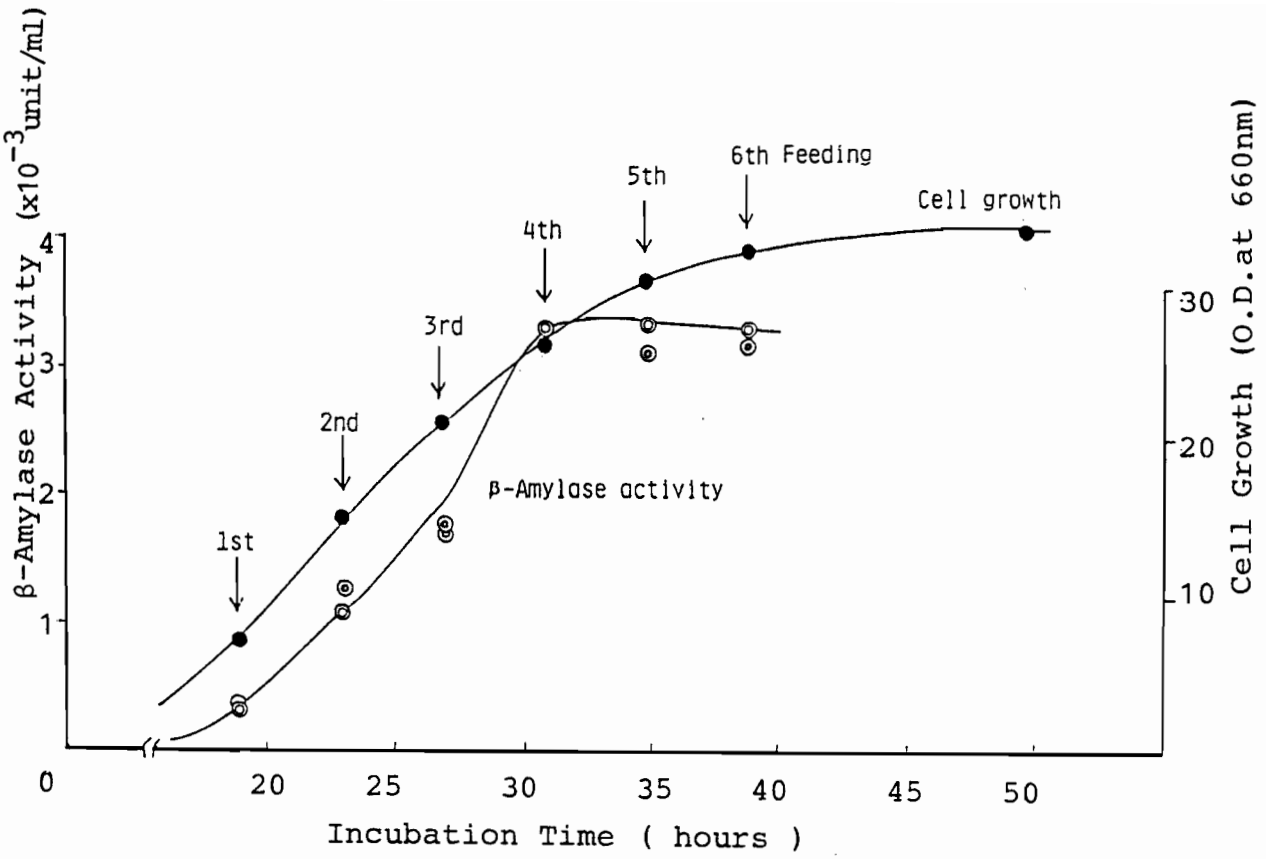


Fig. 3. β-Amylase Production by B.cereus BQ10-S1 SpoII by Feeding the Polypepton Medium.

The culture was carried out with the Polypepton medium at 30°C. At each incubation time shown by the arrow 5 ml of the incubation mixture was taken and 5 ml of the Polypepton medium was newly added.

◎—◎ β-amylase activity ●—● cell growth

Chapter II. Purification and Some Properties of the β-Amylase from B. cereus BQ10-S1 Spo II.

Purification procedure of β-amylases from B.cereus BQ10-S1 and B.cereus BQ10-S1 SpoII and homogeneity tests of them on electrophoresis or analytical ultracentrifugation are mainly described in this Chapter. Some enzymatic properties are also described.

Materials and Methods

Microorganisms and culture methods

B.cereus BQ10-S1, B.cereus BQ10-S1 SpoI, and B.cereus BQ10-S1 SpoII were used for β-amylase production. The microorganisms were cultured in the Polypepton medium under the same conditions mentioned in Chapter I. The cells were removed by centrifugation and the supernatant was used for purification of β-amylase.

Purification procedure

Ammonium sulfate was added to the above supernatant to 60% saturation. After standing for 24 hr the precipitates were harvested by centrifugation, then dissolved in 50mM acetate buffer (pH 5.0) and the insoluble materials were removed by centrifugation. The supernatant obtained was loaded on a Sephadex G-100 column and eluted with the same buffer.
The β-amylase fractions were further loaded on a CM-Sephadex C-50 column equilibrated with the same buffer (pH 5.0). Gradient elution was performed with increasing NaCl concentration.

The β-amylase fractions were dialyzed against deionized water. The enzyme thus purified was lyophilized and used for subsequent experiments.

Homogeneity test

Homogeneity of the purified enzyme was tested by polyacrylamide gel electrophoresis (PAGE) and ultracentrifugation. PAGE was performed by Davis' method[54] using 7% gel at pH 9.5. Centrifugal analysis was performed with a Beckman-Spinco centrifuge (Model E type) at 52,000rpm at 20°C. SDS-PAGE was performed by Osborn's method[55] using 10% polyacrylamide disc gel.

Effects of pCMB and DTNB on β-amylase activity

The β-amylase solution (50mM phosphate buffer, pH 7.2) from *B.cereus* BQ10-S1 SpoII was incubated with pCMB and DTNB at various concentrations at 30°C for 20 min. Then, the remaining activity was measured at 40°C for 3 min.

Degradation of raw starch by the β-amylase

Each 200mg of sweet potato, potato, corn, and wheat starches was suspended in 20ml of 50mM phosphate buffer (pH 7.2) containing 36 units of the β-amylase activity and incubated at 40°C for 6, 10, 20 hr. The reaction mixture was centrifuged at 3,000xg for 10 min and the precipitates were removed, the amounts of reducing sugars in the supernatant was measured according to Somogyi-Nelson method.[56]

Results

As shown in Fig. 4 the β-amylase fractions (Fract. Nos. 35-45) from Sephadex G-100 were loaded on a CM-Sephadex C-50 column and eluted with a linear NaCl gradient at 22°C as shown in Fig. 5. β-Amylase obtained was lyophilized and used for subsequent experiments. A summary of the enzyme purification is shown in Table II, from which it is clear that the enzyme was purified about 86 fold. Fig. 6 shows disc gel electrophoresis of the purified enzyme. The β-amylase from BQ10-S1 was also found to be electrophoretically identical to the enzyme from BQ10-S1 SpoII.

As shown in Fig. 7, the purified enzyme was sedimented as a single symmetric peak on centrifugation. Based on the partial specific volume of 0.74 from the amino acid composition, the sedimentation coefficient was calculated to be 4.8 $S_{20,w}$.

The results of SDS-PAGE of the β-amylases from BQ10-S1 and BQ10-S1 SpoII are shown in Fig. 8, suggesting that both enzymes are identical, each showing a single band on the gel and no subunit structure. The optimum pH and temperature of the purified enzyme from BQ10-S1 were almost the same with those of BQ10-S1 SpoII, about 7.0 and 40°C, respectively (Fig. 9). The Km value for soluble starch was about 0.4% (Fig.10). The β-amylase activity was inhibited by pCMB, but not by DTNB (Fig.11).

The β-amylase from B.cereus BQ10-S1 SpoII could attack wheat and corn starches. The degradation rates of them were calculated to be 5% and 2%,respectively (Fig.12).

Discussion

The β-amylases from B.cereus BQ10-S1 and BQ10-S1 SpoI were purified in the same way as that from BQ10-S1 SpoII. Not only the optimum pH and temperature of these enzymes, but also the degrees of migration in polyacrylamide gel were equal.

The purified enzyme was remarkably inhibited by pCMB. This was in agreement with the cases of other bacterial β-amylases from B.megaterium[20] and B.polymyxa.[29] But the enzyme activity was not inhibited by DTNB. These results will be discussed in the next Chapter.

It is known that the β-amylases from higher plants can hardly attack raw starch.[57] The author examined whether or not the β-amylase from B.cereus BQ10-S1 SpoII could attack raw starch. The degradation rates of wheat and corn starches were calculated to be 5% and 2%,respectively, though they were much lower than those in the cases of α-amylase or glucoamylase I. But the sweet potato starch could hardly be attacked by this β-amylase. Therefore, degradation rates of various raw starches by the β-amylase seem to be varied. Even though the degradation rate of raw starch by barley β-amylase or soybean β-amylase was lower than that by the β-amylase from BQ10-S1 SpoII, they were found to attack raw starches. Sweet potato β-amylase could not attack raw starch. Since crude plant-β-amylase preparations were used in these cases, more detailed experiments should be needed.

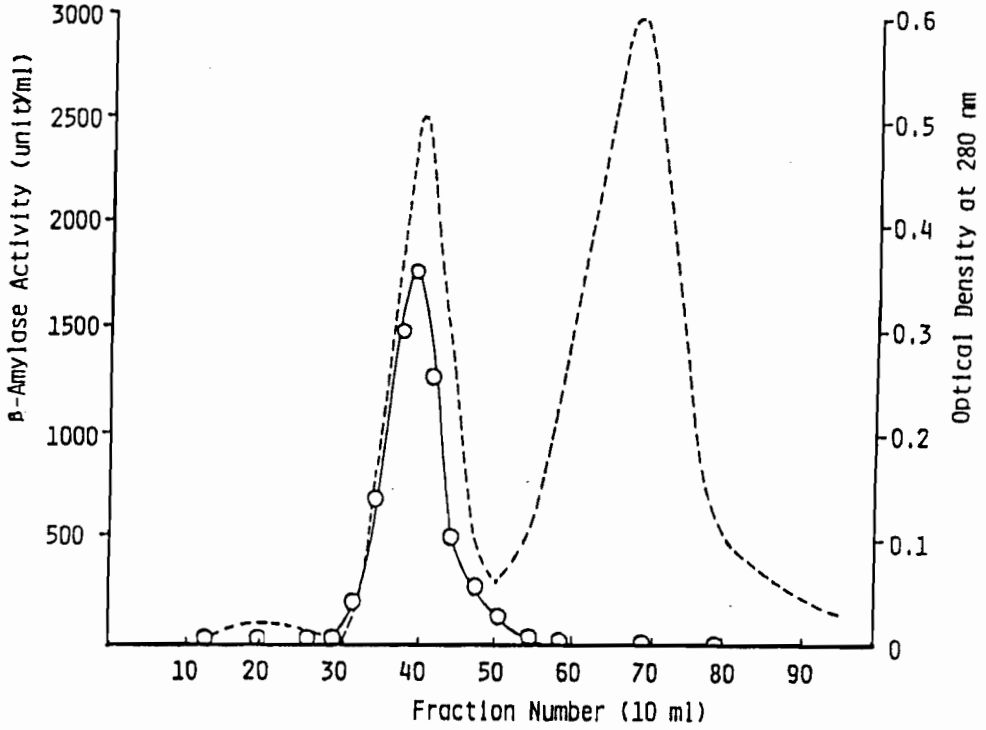


Fig. 4. Gel Filtration of β-Amylase Fraction on Sephadex G-100.

The β-amylase fraction obtained by salting out with ammonium sulfate was loaded on a Sephadex G-100 column (4x55cm) equilibrated with 50mM acetate buffer (pH 5.0) and eluted with the same buffer.

--- protein O—O β-amylase activity

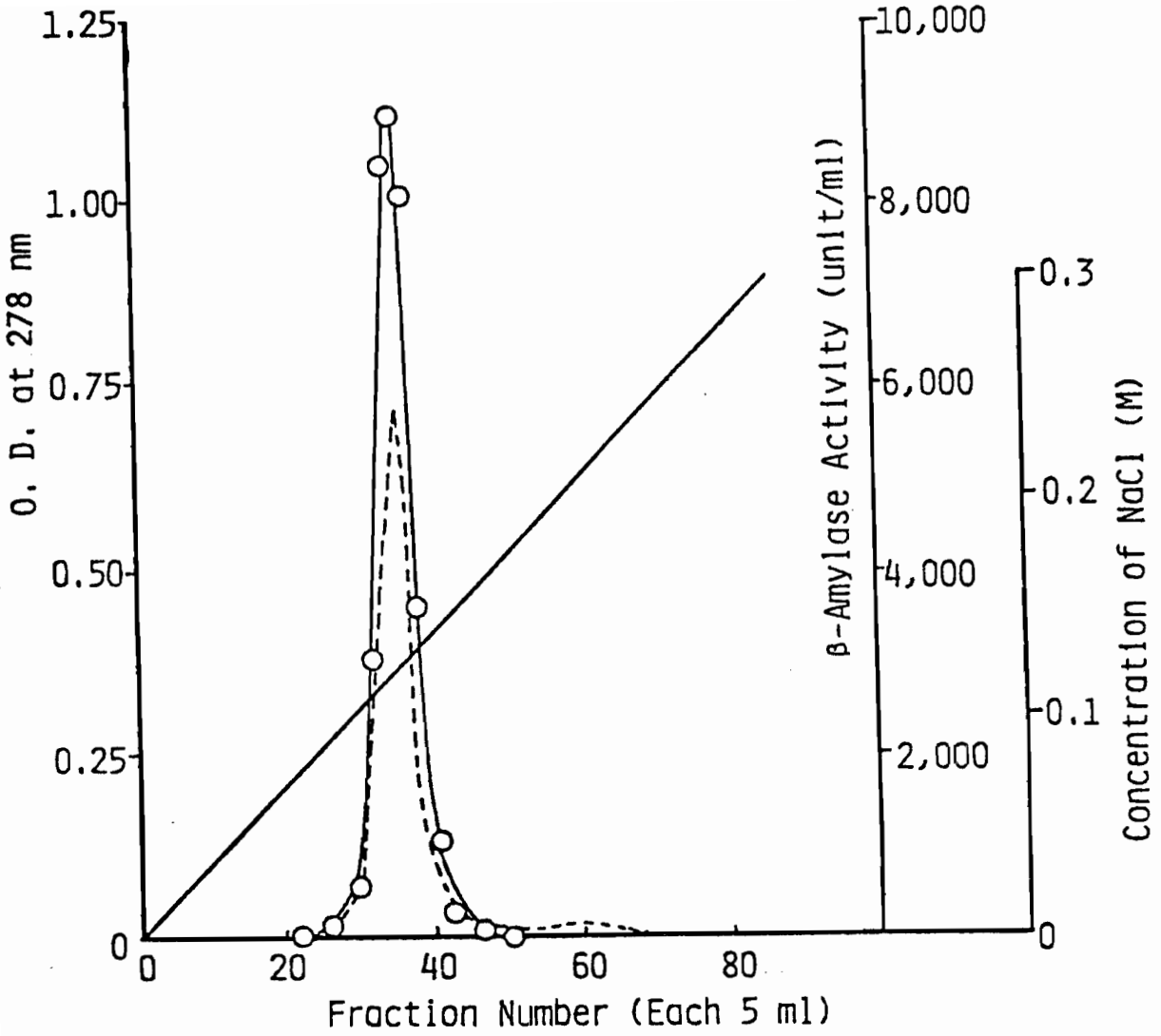


Fig. 5. Chromatography of β-Amylase Fraction on CM-Sephadex C-50. The β-amylase fraction obtained by gel filtration was loaded on a CM-Sephadex C-50 column (3x30cm) and eluted with a linear NaCl gradient (0 to 0.3 M) in 50mM acetate buffer (pH 5.0).
---protein, ○—○ β-amylase activity, —NaCl concentration

Table II. Purification of β-Amylase Produced by B.cereus BQ10-S1 SpoII.

