STUDIES ON STRUCTURE AND FUNCTION OF SOYBEAN β-AMYLASE BY CHEMICAL MODIFICATION OF SULFHYDRYL GROUPS

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CONTENTS

ABBREVIATIONS

CNBr	cyanogen bromide
DTNB	5,5'-dithiobis-(2-nitrobenzoic acid)
EDTA	ethylenediaminetetraacetic acid
MDPS	methyl 2,4-dinitrophenyl disulfide
Mw	molecular weight
NEM	N-ethylmaleimide
NTCB	2-nitro-5-thiocyanobenzoic acid
PCMB	*p*-chloromercuribenzoic acid
2-PDS	2,2'-dithiopyridine
2-PD	2-thiopyridone
SDS	sodium dodecyl sulfate
TNB	5-thio-2-nitrobenzoic acid
Tris	tris(hydroxymethyl)aminomethane

I. INTRODUCTION

β-Amylase [α-1,4-glucan maltohydrolase, EC. 3.2.1.2] catalyzes the liberation of β-anomeric maltose from non-reducing ends of α-1,4-glucan such as starch and glycogen. The enzyme is distributed in higher plants and some microorganisms. The enzyme purified from soybean (*1*, *2*), wheat (*3*) , Japanese-radish (*4*, *5*), rice (*6*), barley (*7*) and malted sorghum (*8*) are monomeric enzymes having molecular weight of 50,000 to 60,000 daltons, whereas only sweet potato β-amylase (*9-11*) is a tetrameric enzyme having four identical subunits of 50,000 daltons. These plant type β-amylases have similar properties, such as amino acid composition, isoelectric point and optimum pH (*11*,*12*). In many higher plants, multiple forms of β-amylase have been reported (*13*,*14*). Some of them have been investigated in a genetic point of view and others in relation to the intracellular regulation of β-amylase activity. In contrast to the plant type enzymes, the properties of β-amylase from bacteria are reported to be different from those of plant type enzymes in molecular dimension, amino acid composition, isoelectric point and optimum pH (*15-18*). In spite of the origins of β-amylase, all of the enzymes purified have been reported to be inactivated by SH-reagents such as *p*-chloromercuribenzoic acid, N-ethylmaleimide and iodoacetamide (*11*,*17*). Therefore, β-amylase has been considered to be an SH-enzyme.

Technological importance of β-amylase is evident in food industries. The product of the enzymatic action, maltose, is widely used in food process as a sweetner-reduced-sweetner and in medicine as an intravenous

sugar supplement. In the industrial production of highly purified maltose from starch, β-amylase is inevitable together with α-amylase and debranching enzymes (*19,20*). As a source of β-amylase in industrial use, wheat bran, sweet potato and defatted soybean meal have been used. In addition to these plant enzymes, recent finding of β-amylase in some microorganisms will bring their industrial application (*20*).

For the excellent application of β-amylase in food industries, including immobilized β-amylase and modification of the enzymatic properties, the knowledge about structure-functional relationship of β-amylase is indispensable. Studies of structure and function of β-amylase have been performed especially on the enzymes from higher plants such as sweet potato, wheat and soybean. Kinetic feature of β-amylase has been studied intensively (*11*), and Hiromi *et al.* elucidated the subsite affinities of β-amylase from wheat (*21*) and soybean (*22*). In spite of the accumulated knowledge about the kinetic properties of β-amylase, primary or the three dimentional structure of the enzyme has not been solved nor clarified the amino acid residues concerning the catalytic step of the enzyme action. From the studies of pH-activity relationship of β-amylase from sweet potato (*23*), barley (*24*) and soybean (*25,26*), carboxyl group and histidyl or sulfhydryl group were assumed to be involved in the catalytic action. However, none of these amino acid residues were identified to be essential for the enzymatic action by chemical modification (*27,28*). The chemical modification of enzyme is an important tool in the deter-

mination of the nature and biological function of amino acid residues in the active center. In the case of β-amylase, chemical modification studies have been concentrated on sulfhydryl(SH) groups because their modification with various SH-reagents caused almost complete inactivation of the enzyme. However, there are some controversy about the role of SH groups of β-amylase. Gertler and Birk (*25*) suggested that the SH groups of soybean β-amylase participated either in the catalytic step of the enzymatic action or in the stabilization of the active structure of the enzyme, while Thoma *et al.*(*23,28*) concluded that the SH groups of sweet potato β-amylase were not directly concerned in its catalytic step. Since these investigators did not distinguish the SH group that participated in the inactivation from other SH groups, the role of SH group of β-amylase still remained to be unsolved.

Among plant type β-amylase, the enzyme from soybean is the most suitable for the enzymatic studies. Soybean β-amylase is composed of a single polypeptide chain containing no carbohydrates (*1,2*). The enzyme is stable and easily prepared from commercial defatted soybean meal by conventional purification methods (*29*). Soybean β-amylase was first crystallized by Fukumoto and Tsujisaka (*30*) and some enzymatic properties were also reported by the same authors (*31*), Peat *et al.* (*32,33*) and Gertler and Birk (*1,24*). Later, Morita and Yagi (*34*) found four components of soybean β-amylase by isoelectric focusing, and Morita *et al.*(*2,29*) crystallized two major components and reported their properties and preliminaly X-ray crystallo-

graphic investigation. The X-ray crystallographic studies of soybean β-amylase using isomorphous replacement with K_2PtCl_4 and *p*-chloromercuribenzene sulfonate is now in progress by Morita *et al.*(*29*,*35*) to elucidate the three-dimentional structure of the enzyme.