	Total Volume (ml)	Activity/ml (unit.ml)	Total Activity (unit)	Specific Activity (unit/O.D. 280nm)	Yield (%)	Rate of Purification
Culture filtrate	835	1023	854,205	85	100	1
Salting out with $(NH_4)_2SO_4$ 0.6 sat.	26	11,700	304,200	1,772	35	21
Gel filtration on Sephadex G-100	130	1,260	163,800	5,727	19.1	67
CM-Sephadex C-50	90	1,680	151,200	7,304	17.7	86

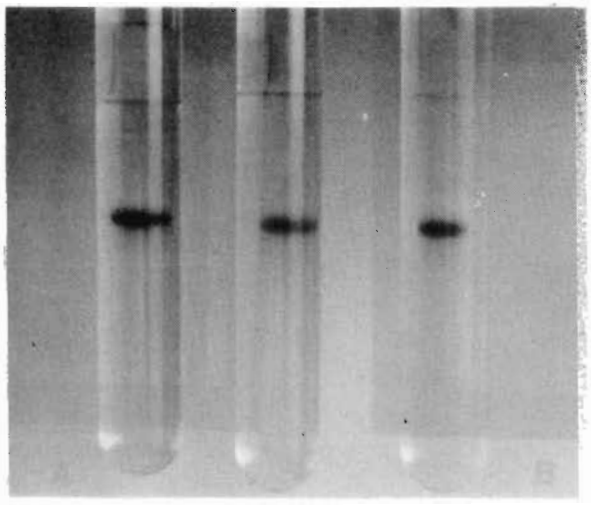
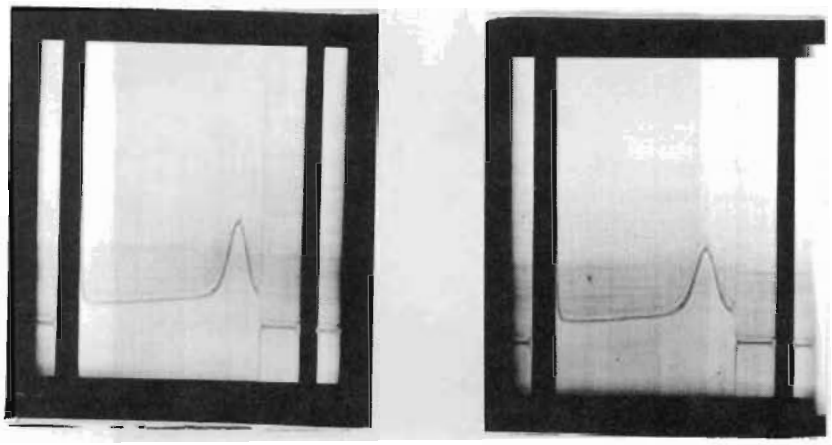


Fig. 6. Polyacrylamide Gel Electrophoresis ofβ-Amylases. Each purified β-amylase (about 50μg) was run on 7.5% polyacrylamide gel at pH 8.9. Each gel was stained with Amide Black 10B. A: β-amylase from BQ10-S1 B: β-amylase from BQ10-S1 SpoII C: mixture of A and B



A B

Fig. 7. Schlieren Patterns of β-Amylase on Analytical Centrifugation.

The β-amylase solution contained 0.1 M NaCl and 25mM phosphate buffer (pH 7.0) and the protein concentration was about 5.0mg/ml. The Schlieren patterns were photographed at a bar angle of 65°.

A: Taken at 20 min after reaching 52,000 rpm.

B: Taken at 28 min after reaching 52,000 rpm.

The sedimentation coefficient was calculated to be $4.8S_{20,w}$.

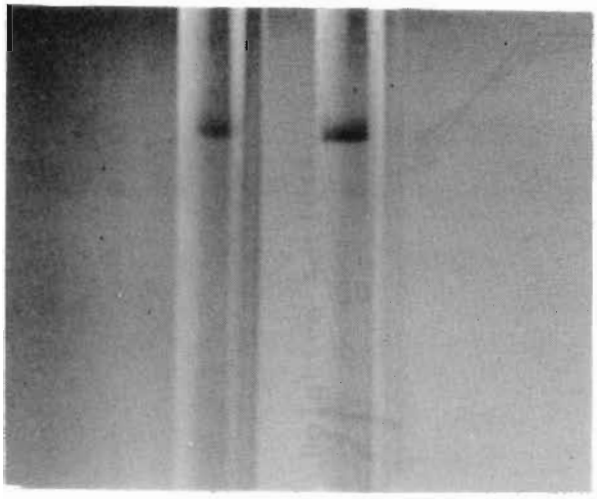


Fig. 8. SDS-polyacrylamide Gel Electrophoresis of β-Amylases.

Gel electrophresis was performed by Osborn's method using 10% acrylamide gel.

A: β-amylase from BQ10-S1

B: β-amylase from BQ10-S1 SpoII

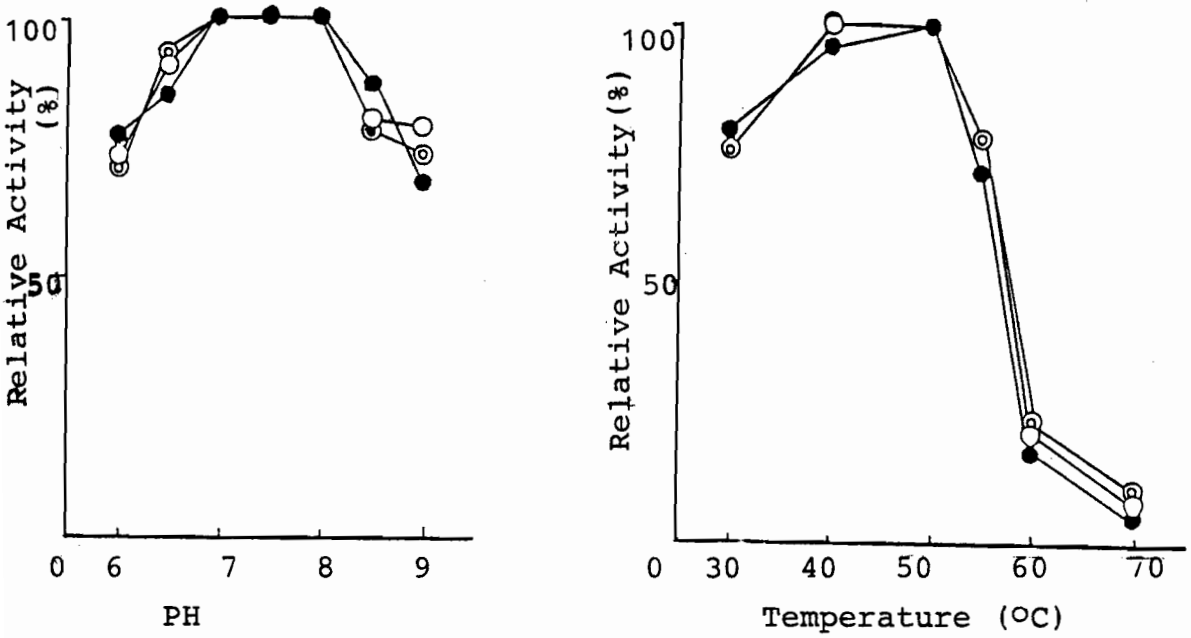


Fig. 9. Effects of pH and Temperature on β-Amylases from BQ10-S1, BQ10-S1 SpoI and BQ10-S1 SpoII.

The reactions were performed for 3min at various pH's at 40°C and at various temperatures at pH 7.0, respectively.

●—● β-amylase from BQ10-S1, ○—○ β-amylase from BQ10-S1 SpoI, ◎—◎ β-amylase from BQ10-S1 SpoII.

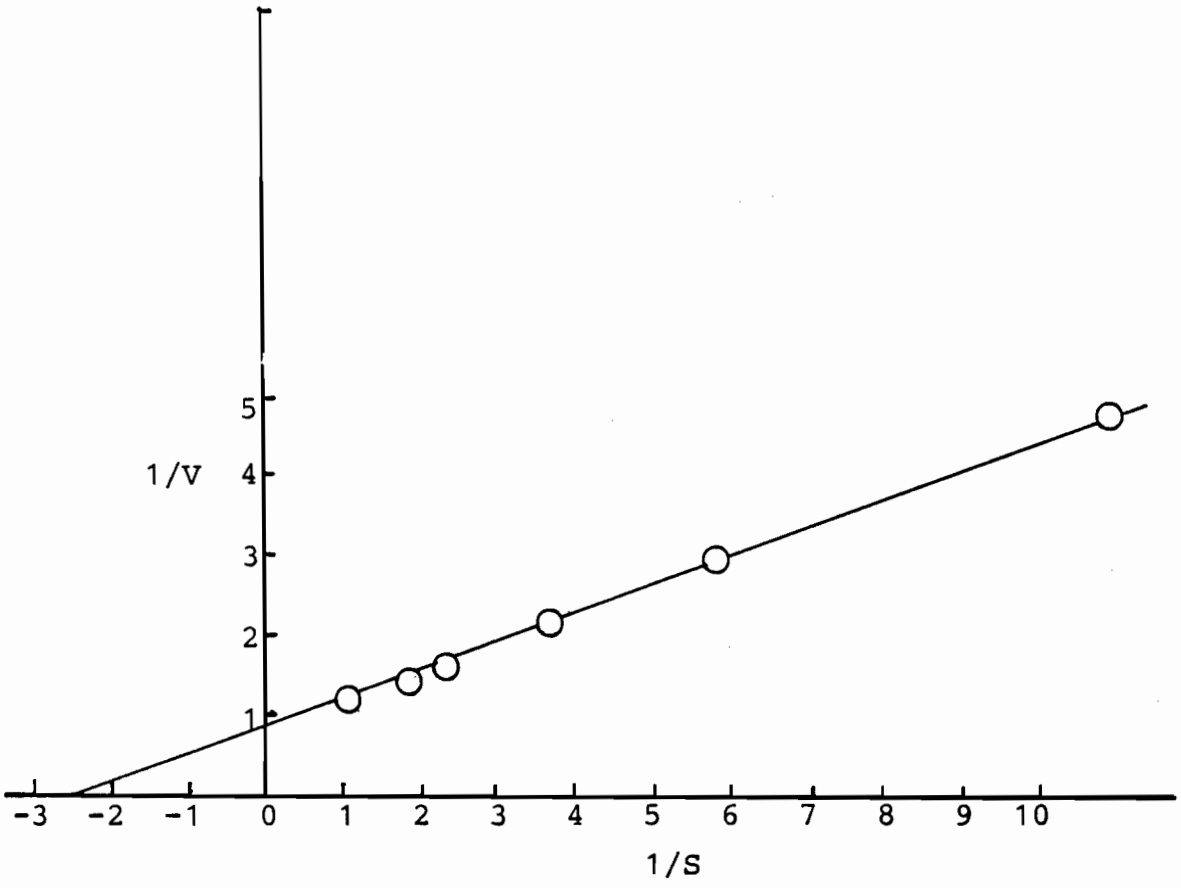


Fig.10. Lineweaver-Burk Plot for β-Amylase.
The reactions were performed for 3 min at various concentrations of soluble starch in 50mM phosphate buffer (pH 7.0) with 30 units/ml (%) of β-amylase.

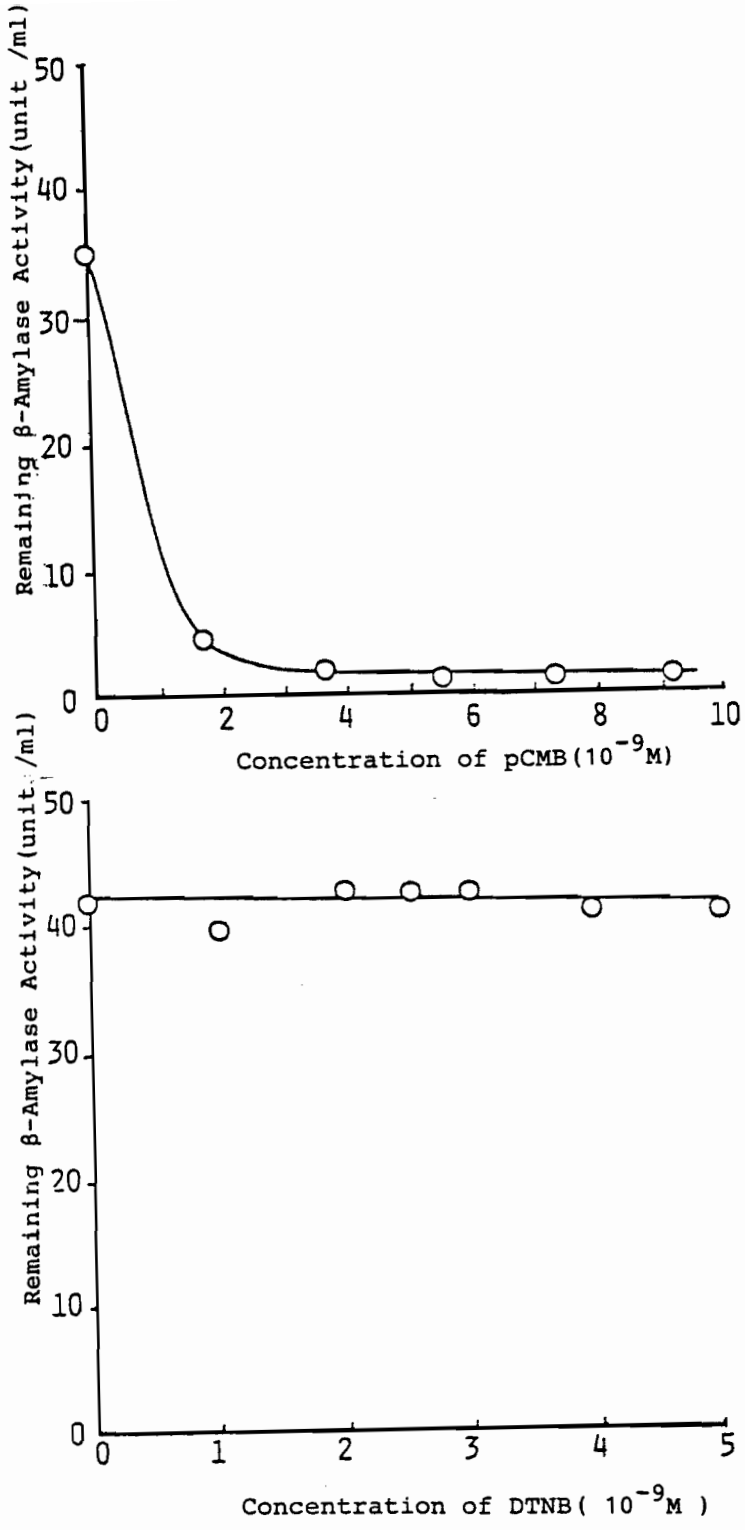


Fig.11. Effects of pCMB and DTNB on β-Amylase Activity.

The β-amylase from BQ10-S1 SpoII was incubated with pCMB and DTNB at various concentrations at 30°C for 20min. Then, the remaining activity was measured at 40°C for 30min.

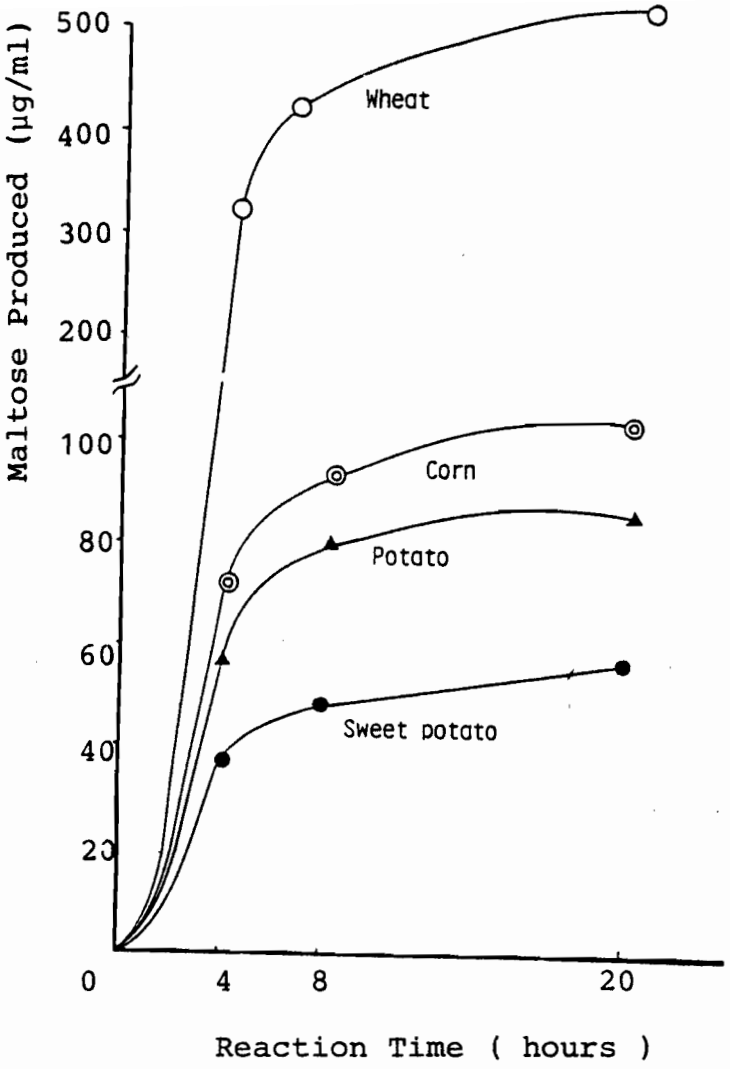


Fig.12. Degradation of Raw Starch by β-Amylase from B.cereus BQ10-S1 SpoII.

Each 200mg of various raw starches was suspended in 20ml of phosphate buffer (pH 7.2) containing the β-amylase (36units) and incubated at 40°C. After centrifugation(^{3},000×g , 10min), the amount of reduced sugars of its supernatant was measured by Somogyi-Nelson method.

Chapter III. Physicochemical Properties of the β-Amylase from *B. cereus* BQ10-S1 Spo II.

The measurement of molecular weight of the β-amylase and the determination of amino acid composition are described in this Chapter. The physico-chemical properties of bacterial β-amylase will be compared with those of plant-β-amylases. The author also deals with detection of sugars in the β-amylase molecule.

Materials and Methods

Calculation of molecular weight

The molecular weight of the purified β-amylase was measured by sedimentation equilibrium, gel filtration, SDS-PAGE and amino acid analysis. i) Sedimentation equilibrium. A Beckman-Spinco centrifuge (Model E type) was used at 12,000rpm. It tooks 20 hr to reach the equilibrium and the initial concentration was determined using a synthetic boundary cell and Rayleigh's interference system. ii) Gel filtration. The standard proteins and β-amylase were denatured in 6M guanidine HCl and 0.1M 2-mercaptoethanol (2-ME) solution and then carboxymethylated with monoiodoacetate. The denatured proteins were loaded on a Sephacryl S-200 column equilibrated with 6M guanidine HCl and eluted with the same solution. iii) SDS-PAGE. SDS-PAGE was performed by Osborn's method[55] using slab gel (7.5%-15% acrylamide gradient gel). iv) Amino acids analysis. Amino acid analysis was performed with a Hitachi 034 analyzer after hydrolysis of β-amylase with 6N HCl at 110°C for 24, 48, and 72 hr, respectively. Cysteine residues were determined by performic acid oxidation and tryptophan

residues by using thioglycollate as antioxidant.

Determination of sulfhydryl groups

Sulfhydryl groups of β-amylase were determined with DTNB and pCMB, by Elluman's[58] and Riordan's methods.[59] The reaction with DTNB was performed in the presence of 0.2% SDS.

Determination of isoelectric point

Isoelectric focusing on a slab polyacrylamide gel was performed with an Atto isoelectric focusing apparatus (SJ-1071) with 5% acrylamide gel containing 2% Ampholite (pH 3.5-10) (LKB Laboratory). Fifteen μl of the enzyme solution was charged to the slab gel and isoelectric focusing was carried out at a constant current of 5mM. For staining of gel 0.2% Coomassie Brilliant Blue R (Shigma Chemical Co.) was used.

Detection of sugars in the β-amylase molecule[60]

Electrophoresis of the purified β-amylase was performed on acrylamide slab gel(7.5%-15% gradient gel) and then the gel was soaked in 40% methanol and 7% acetic acid solution in order to remove SDS and fix the protein. Next it was incubated in 1% periodic acid and 7% acetic acid for 1 hr under the dark condition and washed with 7% acetic acid. The glycoprotein in the gel was stained with Schiff's reagent in the dark.

Results

The molecular weight was calculated to be approximately 6.2×10^4 by gel filtration on Sephacryl S-200 in the presence of 6M guanidine HCl (Fig.13), and 6.0×10^4 by SDS-PAGE (Fig.14). It was also estimated to be 6.3×10^4 by sedimentation equilibrium.

Fig.15 shows the linear relationship between log(fringe displacement) and $(radius)^2$. Table III shows the amino acid compositions of β-amylases from BQ10-S1, BQ10-S1 SpoI and BQ10-S1 SpoII based on one cysteine group per molecule from the results of performic acid oxidation. Each molecular weight calculated from the amino acid composition was 5.5×10^4.

As shown in Table IV, the enzyme could only react with DTNB in the presence of 0.2% SDS. Based on the molecular weight of 6.0×10^4, only one sulfhydryl group was determined by both methods.

The isoelectric point of the purified enzyme was determined to be 8.3 and those of the β-amylases from BQ10-S1 abd BQ10-S1 SpoI were identical with it.

Since the gel was stained by Schiff's reagent, it was clear that carbohydrates associated in the β-amylase molecule (Fig.16).

Discussion

The molecular weights of bacterial β-amylases so far reported seem to be lower than those of plant β-amylases.[19), 27)-29)] We previously found the molecular weight of β-amylases to be about 3.6×10^4 by gel filtration on a Sephadex column equilibrated with 60mM phosphate buffer (pH 7.0).[27)] We did not question the results until a sedimentation coefficient of $4.8S_{20,w}$ was obtained and the molecular weight by SDS-PAGE turned out to be 6.0×10^4. So we reexamined the molecular weights of β-amylases from BQ10-S1, BQ10-S1 SpoI and BQ10-S1 SpoII by gel filtration at high ionic strength and obtained a higher value of 4.4×10^4 in each case. The enzymes denatured with 6M guanidine HCl and 0.1M 2-ME were further subjected to gel filtration with standard proteins and the molecular weights

were about 6.2×10^4. These results suggest that molecular weight of bacterial β-amylases in the native state may not be correctly determined by gel filtration. Andrews[61] and Wilding[62] also pointed out the same problem in estimating molecular weights of enzyme capable of reacting with carbohydrates. Taking into account the results obtained above, it can be concluded that the molecular weight of the purified enzyme from BQ10-S1 SpoII is about 6.0×10^4 [34] (Table V). This value is very close to those of β-amylase from higher plants like soybean,[8] wheat,[11),12] sorghum malt[63] and barley.[5]

Isoelectric point of the β-amylase from B.cereus BQ10-S1 SpoII was not in acidic side like plant-β-amylases, but was 8.3 (TableVI).

The molecular weights of the β-amylases from B.megaterium strain No 32 and B.polymyxa No 72, which were kindly given by Dr. Okada and Dr. Murao, were measured on SDS-PAGE and calculated to be about 6.0×10^4 and 6.7×10^4, respectively.

Sugars were also detected in these β-amylases. This is the first finding in bacterial β-amylase.

The relationship between sulfhydryl group and β-amylase activity has so far been discussed. In plant-β-amylase molecules five cysteine residues were found, but in the case of B.cereus BQ10-S1 SpoII, only one cysteine residue was determined (Table VII). This sulfhydryl group essential for the activity did not seem

to locate on the surface of the molecule because of the less reactivity with DTNB, which was in agreement with the case of plant-β-amylases.[13),14)]

Some comparative experiments on the β-amylases from BQ10-S1, BQ10-S1 SpoI and BQ10-S1 SpoII were performed and the results obtained have shown that all the enzymes are identical (Table III and Fig.6,8,11). Therefore, the high β-amylase production by BQ10-S1 SpoII as in the case of BQ10-S1 SpoI, may be ascribable to a quantitative increase in the same enzyme protein as that of BQ10-S1.

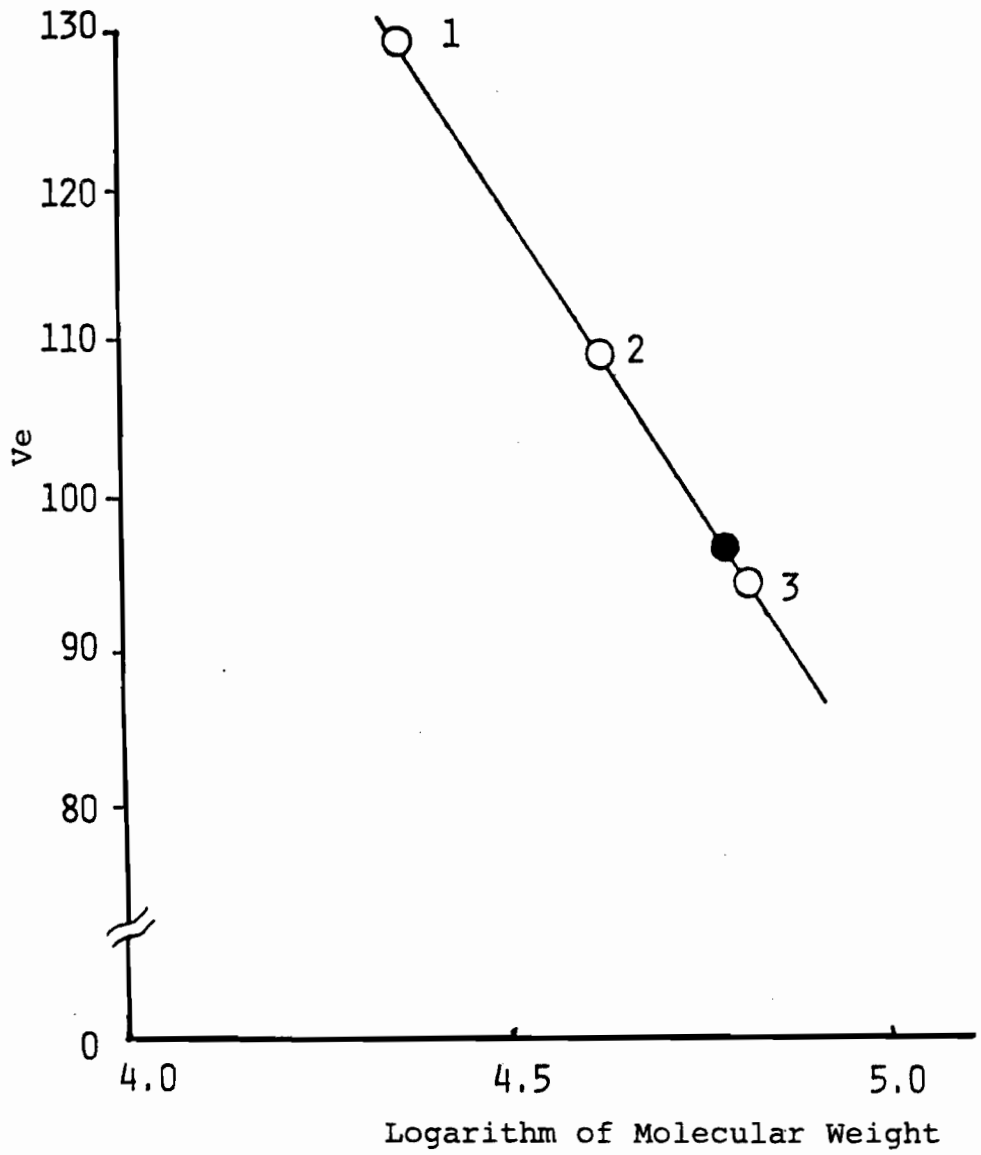


Fig.13. Estimation of Molecular Weights of β-Amylases by Gel Filtration on Sephacryl S-200.