In this thesis, the author elucidated the structure-functional relationship of soybean β-amylase by chemical modification of its SH groups. Prior to the chemical modification studies, the author studied the distribution and properties of soybean β-amylase isozymes. The second deals with the chemical modification of SH groups of soybean β-amylase and the role of the SH groups in the action of the enzyme. Thirdly, the interaction of native and SH-modified soybean β-amylase with cyclohexadextrin and maltose was investigated. Finally, soybean β-amylase was cleaved at the SH groups and the location of five SH groups along the polypeptide chain of the enzyme was determined.

II. MULTIPLE FORMS OF SOYBEAN β-AMYLASE

For the study of structure and function of soybean β-amylase, it is important to clarify the structural differences among the multiple components or isozymes of the enzyme.

Besides the multiplicity of β-amylase from matured soybean seeds, multiplicity of β-amylase has been reported in many plants (*13*). β-Amylase from barley (*36-39*), wheat (*40,41*), maize (*42*), rice (*43,44*) and pea (*45*) were demonstrated to show multiple forms on polyacrylamide gel electrophoresis, starch gel electrophoresis or isoelectric focusing, and the multiple forms of the enzyme from barley, wheat and rye were found to have different molecular dimensions (*7,14*). The latter multiplicity was partly explained as the *in vivo* system of condensed or latant forms, which were converted to active β-amylase by treatment with papain or 2-mercaptoethanol (*46,47*). On the other hand, the multiplicity of β-amylase in maize (*42*) and barley (*39*) was explained by genetic variants controlled by a pair of alleles based on the experimental results of genetic analyses. However, none of the multiple β-amylase components from various origines have been demonstrated as to their structural differences. Post translational modifications, such as phospholylation and trimethylation, may be responsible for electrophoretic variants, as pointed out on multiple forms of wheat α-amylase (*48*).

The multiplicity of β-amylase in soybean seeds was shown by Morita *et al.*(*29*) and Morita and Yagi (*34*). Four components (1 to 4) were found in seeds of soybean and related species by using column isoelec-

tric focusing in a glycerol gradient (*34*). Among them, component 2 and 4 were separated by CM-Sephadex column chromatography and purified to crystalline states (*29*). These two components were simple proteins containing no carbohydrates, and they had the same molecular dimensions (*2*). Their structural differences have not yet been clarified, though properties of component 4 were examined extensively. It was found that component 4 was different from the preparation reported by Gertler and Birk (*1*) as to the C-terminal residue.

In this chapter, first, the author presented multiple forms of β-amylase from 48 varieties of soybean seeds and 2 related species by using polyacrylamide gel isoelectric focusing, because this method had higher resolution and sensitivity than the column isoelectric focusing for detecting minor components. Seven components of soybean β-amylase (1', 1, 2, 3, 4, 5 and 6) were detected; components 1', 5 and 6 were minor and newly detected and the other components were consistent with those reported by Morita and Yagi (*34*). Second, component 6 was purified to homogeneity and its properties were compared with those of previously purified components 1, 2 and 4 to elucidate their structural differences(*49*).

1. Multiple Forms of β-Amylase in Soybean Seeds

In order to examine the multiplicity of β-amylase in soybean and related species, the extract was analyzed by polyacrylamide slab gel isoelectric focusing, and amylase activity was detected by staining the gel with I_2-KI solution. Figure 1 shows typical isoelectric fo-

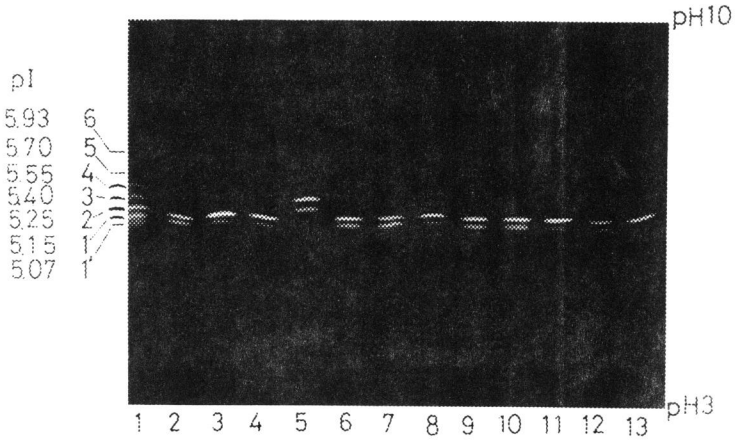

Fig. 1. Isoelectric Focusing Patterns of Amylase from 12 Different Varieties of Soybean Seeds and Commercial Defatted Soybean Meal.

1, Extract from commercial defatted soybean meal; 2, Corsoy; 3, Oodate 1; 4, Kinzu; 5, Bonus; 6, Hill; 7, Hark; 8, Forrest; 9, Dare; 10, Raiden; 11, Tokachinagaha; 12, Matsuura; 13, Sakagami 2. Isoelectric focusing on a slab polyacrylamide gel was performed by the method of Vesterberg (*50*). Pharmarite, pH 3-10 was used as a 2% solution. The concentration of acrylamide and bis-acrylamide were 5 and 7%, respectively. The enzyme solution was prepared from soybean seeds as described by Morita and Yagi (*34*). Fifteen µl of each enzyme solution was charged on the slab gel on a Pharmacia Flat Bed Apparatus FBE-3000. Isoelectric focusing was carried out at a constant current of about 5 mA from 200 to 900 V at 0°C. After focusing the gel was immersed in 20 volums of 0.5 M acetate buffer, pH 5.4, for 10 min, and the gel was immersed in 15 volums of 0.2 M acetate buffer, pH 5.4, containing 1% soluble starch for 12 min at room temperature. Next, the gel was placed in a solution of 0.2% iodine and 4% potassium iodide. The gel was stained dark brown and amylase activity was detected as a colorless band.