β-Amylases from BQ10-S1, BQ10-S1 SpoI and BQ10-S1 SpoII and standard proteins were denatured with 6M guanidine HCl and 0.1M 2-ME and carboxymethylated with monoiodoacetate. The denatured proteins were loaded on a Sephacryl S-200 column (2x50cm) equilibrated with 6M guanidine HCl and eluted with 6M guanidine HCl at 22°C.

1: chymotrypsinogen (25,000) 2: ovalbumin (45,000)

3: bovine serum albumin (67,000)

●: β-amylases from BQ10-S1, BQ10-S1 SpoI and BQ10-S1 SpoII (estimated to be 62,000)

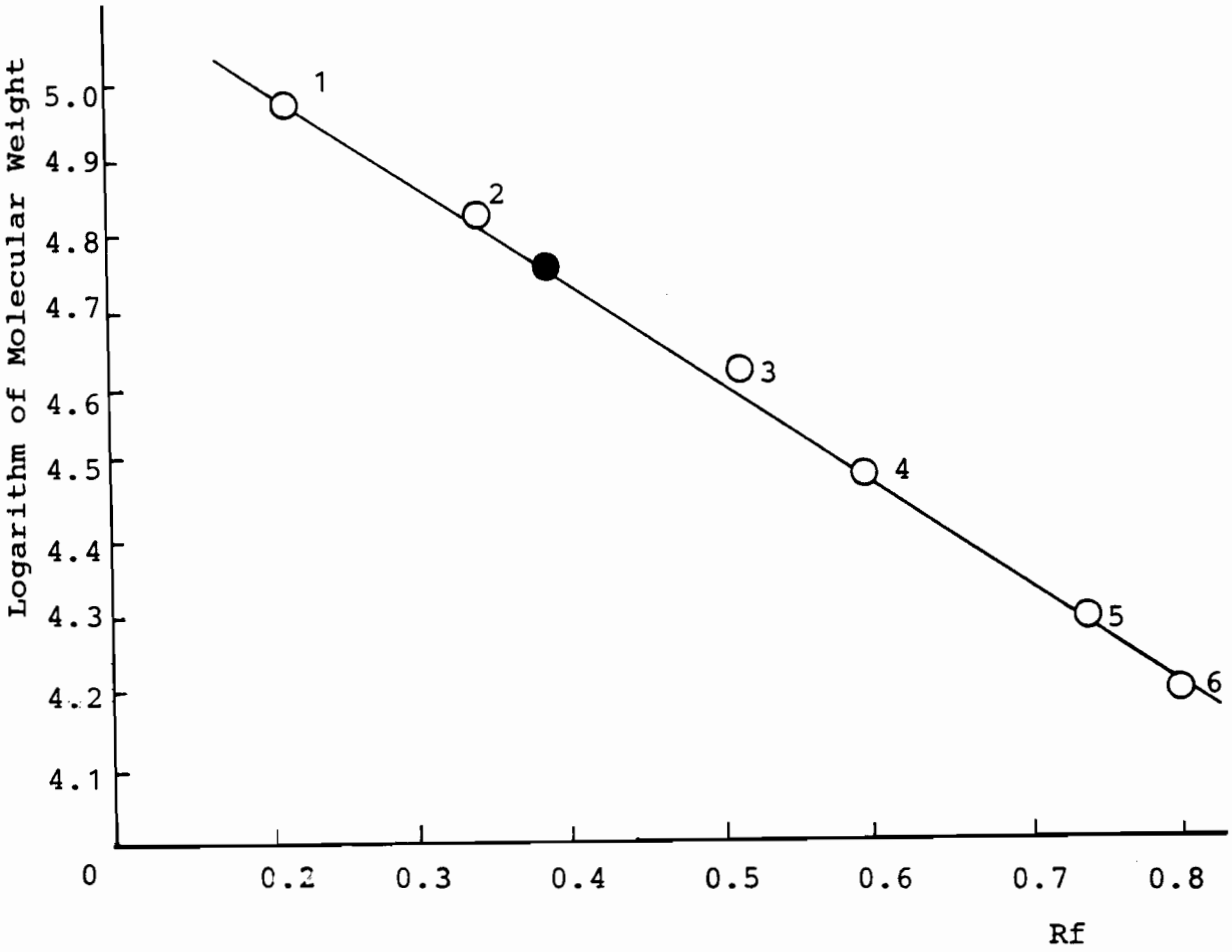


Fig.14. Estimation of Molecular Weights of β-Amylases by SDS-PAGE. Marker proteins (Pharmacia Fine Chemicals) and β-Amylases from BQ10-S1, BQ10-S1 SpoI and BQ10-S1 SpoII were denatured with 1% SDS and 1% 2-ME at 100°C for 3 min and run on 7.5-15% gradient polyacrylamide gel(slab gel). 1: phosphorylase b (94,000). 2: bovine serum albumin (67,000) 3: ovalbumin (43,000) 4: carbonic anhydrase (30,000) 5: trypsin inhibitor(20,000) 6: α-lactalbumin (14,400) ●: β-amylases from BQ10-S1, BQ10-S1 SpoI and BQ10-S1 SpoII (estimated to be 60,000)

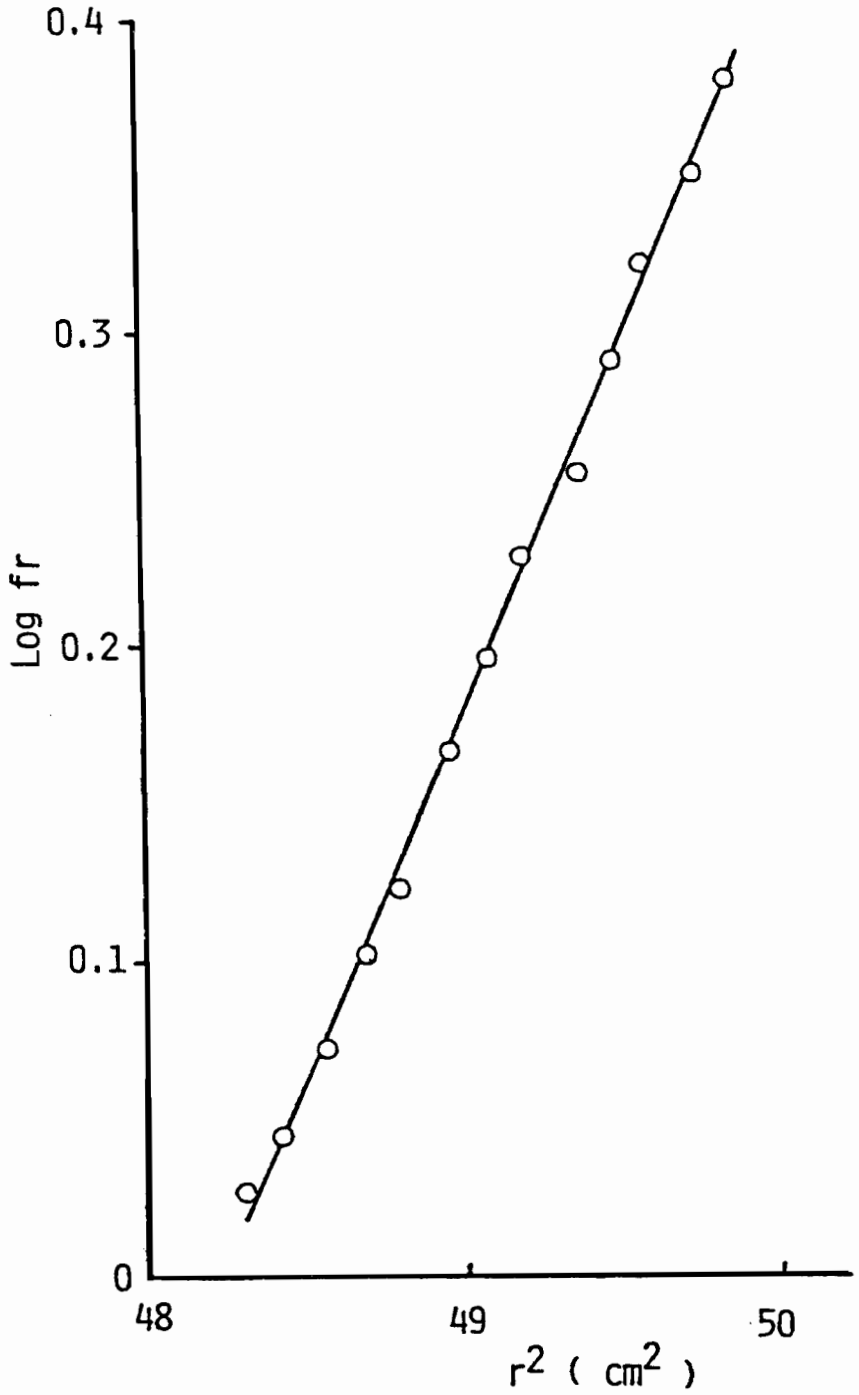


Fig.15. The Linear Relationship between (Radium : r)^2 and $\log_{10}$(Fringe Displacement : fr) on Analytical Ultracentrifugation.

Ultracentrifugation was carried out as described in Methods.

Table III. Amino Acid Compositions of β-Amylases from BQ10-S1, BQ10-S1 SpoI and BQ10-S1 SpoII.

Amino acid	Number of residues estimated by extrapolation		
	BQ10-S1	SpoI	SpoII
Lysine	43	42	42
Histidine	7	7	7
Arginine	11	11	11
Aspartic acid	61	62	60
Threonine	32	32	32
Serine	29	30	29
Glutamic acid	45	45	45
Proline	24	24	24
Glycine	41	41	41
Alanine	35	35	35
½Cystine*	1	1	1
Valine	24	24	24
Methionine	11	10	12
Isoleucine	20	20	20
Leucine	36	36	36
Tyrosine	30	30	30
Phenylalanine	21	21	21
Tryprophan**	16	16	16
Total	487	487	486

* Estimated by the performic acid oxidation method, Elluman's method and Riordan's method.

** Thioglycollate was used as antioxidant.

Table IV. Determination of Sulfhydryl Groups of β-Amylase from *B.cereus* BQ10-S1 SpoII.

Method	Conditions	Number of SH group detected
Amino acid analysis	After performic acid oxidation	0.92
DTNB	In 0.1 M Tris-HCl buffer (pH 8.0)	0
DTNB	IN 0.1 M Tris-HCl buffer (pH 8.0) in presence of SDS	0.92
pCMB	In 0.1 M phosphate buffer (pH 7.0)	0.88

The amino acid analysis was carried out after performic acid oxidation of the enzyme protein. Determination by Elluman's method in the presence of 0.2% SDS and by Riordan's method was based on the molecular weight of 6.0×10^4 of the enzyme.

Table V. Estimation of Molecular Weight of β-Amylase by Various Methods.

Gel filtration	Sephadex G-100	Native state 60mM phosphate buffer(pH 7.0)	3.6×10^4
	Sephacryl S-200	Denatured state 6M guanidine HCl and 2-ME	6.2×10^4
Electrophoresis	SDS-PAGE	Denatured state 1% SDS and 1% 2-ME	6.0×10^4
Sedimentation equilibrium	Beckman-Spinco Model E type	52,000rpm or 10,000rpm 25mM phosphate buffer(pH 7.0) 0.1M NaCl	$4.8\ S_{20,w}$ 6.3×10^4
Amino acid composition	Hitachi Amino Acid Analyzer	1/2 Cys: Performic acid oxdation Try: Thioglycollate as antioxidant	5.5×10^4

Table VI. Pysicochemical properties of various β-Amylases.

	Soybean II[15]	Soybean[8]	Barley[5]	Malted Sorghum[63]	Wheat[11),12)]	Sweet Potato
Molecular weight	57,000	61,700	57,200	55,900	64,200	197,000
$S_{20,w}$	4.50	4.67	4.43	4.24	4.58	8.90
Isoelectric point	5.52	5.85	4.65-5.9		4.8-5.8	4.8

	B.cereus BQ10-S1 SpoII[34)]	B.cereus var. mycoides[28)]	B.megaterium[19),20)]	B.polymyxa[29)]
Molecular weight	60,000	(35,000±5,000)	(36,000-38,000) 60,000(SDS-PAGE)	(44,000) 67,000(SDS-PAGE)
$S_{20,w}$	4.8			
Isoelectric point	8.3		9.1	8.35, 8.59

( ): molecular weight estimated by gel filtration(native state).

Table VII. Comparison of Amino Acid Compositions of Various β-Amylases.

	Sweet potato[13]	Barley[5]	Soybean[8]	Soybean[16] 2	4	6	B.cereus BQ10-S1 SpoII[34]
Lysine	25	19.4	32	29	30	31	42
Histidine	7	14.7	12	11	10	11	7
Arginine	15	24.1	20	18	19	19	11
Aspartic acid	48	57.4	83	65	63	62	60
Threonine	12	16.6	23	18	17	17	32
Serine	14	21.8	33	27	24	27	29
Glutamic acid	34	61.5	70	51	52	50	45
Proline	23	32.0	40	30	30	30	24
Glycine	31	48.7	53	40	39	40	41
Alanine	30	41.8	42	33	32	33	35
½Cystine	5	4.4	5	5	5	5	1
Valine	25	34.9	38	31	31	31	24
Methionine	14	15.0	9	9	12	9	12
Isoleucine	18	19.3	31	26	26	26	20
Leucine	29	38.8	62	46	45	46	36
Tyrosine	17	24.7	30	25	26	25	30
Phenylalanine	17	24.3	22	21	21	21	21
Tryptophan	10	9.4	12	12	12	12	16
Total residues	347		617	497	494	495	486

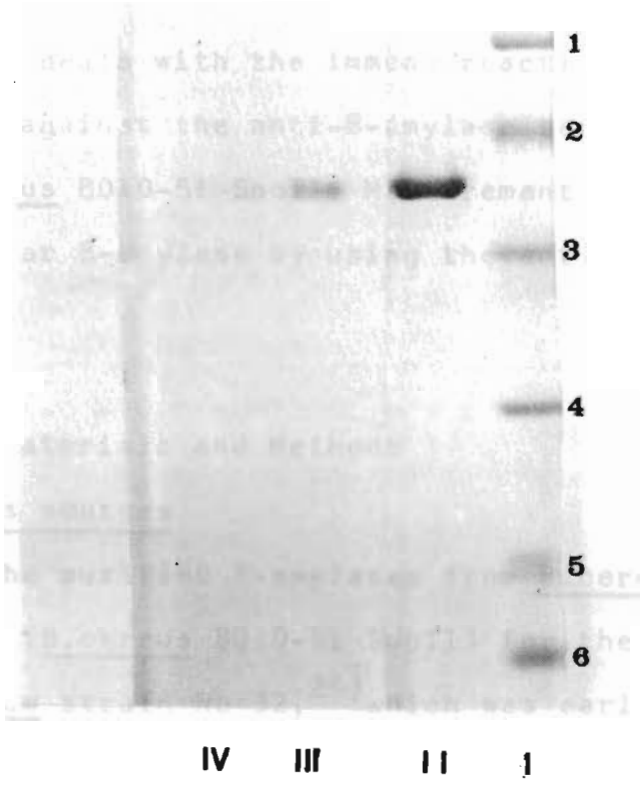


Fig.16. Detection of Sugars in the β-Amylase Molecule Purified from the Culture of B.cereus BQ10-S1 SpoII.

Each 10μg, 20μg of the purified β-amylase and marker proteins (Pharmacia, Fine Chemicals) were dissolved in 1% SDS and 2-ME solution and placed in boiling water for 3 min.

They were run on 7.5-15% polyacrylamide gradient gel (slab). Marker proteins and purified β-amylase (lane I,II) were stained by Coomassie Brilliant Blue R (Shigma Chemical Co.). Others (lane III,IV) were stained in the gel by Schiff's reagent as described in Methods.

lane I: 1. 94,000 2. 67,000 3. 43,000 4. 30,000 5. 20,100 6.14,400

lane II, III, IV: purified β-amylase (20μg, 20μg, and 10μg)

Chapter IV. Immunological Studies for Bacterial β-Amylases.

The author deals with the immuno-reaction of various β-amylases against the anti-β-amylase serum of the β-amylase from B.cereus BQ10-S1 SpoII. Measurement of the amounts of extracellular β-amylase by using the anti-β-amylase serum is also described.

Materials and Methods

β-Amylases sources

We used the purified β-amylases from B.cereus BQ10-S1 and its mutant (B.cereus BQ10-S1 SpoII) for the following experiments. B.megaterium strain No 32,[20] which was early reported as β-amylase-producing bacteria, was kindly given by Dr. Okada (Osaka Municipal Technical Reserch Institute.). The extracellular β-amylase from the bacterium was purified according to his report.[20] The purified β-amylase from B.polymyxa No 72[29] was kindly supplied by Professor Murao (University of Osaka Prefecture). The purified β-amylases from barley (Sigma Co, Ltd), sweet potato (Seikagaku Kogyo Co, Ltd) and soybean (Wako Chemical Co, Ltd) were obtained commercially.

Preparation of anti-β-amylase serum and immunotitration

β-Amylase solution (0.5ml) was mixed with an equal volume of Adjuvant Freund (Nakarai Chemical. Co, Ltd) and injected into

rabbit derm five times at a week interval.(8mg of β-amylase was injected totally). Various volumes of anti-β-amylase serum were added to the β-amylase solution (36μg). After the reaction at 35°C for 30 min, the immunoprecipitates were removed by centrifugation and the β-amylase activity of the supernatants was measured by Bernfeld's method . The protein contents of the antiserum were measured by Lowry's method.

Determination of the amounts of the secreted β-amylase

Each volume of anti-β-amylase serum was added onto the solution of β-amylase (36μg), until the immunoprecipitate gave constant value. The relation between amounts of antigen and immunoprecipitate was represented according to Heidelberger,[64] from which unknown amounts of extracellular β-amylase were estimated.

Immunological comparison of various β-amylases

Immunological comparison of β-amylase from B.cereus BQ10-S1 SpoII with other β-amylases was performed by Micro-Ouchterlony method.[65]

Results

As shown in Fig.17, the activity of the purified β-amylase decreased together with added volumes of the antiserum increased, confirming that the anti-β-amylase IgG was formed.

Fig.18 depicts an experiment in which antiserum prepared to B.cereus BQ10-S1 SpoII β-amylase was allowed to react in Ouchterlony double-diffusion plate with parent, BQ10-S1 SpoI and BQ10-S1 SpoII. It was shown that complete fusion of the

precipitin bands from the three antigen-antibody complexes occurred, confirming that β-amylase obtained from three species is identical. Spur formation was indicated in precipitin bands of β-amylase from B.cereus BQ10-S1 SpoII and that from B.megaterium strain No 32 (Fig.19).

But immuno-reaction didn't occur with the β-amylase from B.polymyxa No 72 and soybean-β-amylase (Fig.20), as in the case of other higher plant β-amylases (data not shown).

The amounts of extracellular TCA-insoluble materials were measured to be about 75μg/ml for BQ10-S1 and 300μg/ml for BQ10-S1 SpoII respectively. On the other hand, those of extracellular β-amylases from BQ10-S1 and BQ10-S1 SpoII were measured to be 25μg/ml and 95μg/ml, respectively (Fig.21, Table VIII).

Discussion

The author prepared an antiserum to the β-amylase from B.cereus BQ10-S1 SpoII and carried out the immunological study on β-amylases by Micro-Ouchterlony method. The similar immunological experiment has been carried out on α-amylases from Bacillus.[66] α-Amylase from B.subtilis Marburg cross-reacted with anti-α-amylase serum against that from B.subtilis var. amylosaccharicus but did not react with α-amylase from B.amyloliquefaciens.

On the other hand, it is interesting in the case of Himalaya Barley that the isozymes of α-amylase produced in aleurone layers were immunologically different.[67]

β-Amylases of B.cereus BQ10-S1 SpoII and B.megaterium strain No 32 were found to maintain similar conformation which determined the antigen-antibody reaction, suggesting a similar genetic evolution. The molecular weights of these β-amylases were also calculated to be equal on SDS-PAGE. Contrariwise, the β-amylases from B.polymyxa No 72 and higher plants did not react with the antiserum.

The amounts of extracellular protein were estimated to be 75μg/ml for BQ10-S1 and 300μg/ml for BQ10-S1 SpoII, and the amounts of extracellular β-amylases were also to be 25μg/ml and 95μg/ml, respectively. These results show that this mutant is capable of secreting more amount of β-amylase and also of other extracellular proteins. It is concluded,therefore, that the increased amounts (about 4 times) of secreted β-amylase of B.cereus BQ10-S1 SpoII result in the higher β-amylase activity (4 times) than that of its parent strain (B.cereus BQ10-S1).

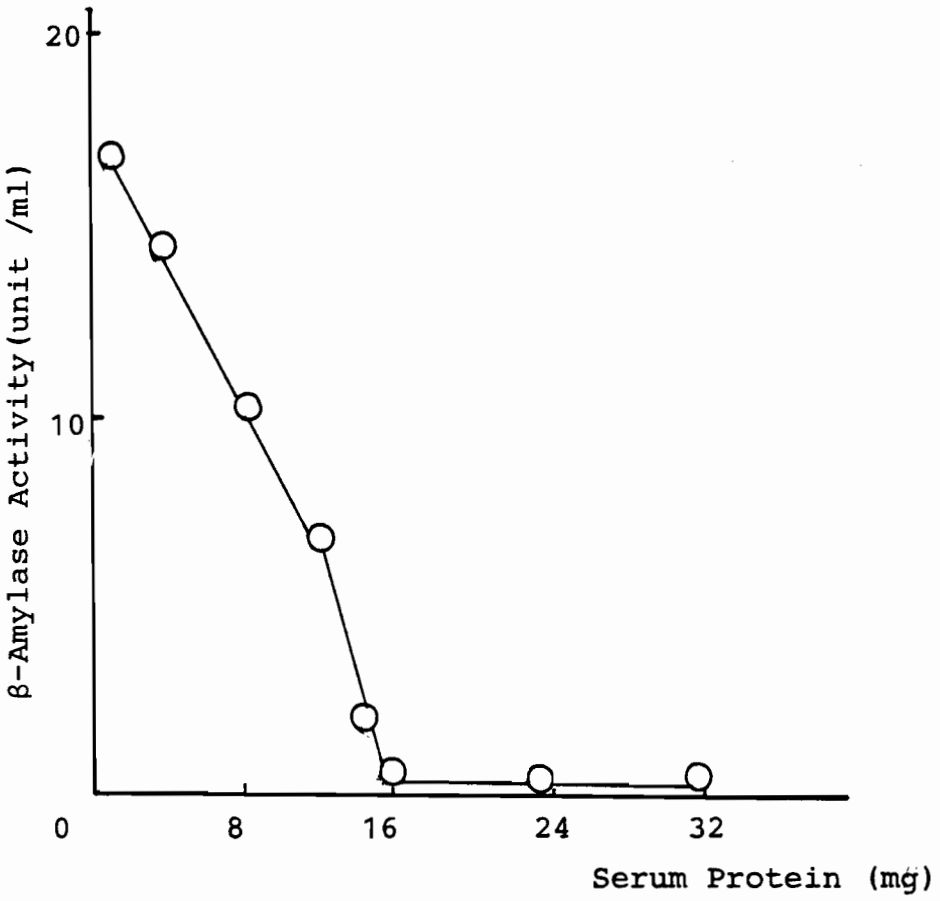


Fig.17. Immunotitration Pattern of β-Amylase by Its Rabbit Antiserum.

Various volumes of anti-β-amylase serum were added to the β-amylase (36μg) dissolved in 50mM phosphate buffer (pH 7.2) and the mixture was incubated at 35°C for 3 min. The formed immunoprecipitates were removed by centrifugation (3,000×g , 5 min). The β-amylase activity of the supernatant was measured. The protein content of the antiserum was measured by Lowry's method.

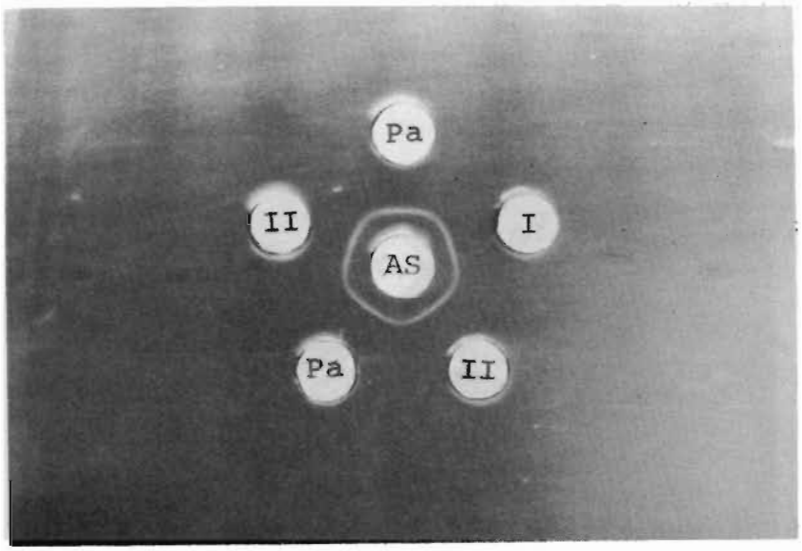


Fig.18. Immunological Comparison of β-Amylases from B.cereus BQ10-S1, BQ10-S1 SpoI and BQ10-S1 SpoII.

AS: Antiserum against β-amylase from B.cereus BQ10-S1 SpoII was added. Pa: β-Amylase from BQ10-S1 was added.

I: β-Amylase from BQ10-S1 SpoI was added.

II: β-Amylase from BQ10-S1 SpoII was added.