Table I. Distribution of Seven β-Amylase Components in Soybean Seeds.

Species and varieties	Components of β-amylase and activity ratio							Type
	1′	1	2	3	4	5	6	
Glycine max (Japanese varieties)								
Akasaya	+	0.8	1.0	+	+			L
Aodaizu	+	0.9	1.0	+	+			L
Cha shoryu	+	0.6	1.0	+	+			L
Daihachirin	+	0.7	1.0	+	+			L
Houtenhakubi	+	0.7	1.0	+	+			L
Iyodaizu	+	0.6	1.0	+	+			L
Kimusume 77	+	0.6	1.0	+	+			L
Kurodaizu			+	0.4	1.0	+	+	H
Kurodaizu shoryu			+	0.5	1.0	+	+	H
Matsuura	+	0.6	1.0	+	+			L
Mizukuguri	+	0.6	1.0	+	+			L
Oodate 1	+	0.4	1.0	+	+			L
Ohoshokuakidaizu	+	0.7	1.0	+	+			L
Raiden	+	0.9	1.0	+	+			L
Sakagami 2	+	0.4	1.0	+	+			L
Shakujo	+	0.7	1.0	+	+			L
Shakkinnashi	+	0.6	1.0	+	+			L
Shirodaizu	+	0.7	1.0	+	+			L
Shimokusano	+	0.5	1.0	+	+			L
Shika 1	+	0.8	1.0	+	+			L
Tamanishiki	+	0.4	1.0	+	+			L
Tanbaguro	+	0.5	1.0	+	0.6			M
Tokachinagaha	+	0.5	1.0	+	+			L
Tsurunoko	+	0.9	1.0	+	+			L
Waseoiran	+	0.7	1.0	+	+			L
Yudagoshaku	+	0.7	1.0	+	+			L
Glycine max (foreign varieties)								
Beeson		+	+	0.8	1.0	+	+	H
Bilox	+	0.25	1.0	+	+			L
Black Eye Brow	+	1.0	1.0	+	+			L
Bonus		+	0.2	0.8	1.0	+	+	H
Corsoy	+	0.7	1.0	+	+			L
Dare	+	0.9	1.0	+	+			L
Ford	+	1.0	1.0	+	+			L
Forrest	+	0.4	1.0	+	+			L
Hakubyosen	+	0.7	1.0	+	+			L
Hark	+	0.9	1.0	+	+			L
Hill	+	0.6	1.0	+	+			L
Huk-Tae		+	+	0.9	1.0	+	+	H
Improved Pelican	+	0.3	1.0	+	+			L
Kinzu	+	0.4	1.0	+	+			L
PI	+	0.5	1.0	+	+			L
Pine Dell	+	0.6	1.0	+	+			L
Sooty	+	0.7	1.0	+	+			L
Steele			+	0.9	1.0	+	+	H
Tokio Noir	+	0.4	1.0	+	+			L
Yellow Bilox Hybrid	+	0.8	1.0	+	+			L
Yokoten	+	0.5	1.0	+	+			L
York	+	0.6	1.0	+	+			L
Glycine gracilis T-34	+	0.6	1.0	+	+			L
Glycine ussuriensis 303246			+	0.5	1.0	+	+	H

cusing patterns and pI values for β-amylase components from 12 varieties and commercial defatted soybean meal. Seven active bands were detected, and their isoelectric points (1' to 6) were 5.07, 5.15, 5.25, 5.40, 5.55, 5.70 and 5.93 ± 0.04, respectively. The isoelectric points of the major components (1 to 4) are consistent with those reported by Morita and Yagi (*34*). The seven detected bands represent β-amylase components because no α-amylase bands were found using 1% β-limit dextrin in place of soluble starch as a substrate. These bands did not show quantitative change in their patterns on storage of the extract at 4°C or 30°C for a week. Protease inhibitors such as diisopropylfluorophosphate and pepstatin also showed no effect. Thus the seven active bands correspond to β-amylase components of intact soybean seeds and no artifacts. Table I represents the number of multiple forms and the ratio of major components in the examined seeds of 48 varieties of soybean and two related species. Soybean and related species can be divided into two types by the multiplicity of β-amylase from Table I. Thus the low pI type contains

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Densitometry of β-amylase activity was carried out using photographic film of isoelectric focusing gel (Fig. 1) with an Aska model OZ-700 densitometer. Activity ratio is represented as the ratio to the strongest band and minor components are shown as +. L, H and M represent the low, high and intermediate pI types. Seeds of 48 varieties of soybean, one variety of *Glycine gracilis* and one variety of *Glycine ussuriensis* were obtained from the national Institute of Agricultural Science, Hiratsuka, Kanagawa Pref., the Crop Research Laboratory, Department of Agriculture, Kyoto University and kindly supplied by Dr. T. Mori, Associate Professer of the Reseach Institute for Food Science, Kyoto University.

components 1 and 2 predominantly and components 1', 3 and 4 are the minor ones, but the high pI type contains components 3 and 4 predominantly and components 2, 5 and 6, or occasionally 1, 2, 5 and 6 are minor ones. Most soybean varieties (41 out of 48) and *Glycine gracilis* belong to the low pI type and 6 soybean varieties out of 48 and *Glycine ussuriensis* belong to the high pI type. Most Japanese soybean seeds (25 out of 28) were the low pI type. Only Kurodaizu and Kurodaizu shoryu were the high pI type. Another one, Tanbaguro, showed an intermediate pattern between these two types. It contains components 1, 2 and 4 mainly and it seems to be a hybrid of high and low pI types. The black color of the episperm of these three soybean seeds, Kurodaizu, Kurodaizu shoryu and Tanbaguro, was consistent with the presence of component 4 as one of the major components. No other Japanese soybean seed examined had black colored episperm and component 4 as a major component. A semicultivated species, *Glycine gracilis*, belongs to the low pI type, and a wild species, *Glycine ussuriensis*, belongs to the high pI type. These results suggest that both *Glycine gracilis* and *Glycine ussuriensis* may be equivalent ancestral species of *Glycine max*.

Gorman and Kiang (*51*,*52*) previously found that seeds of most soybean cultivars possess three kind of amylases (AM-1, AM-2 and AM-3) detectable by a slab polyacrylamide gel electrophoresis. AM-3 was the main activity band of amylase and it had two variants, slow AM and fast AM, named on the basis of their electrophoretic mobility. By crossing variants having fast AM and slow AM, it was hypothesized

that they are controlled by a pair of co-dominant alleles at a single locus, but they concluded that AM-3 represents α-amylase. Recently, Hildebrand and Hymowitz (*53*) reported that the AM-3 bands of Gorman and Kiang represent β-amylase and that the two variant bands, slow AM and fast AM are the same as the Sp_1^a and Sp_1^b soybean protein reported by Larsen (*54*) and Larsen and Coldwell (*55*). In connection with these investigations, the two types, low pI type (L) and high pI type (H), seems to be compatible with the fast AM and slow AM of Golman and Kiang, and with Sp_1^b and Sp_1^a protein of Larsen, respectively, because the electrophoretic mobility of the variant bands of these investigators corresponds to the apparent pI values of the two pI types. Moreover, the pI type of some cultivars (Table I) are consistent with the reported variant types without exceptions: Beeson (H), fast AM; PI (L), slow AM; Ford (L), Sp_1^a; Dare (L), Sp_1^a; Hill (L), Sp_1^a. However, the investigators did not mention to the heterogeneity of each AM-3 band or that of the two protein bands owing to incomplete separation of the components between 1 and 2 or between 3 and 4.

2. Purification of Component 6

Component 6 of soybean β-amylase was purified from 20.8 Kg of defatted soybean flour which was generously supplied by Honen Seiyu Co., Tokyo, according to the method of Morita *et al.* (*29*) with some modification for the separation of minor components. The flour was milled and suspended in 10 mM acetate buffer, pH 5.4. After the

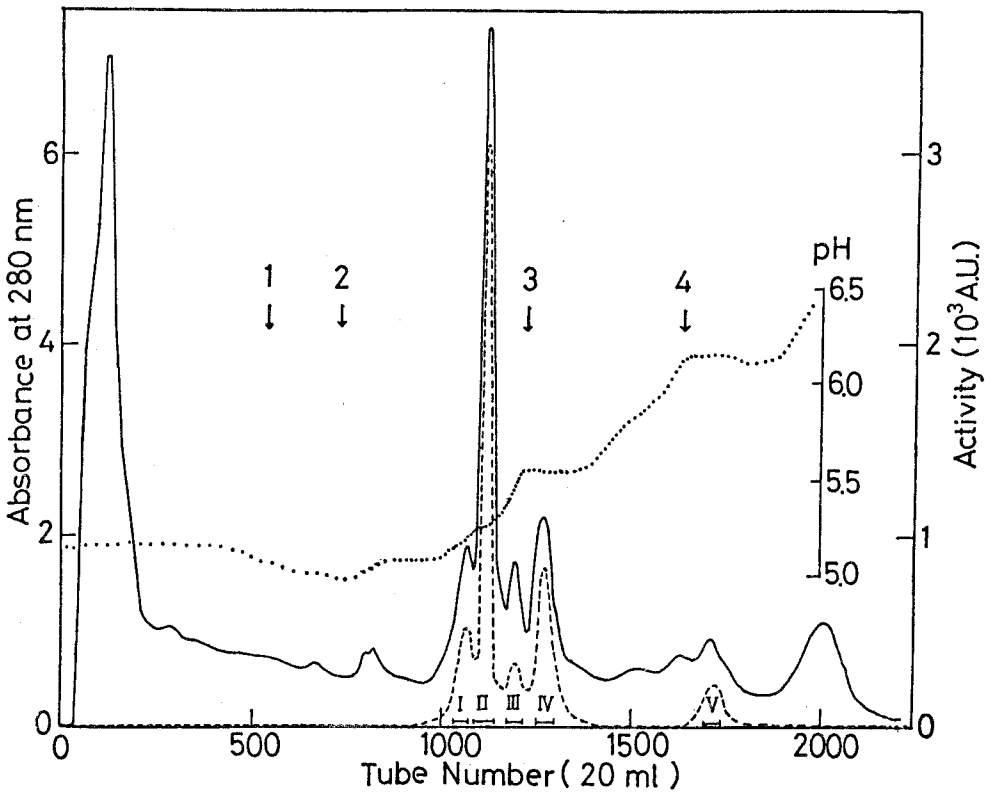

Fig. 2. Separation of Soybean β-Amylase Components on a CM-Sephadex C-50 Column.