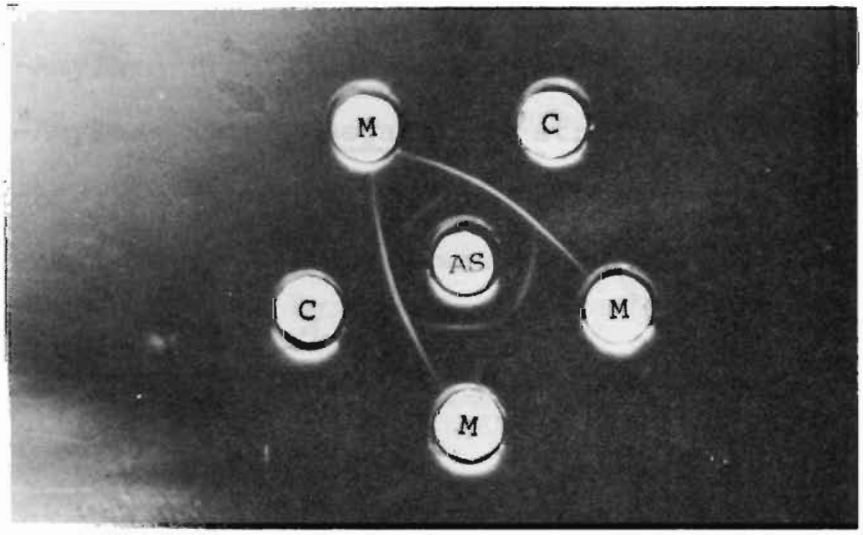


Fig.19. Immunological Comparison of β-Amylase from B.cereus BQ10-S1 SpoII with that from B.megaterium strain No 32.

AS: Antiserum against β-amylase from B.cereus BQ10-S1 SpoII was added. C: β-Amylase from B.cereus BQ10-S1 SpoII was added.

M: β-Amylase from B.megaterium strain No 32 was added.

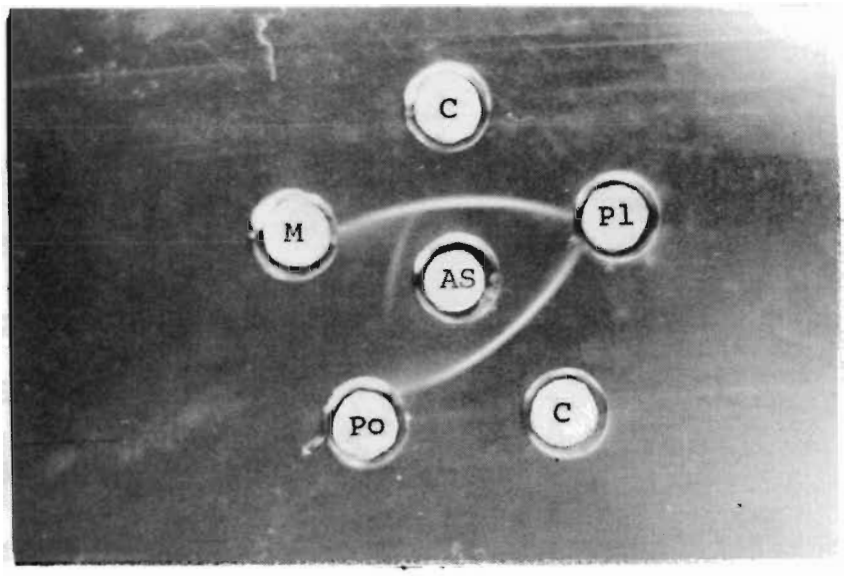


Fig.20. Immunological Comparison of Various β-Amylases.

AS: Antiserum against β-amylase from B.cereus BQ10-S1 SpoII was added.

C: β-Amylase from B.cereus BQ10-S1 SpoII was added.

M: β-Amylase from B.megaterium strain No 32 was added.

Po: β-Amylase from B.polymyxa No 72 was added.

Pl: β-Amylase from soybean was added.

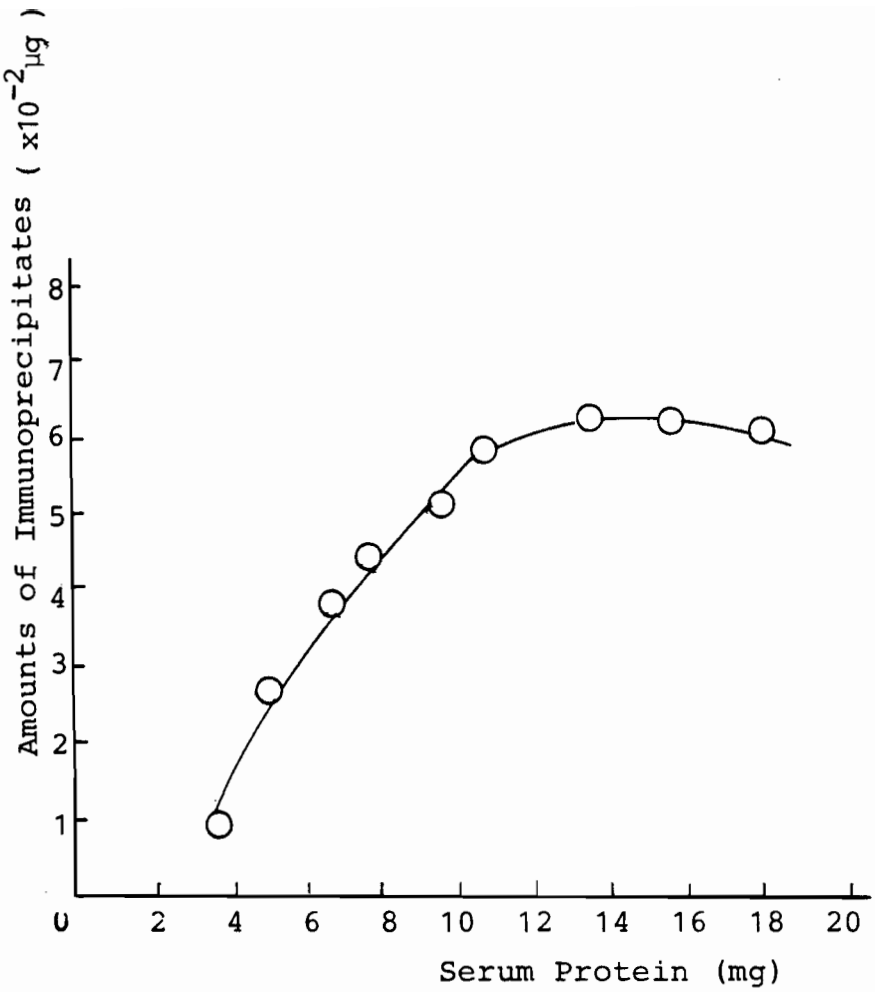


Fig.21. The Quantitative Changes of Immunoprecipitates.
Each volume of anti-β-amylase serum was added to 50mM phosphate buffer containing 32.5µg of β-amylase. These mixtures were incubated at 35°C and centrifuged (3,000 x g , 5 min). Protein contents of immunoprecipitates were measured by Lowry's method.

Table VIII. Determination of Extracellular β-Amylases from *B.cereus* BQ10-S1 and *B.cereus* BQ10-S1 SpoII.

	TCA-insoluble protein	Extracellular β-amylase
BQ10-S1	75μg/ml	25μg/ml
BQ10-S1 SpoII	300μg/ml	95μg/ml

Chapter V. Studies on Mechanism of β-Amylase Secretion.

In this Chapter, the author describes the identification of the extracellular and intracellular proteins which reacted immunologically with the anti-β-amylase serum, and the detection of a protein translated from β-amylase mRNA from B.cereus BQ10-S1 SpoII in a cell-free system.

Materials and Methods.

Preparation of extracellular, and intracellular immunoprecipitates

Extracellular: To the culture filtrate of B.cereus BQ10-S1 SpoII, 0.5ml of the unsensed rabbit serum was added in order to avoid the non-specific reactions. After centrifugation, equal volumes of 0.2% SDS and the anti-β-amylase serum (0.2ml) were added to the supernatant and incubated for 30 min at 35°C. Then the immunoprecipitates formed were harvested by centrifugation (3,000xg, 10 min) and washed three times with 0.01M sodium phosphate buffer (pH 7.0) containing 0.9% NaCl, 0.1mM EDTA and 0.01% SDS.[68)]

Intracellular: The harvested cells were washed four times with a saline solution and sonicated. The cell-homogenate was centrifuged (20,000xg, 15 min) and the supernatant was then centrifuged (100,000xg, 1hr). The supernatant was treated in the same manner as extracellular immunoprecipitation.

Determination of secreting phase of β-amylase

^{14}C-Amino acid mixture (protein hydrolysates, 10μCi) was added to the culture of B.cereus BQ10-S1 SpoII during shaking culture at several-hour interval after inoculation, and the culture was continued for 2 hr. The extracellular and intracellular immunoprecipitates were prepared from the culture as described above and the incorporated radioactivities to them were measured by liquid scintillation counter(Aloka LSC-903).

Identification of the immunoprecipitates by Western-Blotting method [69),70)]

The purified β-amylase and the immunoprecipitates of culture broth(cells and medium) were migrated in polyacrylamide gel containing SDS (slab gel) by SDS-PAGE. These proteins in the gel were transferred to a nitrocellulose sheet (Toyoroshi Co Ltd, TM 2) by electrophoresis (40 V, 2hr, at 0°C). The sheet was soaked in 3% bovine serum albumin solution containing 0.1% NaCl for 30 min at room temperature, and incubated with the anti-β-amylase serum for 30 min at room temperature. It was washed three times with 10mM phosphate buffer (pH 7.0) containing 137mM NaCl, 2.7mM HCl, 8mM Na_2HPO_4, and 2mM KH_2PO_4.

The sheet was incubated with fluorescein-conjugated goat anti-rabbit IgG (Cappel Laboratories) containing 3% bovine serum albumin, and washed with the phosphate buffer. The target protein was detected on the sheet by UV-irradiation.

Preparation of RNA fractions from B.cereus BQ10-S1 SpoII

The cells of B.cereus BQ10-S1 SpoII were harvested from the culture in the middle logarithmic phase, and washed with a saline solution. The cells (8g wet weight) were ground in a mortar precooled at -20°C with the equal weight of alumina and 2ml of Buffer I consisted of 0.01M NaPB pH 7.4, 0.01M $MgCl_2$, 0.01M NaCl, 0.01M NaN_3, 1% SDS, 0.1% diethylpyrocarbonate for 4 min, followed by the addition of 5ml of Buffer I and an equal volume of water-saturated m-cresol.[71] The mixture was centrifuged (20,000xg,15 min) and the supernatant was mixed with an equal volume of water-saturated phenol and centrifuged (20,000xg, 15 min). NaCl (final concentration to 0.1M) and 2 volumes of ethanol were added to the supernatant and stored at -20°C for several hours. The RNA fraction was collected by centrifugation and stored at -70°C.

Preparation of S-30 and S-100 fractions from E.coli K-12

Frozen cells of E.coli K-12 (5g) were ground in a mortar with 2 amounts of alumina for 2 min and then DNase (Worthington) was added (3μg/ml), and ground again for about 5 min. The cell homogenate was mixed with 2 volumes of Buffer A [71] (10mM Tris-HCl pH 7.8, 60mM NH_4Cl, 10mM Mg-Acetate, 6mM 2-ME, 3.45mM PMSF, 0.2mM DFP) and centrifuged (3,000xg, 20 min). The supernatant was centrifuged once more (30,000xg, 30 min). A part of the supernatant was dialyzed against 500 volumes of Buffer A and stored at -70°C (S-30 fraction). The other part was ultracentrifuged(100,000xg,3 hr) and the supernatant was stored at -70°C (S-100 fraction).

Reaction mixture for cell-free protein synthesis

S-30 fraction (0.1ml) preincubated at 35°C for 7 min was mixed with 0.1ml of 50mM Tris-HCl buffer (pH 7.8) containing 60mM NH_4Cl, 10mM Mg-acetate, 1mM dithiothreitol, 1mM ATP, 0.2mM GTP, 5mM phosphoenolpyruvate, 30μg/ml of pyruvate kinase, 2μCi/ml of ^{3}H-leucine and other nineteen kinds of amino acids which composed a protein. Then S-100 fraction (0.05ml) and RNA fraction were mixed and incubated at 37°C for 20 min.

Preparation of immunoprecipitates from synthesized proteins and its identification by SDS-PAGE

The unsensed rabbit serum (0.1ml) was added to *in vitro* β-amylase synthetic system, as described above and centrifuged (3,000xg, 5 min). To the supernatant, the anti-β-amylase serum (0.1ml) was added and incubated at 35°C. After 10 min the anti-rabbit IgG (Cappel Laboratories) was added (50mg) and coprecipitated by incubating for 10 min at 35°C. The precipitate obtained by centrifugation of antigen-antibody reaction mixture was washed 4 times with the 0.01M sodium phosphate buffer (pH 7.0) containing 0.9% NaCl, 0.1mM EDTA and 0.01% SDS. The immunoprecipitate obtained was dissolved in the solution containing 1% SDS and 1% 2-ME, and migrated in polyacrylamide gel on SDS-PAGE. After electrophoresis, the dried gel was cut at 2.5mm interval and each slice was dissolved in 0.5ml H_2O_2. The radioactivity in each slice was measured by a liquid scintillation counter.

Results

The radioactivity incorporated to both extracellular and intracellular β-amylase immunoprecipitin showed the highest level during 6-8 hr after inoculum under the conditions tested. These results show that β-amylase is mainly secreted during the period from the middle to the late logarithmic phase (Fig.22).

A single protein band with molecular weight corresponding to the purified β-amylase was detected to react with fluorescein-conjugated goat anti-rabbit IgG on the nitrocellulose sheet transferred by Western-Blotting method from SDS-slab gel electrogram of the extracellular immunoprecipitates.

However, two proteins, one of which molecular weights was calculated to be 6.0×10^4 and the other to be $11\text{-}12 \times 10^4$, were detected in intracellular immunoprecipitates (Fig.23).

The radioactivity of ^{3}H-leucine was also detected in the TCA-insoluble fraction and immunoprecipitates of a protein synthesized by *in vitro* protein synthetic system with *B.cereus* BQ10-S1 SpoII RNA fraction. These results suggest that mRNA from the bacterium was actively translated (Table IX). The radioactivity was also detected in a protein fraction, of which molecular weight was estimated to be $10\text{-}11 \times 10^4$, only when RNA fraction from *B.cereus* BQ10-S1 SpoII was added (Fig.25). SDS-PAGE of the immunoprecipitate of extracellular fraction revealed that a single peak of radioactivity corresponded to the β-amylase protein (Fig.24).

In immunoprecipitate of intracellular fraction, two peaks of radioactivity were observed; one of them seemed to correspond to that of β-amylase, and the other to a protein fraction with almost the same molecular weight as one obtained from *in vitro* synthesized proteins.