———, absorbance at 280 nm; - - -, activity of β-amylase; and ·····, pH of eluate. A CM-Sephadex C-50 column (5 × 90 cm) was equilibrated with 10 mM acetate buffer, pH 5.1, containing 18 mM 2-mercaptoethanol and 1 mM EDTA. After the enzyme solution was put onto the column, the column was washed with 10 liters of the buffer solution and then elution was performed by increasing the concentration and pH of the buffer as follows : 1, 10 mM acetate buffer, pH 5.1 to 50 mM acetate buffer, pH 5.1 (1 liter each), and then the column was washed with 2 liters of 50 mM acetate buffer, pH 5.1. 2, 50 mM acetate buffer, pH 5.1 to 5.6 (2.5 liters each) and then the column was washed with 4 liters of 50 mM acetate buffer, pH 5.6. 3, 50 mM acetate buffer, pH 5.6 to 6.1 (2.5 liters each) and then the column was washed with 3 liters of 50 mM acetate buffer, pH 6.1. 4, 50 mM acetate buffer, pH 6.1, to 50 mM sodium acetate, pH 7.7, (2.5 liters each) and then the column was washed with 8 liters of 50 mM sodium acetate, pH 7.7. Five active fractions, I, II, III, IV and V, were obtained.

suspension was centrifuged, the pH of the supernatant was adjusted to 5.0 by the addition of 1 M acetate and a clear filtrate (Acid extract) was obtained by filtration in a cold room. Then, ammonium sulfate fractionation (25-60% saturation) was carried out, and the precipitate was dialyzed against 10 mM acetate buffer, pH 5.1, containing 18 mM 2-mercaptoethanol and 1 mM EDTA at 4°C for 10 days. The precipitate formed during dialysis was spun off, and the supernatant was subjected to a column of CM-Sephadex. Figure 2 shows the elution profile from the CM-Sephadex column. Five active fractions, I, II, III, IV and V, were collected. From the results of gel isoelectric focusing, it was found that fraction I contains component 1', 1 and 2, fraction II component 2, fraction III components 2, 3 and 4, fraction IV components 3, 4 and 5, and fraction V component 6. Component 2 was the major component present in the defatted soybean flour used this time, and components 2 and 4 were purified by DEAE-Sephadex and Sephadex G-100 as described by Morita *et al.* (*29*). In order to purify component 6, fraction V was subjected to a second CM-Sephadex chromatography (Fig. 3). The major fraction of component 6 was then subjected to purification on a Sephadex G-100 column and then on a DEAE-Sephadex A-50 column (Fig. 4). The whole purification procedure for component 6 is summarized in Table II. The specific activity of the final preparation of component 6 reached 803 A.U./mg protein, which was higher than the values for components 2 and 4 reported by Morita *et al.* (*29*). However, it was found that the activity of soybean β-amylase decreased gradually to a definite

value during storage and it recovered the full activity after the enzyme was treated with 0.2 M 2-mercaptoethanol. The specific activity of component 2 was found to be 856 after this treatment.

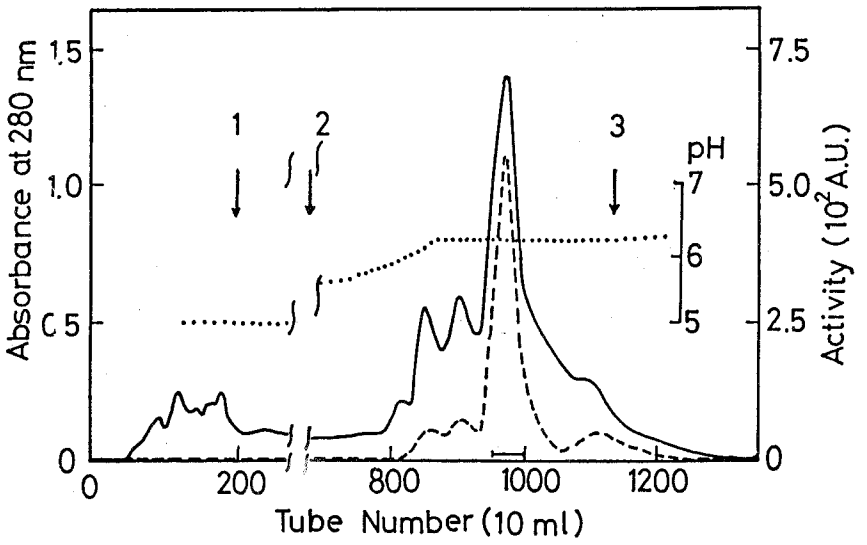

Fig. 3. Elution Profile of Component 6 from the 2nd CM-Sephadex C-50 Column.

——— , absorbance at 280 nm; - - - , activity of β-amylase; and ····· , pH of eluate. The column (5 × 40 cm) was equilibrated with 50 mM acetate buffer, pH 5.0, containing 18 mM 2-mercaptoethanol and 1 mM EDTA. The ammonium sulfate precipitates of component 6 were collected by centrifugation and dialyzed against the above buffer solution at 4°C for 3 days. After the enzyme solution was put onto the column, the column was washed with 1.5 liters of the buffer and the elution was performed by increasing the pH of the buffer: 1, 50 mM acetate buffer, pH 5.0 to 5.6 (1 liter eash) and then the column was washed with 2 liters of 50 mM acetate buffer, pH 5.6. 2, 50 mM acetate buffer, pH 5.6 to 6.2 (1 liter each) and then the column was washed with 6 liters of 50 mM acetate buffer, pH 6.2. 3, 50 mM acetate buffer, pH 6.2, to 50 mM sodium acetate, pH 7.7, (0.5 liters each) and then the column was washed with 1 liter of 50 mM sodium acetate, pH 7.7. The component 6 fractions as shown by a horizontal bar were collected.

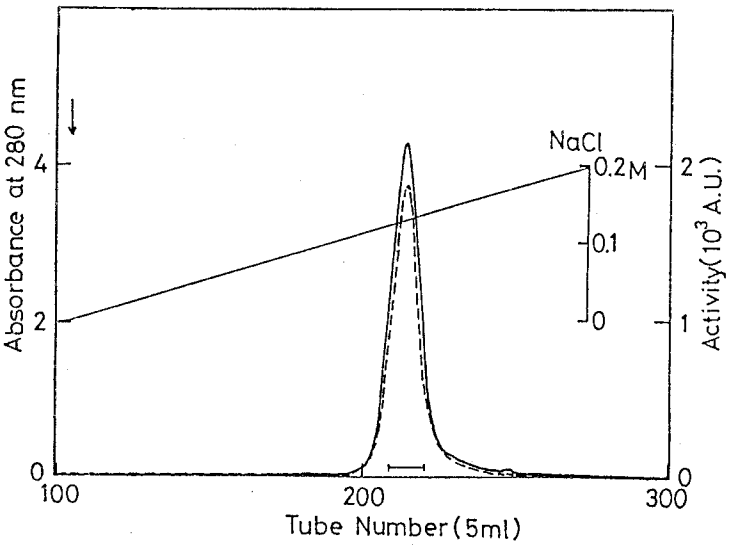

Fig. 4. Elution Profile of Component 6 from a DEAE-Sephadex A-50 Column.

———, absorbance at 280 nm; - - - , activity of β-amylase; and ——— , concentration of NaCl in the elution buffer. The column (2.5 × 40 cm) was equilibrated with 20 mM phosphate buffer, pH 7.6, containing 18 mM 2-mercaptoethanol and 5 mM EDTA. The fractions of component 6 after gel filtration was dialyzed against the same buffer solution for 2 days at 4°C. After the enzyme solution was put onto the column, the column was washed with 500 ml of the buffer, and elution was performed by increasing the NaCl concentration linearly from 0 to 0.2 M in the same buffer (400 ml each).

Table II. Summary of the Purification of Soybean β-Amylase Component 6.

Fraction	Volume (ml)	Total [1] activity (10^3 A.U.)	Total [2] protein (g)	Specific activity (A.U./mg protein)	Yield (%)
Acid extract	166,000	17,900	1,440	12	100
$(NH_4)_2SO_4$ 25~60% satn.	5,700	15,700	329	48	88
Dialysis	4,200	12,200	140	87	68
CM-Sephadex 1st [3]	1,990	455	2.73	167	2.5
CM-Sephadex 2nd	550	187	0.343	546	1.0
Gel filtration	40	109	0.161	677	0.60
DEAE-Sephadex	34	49	0.061	803	0.28

The homogeneity of component 6 was examined by gel isoelectric focusing, and the enzyme was located by amylase activity (Fig. 5A) and by protein staining (Fig. 5B). The results revealed the homogeneity of component 6. Component 6 was also examined by SDS-gel electrophoresis as shown in Fig. 5C. Components 6 and 2 exhibited the same mobility, corresponding to a molecular weight of 57,000 daltons (*2*). The isoelectric point of the purified component 6 was determined to be 5.93 by column isoelectric focusing in a glycerol gradient, and it was consistent with the value obtained by gel isoelectric focusing.

3. Comparison of Soybean β-Amylase Components

Morita *et al.* showed that no α-DNP-amino acid could be detected by Sanger's FDNB method for component 4 (*2*). The present study showed that no N-dansyl amino acid could be detected for both compo-

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1) β-Amylase activity was measured in 0.1 M acetate buffer for 3 min at pH 5.4 and 37°C using 0.5% amylopectin as a substrate, according to the method of Bernfeld (*56*).

2) Protein concentration was determined according to Lowry *et al.*(*57*) using bovine serum albumin as a standard. The protein concentration of the purified enzyme was determined spectrophotometrically using an absorption coefficient of 97 $mM^{-1}cm^{-1}$ at 280 nm, which was determined based on dry weight.

3) The yield of the other components in this step were as follow; components 1+1' (fraction I), 5.4%; component 2 (fraction II), 24.7%; components 2+3+4 (fraction III), 2.2%; and components 4+5 (fraction IV), 8.5%.

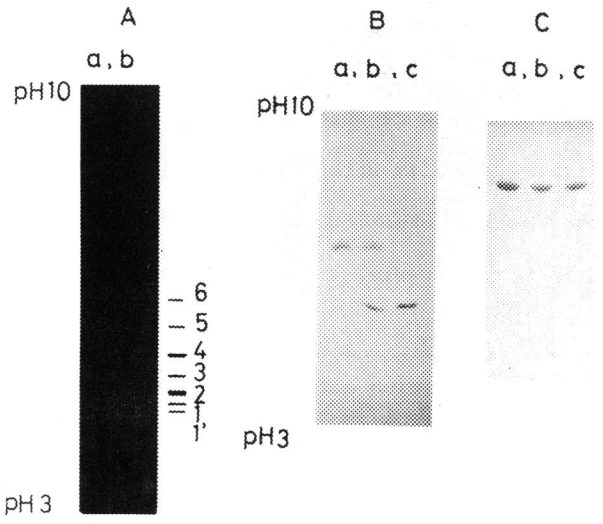


Fig. 5. A, Gel Isoelectric Focusing of Component 6 and the Extract of Defatted Soybean Meal. The gel was stained by activity. a, component 6; b, the extract of commercial defatted soybean meal.