Discussion

The author attempted to elucidate the mechanism of β-amylase secretion in *B.cereus* BQ10-S1 SpoII. Though the bacteria capable of secreting β-amylase were already reported, there was no information on the mechanism of secretion.

As mentioned in Introduction, the mechanism of secretion of α-amylase from *Bacillus* has not been understood completely.[39),40)]

The author evidenced the occurrence of an intracellular β-amylase precursor by the application of the Western-Blotting method.[69),70)]

The purified β-amylase was found in a single band on the position corresponding to that of the protein of which molecular weight was estimated to be 6.0×10^4 by using the Western-Blotting method, supporting immunologically the purity of β-amylase.

Immunoprecipitates of the intracellular fractions gave two antigen-antibody reactive bands; one of them was equal to the β-amylase, and the other had a molecular weight of $11\text{-}12 \times 10^4$. The latter protein band was also detected on the nitrocellulose sheet, even if SDS-PAGE was carried out after the intracellular fractions were treated at $100^{\circ}C$ for 10 min by the solution

containing 1% SDS, 2-ME, and 8M urea, in which was completely dissociated a protein. These suggest that the protein ($11\text{-}12\times10^4$) is not a dimer of the β-amylase (6.0×10^4). But, these proteins were not detected if the nitrocellulose sheet was incubated with the unsensed rabbit serum in place of the anti-β-amylase serum. From these results it seems that the protein ($11\text{-}12\times10^4$) is reactive immunologically with the anti-β-amylase serum and that it might be a precursor-like protein of the β-amylase.

These results were supported by the evidence that when B.cereus mRNA was incubated with in vitro protein synthetic system of E.coli K-12, a protein with molecular weight of $10\text{-}11\times10^4$ which was reactive to anti-β-amylase serum was detected, indicating that β-amylase is translated as proprotein with the molecular weight of $10\text{-}11\times10^4$.

However, whether or not the precursor might contain a signal peptide was not clear. Rational explanation on the fact that the detected precursor from intracellular immunoprecipitates (by Western-Blotting method) was not exactly the same molecular weight as that from the cell-free synthesized protein could not be derived.

The three species of the membrane bound α-amylase of B.subtilis Marburg were separated by gel filtration, and these components seemed to be precursors of the extracellular α-amylase[40] (Mw. 5.5×10^4). But it was not clear whether they were composed of a single polypeptide or bounded with other membrane proteins.

How a β-amylase precursor of B.cereus BQ10-S1 SpoII changes to its extracellular form in the secreting process is not clear.

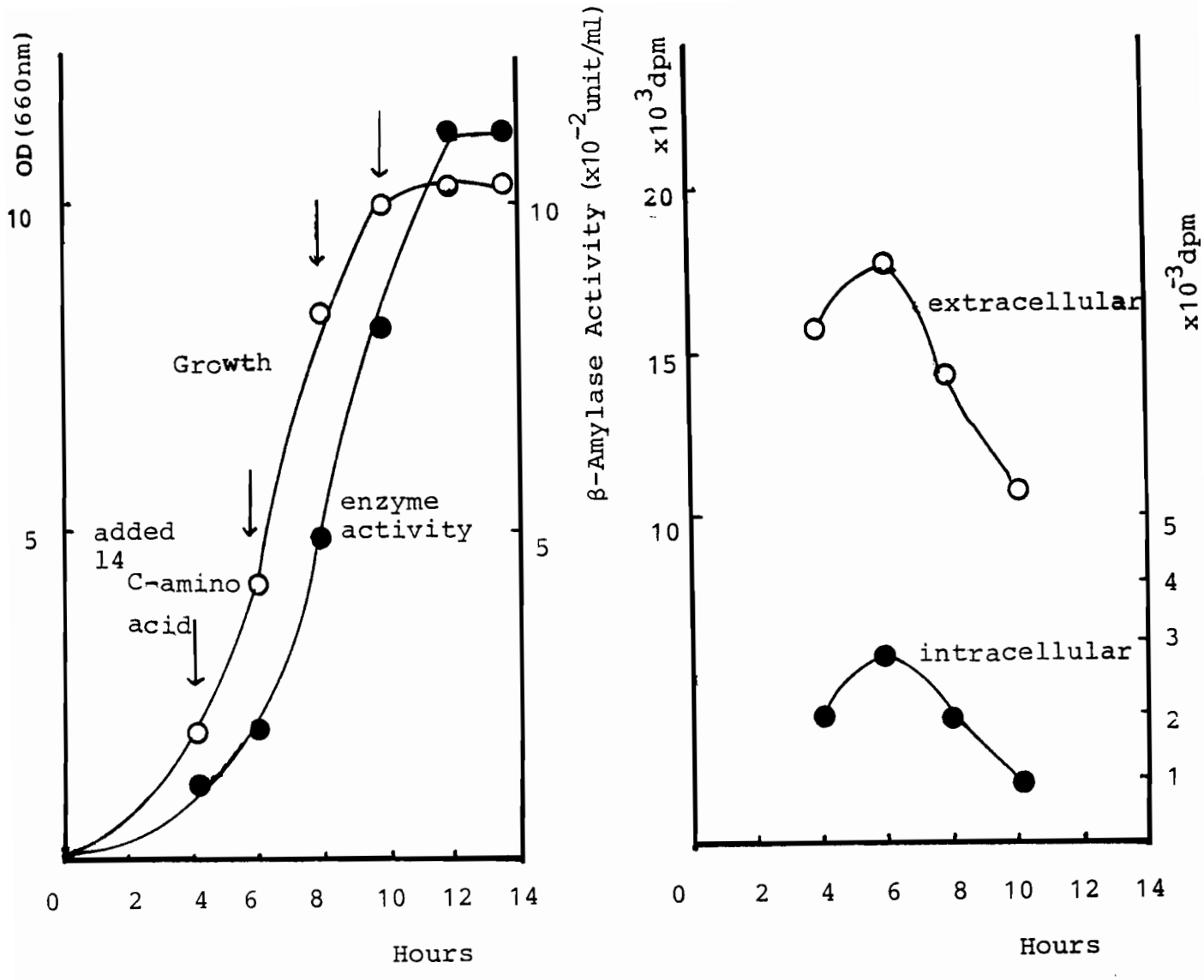
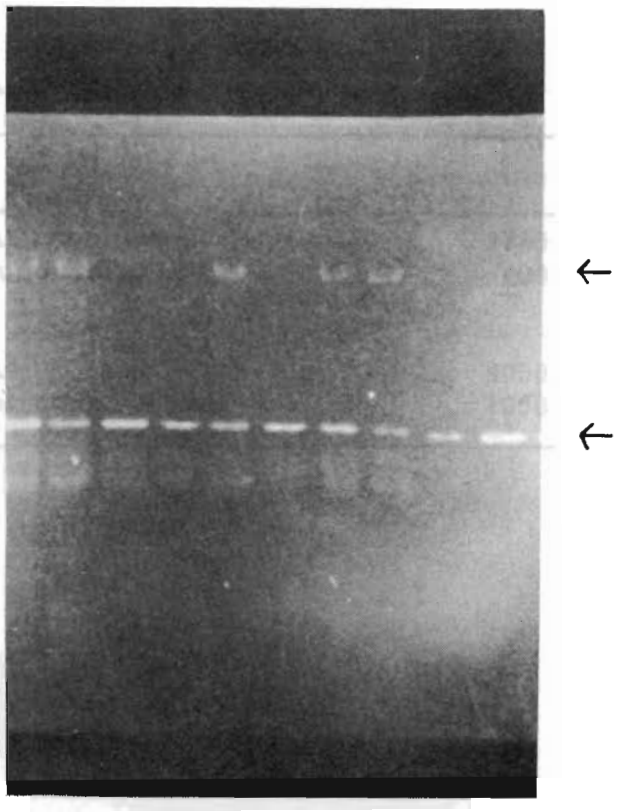


Fig.22. Estimation of the Secretion Period of the β-Amylase during Culture.

^{14}C-Amino acids (protein hydrolysates, 10μCi) were added to the culture of B.cereus BQ10-S1 SpoII during shaking culture at several-hour interval after inoculation (↓), and the culture was continued for 2 more hours. The extracellular and intracellular immunoprecipitates were prepared as described in Materials and Methods. The incorporation of the radioactivity (^{14}C-Amino acids) to them was measured by liquid scintillation counter.



I_5 I_4 E_4 E_3 I_3 E_2 I_2 I_1 E_1 P

Fig.23. Determination of Extracellular and Intracellular Immunoprecipitates on Nitrocellulose Sheet by Western-Brotting method.

Purified β-amylase and extracellular and intracellular immunoprecipitates were run on 7.5-15% polyacrylamide gel (slab gel) and then the proteins in the gel were transferred to the nitrocellulose sheet. The photograph was taken under UV-irradiation to the sheet.

P: Purified β-amylase (5μg).

Extracellular immunoprecipitates: E_1(30μg), E_2(50μg) E_3(70μg), E_4 (100μg). Intracellular: I_1: (30μg), I_2(50μg) I_3(70μg), I_4(80μg), I_5(100μg).

Table IX. Protein-synthesizing Activity in Cell-free Systems Prepared from *E.coli* K-12.

		^{3}H-leucine incorporated (dpm)
-mRNA	TCA-insoluble	3780
	Immunoprecipitates	400
+mRNA	TCA-insoluble	9050
	Immunoprecipitates	1050

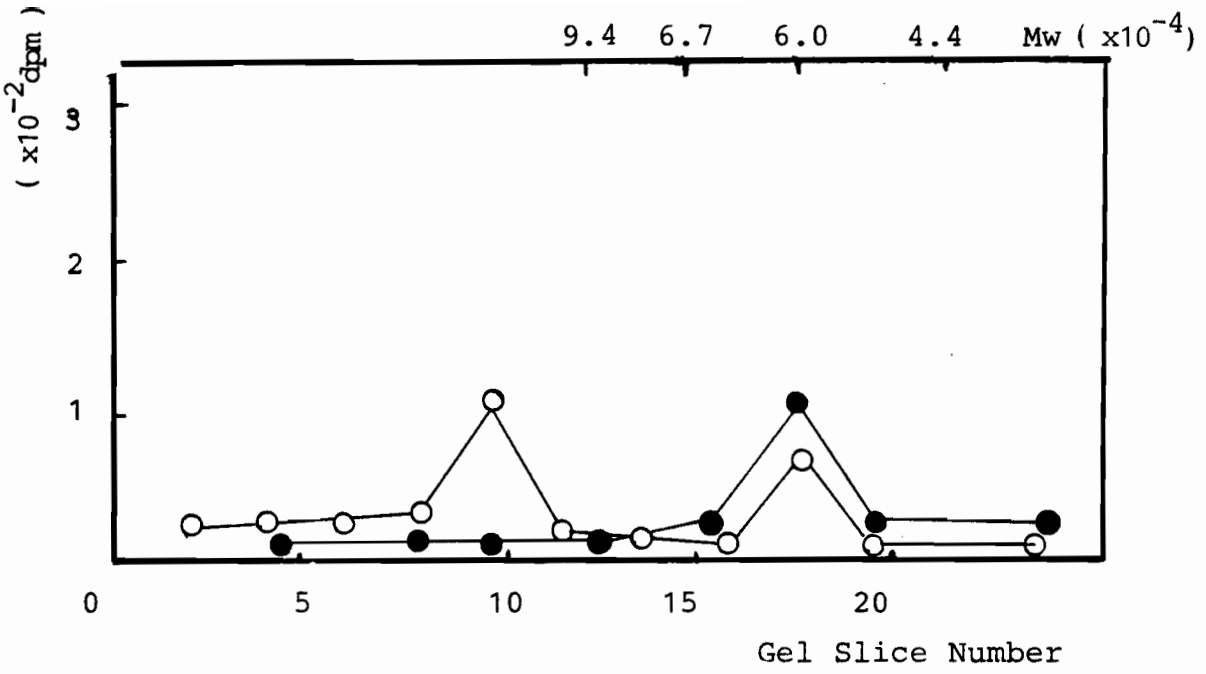


Fig.24. Incorporation of ^{14}C-Amino Acids to Extra- and Intracellular Immunoprecipitates.

^{14}C-Amino acids (protein hydrolysates, 10μCi) were added to the culture of B.cereus BQ10-S1 SpoII during shaking culture at 6 hr after inoculation, and the culture was continued for 2 hr. Then the extracellular and intracellular immunoprecipitates were prepared. They were run on polyacrylamide gel (7.5-15% slab gel). The gel was cut in slices at 2.5mm interval and radioactivity of each of them was measured by liquid scintillation counter.

○—○: intracellular. ●—●: extracellular.

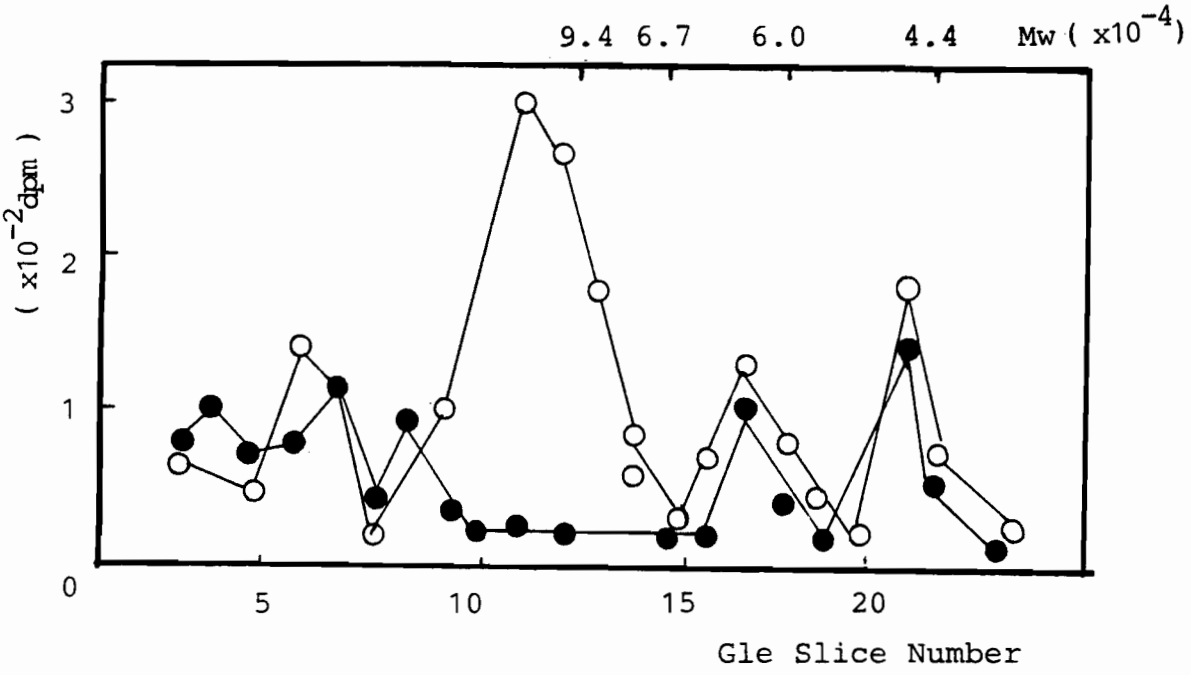


Fig.25. Incorporation of ^{3}H-Leucine to Immunoprecipitates of the Synthesized Proteins in Cell-free System.