B, Gel Isoelectric Focusing of Components 2 and 6. The gel was stained by Coomassie Brilliant Blue G-250 by the method of Brakesky and Boezi (*58*). a, component 6; b, components 2 and 6; c, component 2.

C, SDS-gel Electrophoresis of Components 2 and 6. SDS-polyacrylamide gel electrophoresis was performed according to the method of Laemmli (*59*), and proteins were stained with Coomassie Brilliant Blue R-250. a, component 2; b, components 2 and 6; c, component 6.

nents 2 and 6 by the dansyl chloride method. No α-DNP-amino acid could be detected for component 2, either. These results revealed that the N-termini of all three components were blocked. As to the C-termini, component 2 was digested by carboxypeptidase A, B and Y in the presence or absence of denaturants (4 M urea or 0.056 M SDS) according to the methods of Amber (*60*) and Hayashi *et al.*(*61*), but

Table III. Amino Acid Compositions of Soybean β-Amylase Components.

Nearest integers of residues are shown in parentheses. Amino acid analysis was performed by the method of Spackman *et al.* (*62*) with a Hitachi KLA-5 amino acid analyzer. Samples were hydrolyzed at 110°C in constant boiling HCl in evacuated, sealed tubes for 24, 48 and 72 h. DL-norleucine (*63*) and S-β-(4-pyridylethyl) cysteine (*64*) were used as internal standard.

Amino acid	Mol amino acid per 57,000 g protein		
	component 2	component 4[a]	component 6
Arginine	18.3 (18)	18.8 (19)	19.1 (19)
Lysine	29.1 (29)	29.7 (30)	31.1 (31)
Histidine	11.1 (11)	10.1 (10)	11.1 (11)
Aspartate[b]	65.0 (65)	63.4 (63)	62.1 (62)
Glutamate	51.1 (51)	51.7 (52)	49.8 (50)
Serine[b]	27.3 (27)	23.7 (24)	26.7 (27)
Threonine[b]	18.3 (18)	16.8 (17)	17.1 (17)
Proline	30.0 (30)	29.8 (30)	29.7 (30)
Glycine	40.2 (40)	38.9 (39)	39.9 (40)
Alanine	32.7 (33)	32.1 (32)	33.1 (33)
Valine[c]	31.4 (31)	31.4 (31)	31.2 (31)
Isoleucine[c]	26.2 (26)	25.7 (26)	25.9 (26)
Leucine[c]	46.4 (46)	45.3 (45)	46.3 (46)
Methionine[d]	8.8 (9)	11.6 (12)	8.7 (9)
Cysteine[d]	5.1 (5)	5.4 (5)	5.0 (5)
Tyrosine	25.1 (25)	25.9 (26)	24.9 (25)
Phenylalanine	21.1 (21)	20.7 (21)	20.7 (21)
Tryptophan[e]	11.7 (12)	11.5 (12)	12.1 (12)
Total	(497)	(494)	(495)

a) Values determined by Morita *et al.*(*2*).

b) Extrapolated to zero time from 24, 48 and 72 h hydrolysis.

c) Determined on two 72 h hydrolyzates.

d) Determined on three 22 h hydrolyzates after performic acid oxidation (*65*).

e) Determined by the method of Edelhoch (*66*).

no free amino acid was detected. In contrast with the carboxypeptidase digestion results, glycine was detected by hydrazinolysis for both components 2 and 6. The recovery of glycine was 65 and 74% for component 2 and 6, respectively.

The amino acid compositions of components 2 and 6 of soybean β-amylase were determined. Table III shows the results together with the composition of component 4 (*2*). These three components have very similar amino acid compositions but slight differences beyond experimental error were found between the components. The slight differences in the pI values among the three components may be partly atributed to the differences in the charged groups of these components, though the amide contents were not determined in the present analysis. Five half-cystines were common for all the components and the residues in components 2 and 6 had all free sulfhydryl groups as detected by Ellman's method (*67*) in the presence of 4 M guanidine-HCl, as shown for component 4 by Morita *et. al.* (*2*).

The amino acid compositions of soybean β-amylase components were compared with those of α- and β-amylases from different origins by the method of Marchalonis and Weltman (*68*). The relatedness, SΔQ, is given as follows:

$$S\Delta Q = \sum_i (A_{i,j} - A_{i,k})^2$$

Here $A_{i,j}$ and $A_{i,k}$ are mol per cent of a given amino acid, i, of the particular protein, j and k, which are compared. The SΔQ values among soybean β-amylase components, 2, 4 and 6 were calculated to be within

1.2. The amino acid compositions of soybean β-amylase component 2 was further compared with that of other amylases. Table IV shows the SΔQ values calculated among α- and β-amylases from various origins. The plant type β-amylases have a marked homology one another and they also exhibited less relatedness with β-amylase from *Bacillus cereus*. On the other hand, no homology was found between the plant type β-amylases and β-amylase from *Bacillus polymyxa*. Moreover, it is noted that plant type β-amylases have a weak homology with α-amylases from sorghum malt and porcine pancreas. These results are

Table IV. Relatedness of Amylases Calculated from the Amino Acid Compositions.

SΔQ values were calculated from the reported amino acid compositions of amylases by using 18 amino acids containing tryptophan and half-cystine. The values enclosed by solid and dashed lines indicate groups of amylases with strongly and weakly related amino acid compositions, respectively.