RNA fractions extracted from B.cereus BQ10-S1 SpoII were translated in cell-free mixture prepared from E.coli K-12, as described in Methods. The immunoprecipitates, which were harvested from the mixture as also described in Methods, run on polyacrylamide gel (7.5-15% slab gel). The gel was cut in slices at 2.5mm interval. The radioactivity of each of them was measured by liquid scintillation counter.

○—○ : RNA from B.cereus was added.

●—● : RNA from B.cereus was not added.

Summary

A rifampin-resistant, asporogenous mutant, Bacillus cereus BQ10-S1 SpoII was newly isolated from Bacillus cereus BQ10-S1.

The amounts of secreted β-amylase and proteins from the mutant were estimated to be about 95μg/ml and 300μg/ml, respectively. This mutant had the ability to secret about 4 times as much β-amylase as extracellular proteins. The activity of extracellular protease was also estimated to be 4 times higher than that of its parent strain (BQ10-S1).

β-Amylase produced by B.cereus BQ10-S1 SpoII was purified homogeneously by salting out with ammonium sulfate, and column chromatography on Sephadex G-100 and CM-Sephadex C-50.

The purified enzyme was homogeneous on disc electrophoresis and ultracentrifugation. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of the enzyme showed a single band, suggesting no subunit structure. The sedimentation coefficient was $4.8S_{20,w}$ and the molecular weight was estimated to be 6.0×10^4 by SDS-PAGE, 6.2×10^4 by gel filtration in the presence of 6M guanidine HCl, 6.25×10^4 by sedimentation equilibrium and 5.5×10^4 by amino acid analysis. The optimum pH and temperature were about 7.0 and 40°C, respectively. The Km value for soluble starch was 0.4%. The degradation rate of raw starch of wheat and corn was calculated to be 5% and 2%, respectively. The activity was remarkably inhibited by 5×10^{-9}M pCMB but not by DTNB at the same concentration.

The enzyme could react with DTNB only in the presence of denaturant (0.2% SDS). Only one sulfhydryl residue was detected by amino acid analysis, by Elluman's and Riordan's methods.

It was found that the preparations of bacterial β-amylases associated carbohydrates in their molecules. The isoelectric point of the enzyme was 8.3. The β-amylases from B.cereus BQ10-S1 and B.cereus BQ10-S1 SpoI were also homogeneously purified by the same method as B.cereus BQ10-S1 SpoII. The enzymes from them had the same amino acid composition and reacted to the anti-β-amylase serum equally. The each line of immunoprecipitin of them was fused each other. Spur was formed on Ouchterlony plate between the line of immunoprecipitin of β-amylase from B.cereus BQ10-S1 SpoII and that from B.megaterium strain No 32 against the anti-β-amylase serum. However, immuno-reaction did not occur with the β-amylase from B.polymyxa No 72 and the higher plant β-amylases.

The β-amylase of B.cereus BQ10-S1 SpoII was mainly secreted from middle logarithmic phase to late one.

A single protein band equal to the purified β-amylase was detected on the nitrocellulose sheet transferred by Western-Blotting after SDS-PAGE of immunoprecipitate with the extracellular fraction.

However, two proteins, one of which molecular weights was calculated to be 6.0×10^4 and the other to be $11\text{-}12 \times 10^4$, were detected from the immunoprecipitate with intracellular fraction on the nitrocellulose sheet.

The incorporation of radioactivity of ^{3}H-leucine was detected both in TCA-insoluble fraction and immunoprecipitates from *in vitro* synthesized protein. The activity of *in vitro* protein synthesis increased when RNA fraction from *B.cereus* BQ10-S1 SpoII was added. Radioactivity was detected in a protein, of which molecular weight was estimated to be $10\text{-}11\times10^{4}$, only when RNA fraction from *B.cereus* BQ10-S1 SpoII was added. The protein of higher molecular weight seemed to be a precursor-like protein of the β-amylase of *B.cereus* BQ10-S1 SpoII.

Acknowledgement

The author wishes to express his sincere gratitude to Professor Dr. Hiroshi Nishira, Associate Professor Dr. Ryu Shinke, and Dr. Kenji Aoki of Kobe University for their kind guidance and encouragement throughout the course of this work.

The support and many valuable advices of Professor Dr.Shoichiro Yuki of Kobe University are gratefully acknowledged. It is also a great pleasure to acknowledge their kind guidance and encouragement of Professor Dr. Shozaburo Kitaoka, and Associate Professor Dr. Yoshihisa Nakano of University of Osaka Prefecture.

The author is also grateful to Professor Dr. Sawao Murao of University of Osaka Prefecture and Dr. Shigetaka Okada of The Osaka Municipal Technical Research Institute for their kind supply of purified β-amylase and a β-amylase producing bacterial strain.

Thanks are also due to the members in the Laboratory of Fermentation Technology, Department of Agricultural Chemistry, Kobe University for their kind help and discussion.

References

1) R. Kuhn, *Ber.*, 57B, 1965(1924).

2) A. K. Balls, R. R. Thompson and M. K. Walden, *J. Biol. Chem.*, 163, 571 (1946).

3) E. H. Fisher, K. H. Meyer, G. Noelting and A. Pignest, *Arch. Biochem. Biophys.*, 27, 235(1950).

4) K. H. Meyer, E. H. Fisher and A. Pingest, *Helv. Chim. Acta*, 24, 316(1951).

5) K. Visuri and M. Nummi, *Eur. J. Biochem.*, 28, 555(1972).

6) K. H. Meyer, P. F. Spahr and E. H. Fisher, *Helv. Chim. Acta*, 36, 1924 (1953).

7) K. H. Tipples and R. Thachuk, *Cereal Chem.*, 42, 111(1965).

8) A. Gertler and Y. Birk, *Biochem. J.*, 95, 621 (1965).

9) J. Spradlin and J. A. Thoma, *J. Biol. Chem.*, 245, 117(1970).

10) K. Uehara, T. Mizoguchi and S. Manner, *J. Biochem.*, 68, 359(1970).

11) T. Thachuk and K. H. Tripples, *Cereal Chem.*, 43, 62(1966).

12) M-L. Niku-Paavola, M. Numii, A. Kachkin, J. Daussant and T-M. Enari, *Cereal Chem.*, 49, 580(1972).

13) J. A. Thoma, D. E. Koshland, R. Shinke and J. Ruscica, *Biochemistry*, 4, 714(1965).

14) B. Mikami, S. Aibara and Y. Morita, *J. Biochem.*, 88, 103(1980).

15) Y. Morita, F. Yagi, S. Aibara and H. Yamashita, *J. Biochem.*, 79, 591(1976).

16) B. Mikami, S. Aibara and Y. Morita, *Agric. Biol. Chem.*, 46, 943(1982).

17) J. Robyt and D. French, *Arch. Biochim. Biophys.*, 104, 338(1964).

18) E. B. Tilden and G. S. Hudson, *J. Bacteriol.*, 43, 527(1942).

19) M. Higashihara and S. Okada, *Proc. Symp. Amylase*, p. 39 (1971).

20) M. Higashihara and S. Okada, *Agric. Biol. Chem.*, 38, 1023(1974).

21) J. H. Pazur and S. Okada, *J. Biol. Chem.*, 24, 4146(1966).

22) P. J. Griffin and W. M. Fogarty, *Biochem. Soc. Transactions*, 1, 397(1973).

23) P. J. Griffin and W. M. Fogarty, *Biochem. Soc. Transactions*, 1, 1097(1973).

24) R. Shinke, H. Nishira and N. Mugibayashi, *Agric. Biol. Chem.*, 38, 665 (1974).

25) J. J. Marschall, *FEBS Letters*, 46, 1 (1974).

26) W. H. Fogarty and M. T. O'Reilly, unpublished observation (1974).

27) R. Shinke, K. Aoki, H. Nishira and S. Yuki, *J. Ferment. Technol.*, 57, 53(1979).

28) Y. Takasaki, *Agric. Biol. Chem.*, 40, 1523(1976).

29) S. Murao, K. Ohyama and M. Arai, *Agric. Biol. Chem.*, 43, 719(1979).

30) E. J. Bouke and W. M. Fogarty, *Proc. Soc. Gen. Microbiol.*, 2, 81(1975).

31) R. Shinke, Y. Kunimi, K. Aoki and H. Nishira, *J. Ferment. Technol.*, 55, 103(1977).

32) R. H. Doi, L. R. Brown, G. Rodgers and Y.-P. Hsu, *Proc. Natl. Acad. Sci.*, 662, 404(1970).

33) T. Nanmori, R. Shinke, K. Aoki and H. Nishira, *Agric. Biol. Chem.*, 47, 609(1983).

34) T. Nanmori, R. Shinke, K. Aoki and H. Nishira, *Agric. Biol. Chem.*, 47, 941(1983).

35) S. Akabori, B. Hagihara and T. Ikenaka, *J. Biochem.*, 41, 577(1954).

36) H. Hanabusa, T. Ikenaka and S. Akabori, *J. Biochem.*, 42, 55(1955).

37) J. H. Pazur and S. Okada, *Carbohydrate Res.*, 4, 371(1967).

38) M. Arai, Y. Minoda and K. Yamada, *Agric. Biol. Chem.*, 32, 922(1969).

39) P. Mantsala and H. Zalkin, *J. Biol. Chem.*, 254, 8546(1979).

40) Y. Nagata, K. Yamaguchi and B. Maruo, *J. Bacteriol.*, 119, 425(1974).

41) Y. Nagata, H. Yamaguchi and B. Maruo, *Agric. Biol. Chem.*, 44, 1283(1980).

42) H. Yamaguchi, Y. Nagata and B. Maruo, *Agric. Biol. Chem.*, 44, 1291(1980).

43) T. W. Okita and R. Decaleya, *Plant Physiol.*, 63, 195(1974).

44) S. Yamamoto and J. O. Lampen, *Proc. Natl. Acad. Sci.*, 73, 1457(1976).

45) S. Yamamoto and J. O. Lampen, *J. Biol. Chem.*, 251, 4095(1976).

46) G. Blobel and B. Dobberstein, *J. Cell Biol.*, 67, 835(1975).

47) G. Blobel and B. Dobberstein, *J. Cell Biol.*, 67, 852(1975).

48) L. E. Sacks and G. Alderton, *J. Bacteriol.*, 82, 331(1961).

49) P. Bernfeld, "Methods in Enzymology" Vol. III, ed. by S. P. Colowick and N. O. Kaplan, Academic Press, New York (1955), p. 5.

50) H. Kringstad and J. Kilhord, *Proc. Eur. Brew. Conv.*, Copenhagen, 67(1957).

51) O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, 193, 265(1951).

52) T. Sasaki, M. Yamasaki, B. Maruo, Y. Yoneda, K. Yamane, A. Takatsuki and G. Tamura, *Biochem. Biophys. Res. Commun.*, 70, 125(1976).

53) B. Maruo, K. Yamane, Y. Yoneda and K. Hitotsuyanagi, *Proc. Japan Acad.*, 54, 435(1978).

54) B. J. Davis, *Ann. N. Y. Acad. Sci.*, 121, 404(1964).

55) K. Weber, J. R. Pringel and M. Osborn, "Methods in Enzymology" Vol. 26, Academic Press, New York (1972), p. 3.

56) M. Somogyi, *J. Biol. Chem.*, 195, 19(1952).

57) S. Ueda, *J. Jap. Soc. starch Sci.*, 22, 114(1975).

58) G. L. Elluman, *Arch. Biochem. Biophys.*, 25, 437(1959).

59) B. L. Vallee and J. F. Riordan, *Brookhaven Symp. Biol.*, 21, 91(1968).

60) R. M. Zacharius, T. E. Zell, J. H. Morrison and J. Woodlock, *Anal. Biochem.*, 30, 148(1969).

61) P. Andrews, "Methods of Biochemical Analysis" Vol. 18, Interscience, New York (1970), p. 1.

62) P. Wilding, *Clin. Chim. Acta*, 8, 918(1963).

63) D. P. Botes, F. J. Jonbert and Y. Novellie, *J. Sci. Food Agr.*, 18, 409(1967).

64) M. Heidelberger and F. E. Kendall, *J. Exp. Med.*, 62, 697(1935).

65) O. Ouchterlony, "Progress in Allergy", ed. by P. Kallos, Karger, Basel-London (1958), p. 1.

66) K. Yamaguchi, Y. Nagata and B. Maruo, *J. Bacteriol.*, 119, 416(1974).

67) J. Jacobsen, J. Thomas and V. Higgines, *Plant Physiol.*, 70, 1647(1982).

68) S. Halegoua, A. Hirashima and M. Inouye, *J. Bacteriol.*, 120, 1204(1974).

69) H. Towbin, T. Stachelin and J. Fordon, *Proc. Natl. Acad. Sci.*, 76, 4354(1979).

70) R. T. M. J. Vaessen, J. Kreike and G. S. P. Groot, *FEBS Letters*,

124, 193(1981).

71) L. Legault-Demare and G. H. Chambliss, *J. Bacteriol.*, 120, 1300(1974).