No	Amylase	Ref.	1	2	3	4	5	6	7	8	9
1	Soybean β comp. 2	-	-								
2	Sweet potato β	(*69*)	11.8	-							
3	Sorghum malt β	(*8*)	21.5	13.5	-						
4	Wheat β	(*3*)	24.0	23.6	24.5	-					
5	*Bacillus cereus* BQ10 β	(*18*)	32.1	33.0	45.8	76.0	-				
6	*Bacillus polymyxa* β	(*17*)	160.5	158.3	197.0	207.1	102.0	-			
7	Sorghum malt α	(*70*)	36.6	49.8	52.0	49.9	79.0	152.4	-		
8	Porcine pancreas α-I	(*71*)	53.6	44.4	70.6	60.5	71.8	115.2	79.0	-	
9	Taka-amylase A	(*72*)	66.2	76.0	81.4	111.4	51.9	109.3	86.5	61.0	-

important in the estimation of the evolutionary relationship of the amylases.

Figure 6 shows the result of Ouchterlony double immunodiffusion of components 2 and 6. The fused spars show that these components have the same higher structure at least at the site of antibody formation. Km values for amylopectin were determined to be 2.25 and 1.65 mg/ml amylopectin for component 2 and 6, respectively. Experiments on the effect of pH (pH 2.5 to 11) on the enzyme activity or stability showed that the difference between component 2 and 6 was within the experimental error. Each component has an optimum pH at

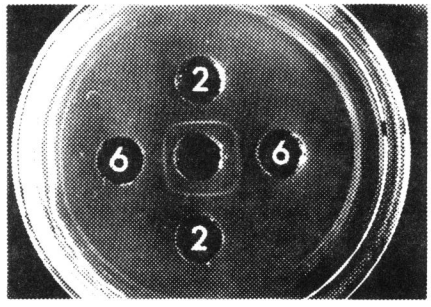


Fig. 6. Ouchterlony Double Immunodiffusion of Soybean β-Amylase Components and Antibody for Component 2.

Center well, immunogloblin (40 μg); 2, purified component 2; 6, purified component 6. Four μg of each component was applied. Antisera against purified component 2 were obtained by the immunization of young adult rabbits at two weekly intervals with 3 injections each containing 8-10 mg protein. The injections were given with complete Freund's adjuvant. The γ-globulin was obtained by repeating precipitation with ammonium sulfate at 33% saturation.

5.4. The effect of neutral salt (NaCl from 0.01 M to 1 M) on the enzyme activity or stability was also confirmed to be almost the same for both components 2 and 6. Heat stability curves, however, showed a little difference between components 2 and 6. The activity of component 6 decreased more rapidly than that of component 2 on heating. After incubation in 0.1 M acetate buffer, pH 5.4, at 60°C for 90 min, the activity of component 2 decreased to 62% of the initial activity. However, the activity of component 6 decreased to 45% of the initial activity. In each case, the decrease of activity followed first order kinetics.

Table V summarizes the properties of the soybean β-amylase components so far investigated. Components 1, 2, 4 and 6 have the same immunological properties and the same molecular weight. In components 2, 4 and 6, the N-termini seem to be blocked as a pyrolidone carboxyl group or by acetylation as suggested for component 4 (*2*). Components 2 and 6 have glycine as their C-terminal amino acid residues. This is remarkably different from component 4 which has alanine as the C-terminus. Component 2 is the major component of β-amylase in most soybean seeds. In these respects, component 2 seems to be identical with the β-amylase purified by Gertler and Birk (*1*) which has glycine as the C-terminal amino acid residue. The properties between components 2 and 6 were very close except for the Km values for amylopectin and stability against heat. The results of amino acid analysis (Table III) showed slight definite

differences between the three components. This clearly demonstrates that they are genetic variants. Characterization of the other components (1', 3 and 5) was not performed, as they were not sufficiently homogeneous to be analyzed in the present investigation. However, the results of Table I and V suggest that the seven components of soybean β-amylase will be isozymes caused by genetic variation.

Table V. Comparison of Properties of Soybean β-Amylase Components.

Component	pI [a]	M_w	N-Terminal amino acid	C-Terminal amino acid	*Km* (mg/ml amylopectin)	Immuno-precipitation (for antibody 2)	Ref.
1	5.15	57,000				+	73
2	5.25	57,000	Blocked [b,c]	-Gly [d]	2.25	+	
4	5.55	57,000	Blocked	-Val-Ala	2.25	+	2
6	5.93	57,000	Blocked [b]	-Gly	1.65	+	

a) For the determination of pI values, column isoelectric focusing in a glycerol gradient was performed according to the method of Vesterberg and Svensson (*74*) with a column volume of 30 ml as described by Doi and Ohtsuru (75) using Pharmarite pH 4-6 or 5-7.

b) The N-terminal amino acid was analyzed by the 1-dimethylaminonaphthalene-5-sulfonyl chloride (DNS) method of Hartley and Massay (*76*) as modified by Gray (*77*).

c) The N-terminal amino acid was analyzed by the 1-fluoro-2,4-dinitrobenzene (FDNB) method of Sanger (*78*).

d) The C-terminal amino acid was analyzed by hydrazinolysis (79). Each 5 mg protein was treated with 2 ml anhydrous hydrazine with 50 mg Amberlite CG-50 for 23 h at 60°C in evacuated, sealed tubes. Amino acid hydrazide was removed from free amino acids by extraction into benzaldehyde and the C-terminal amino acids released were analyzed by amino acid analyzer.

III. SULFHYDRYL (SH) GROUPS OF SOYBEAN β-AMYLASE

Chemical modification is one of the powerful methods for the study of the structure and function of enzyme. Catalytically important amino acid residues have been identified with the method, but there are some difficulties included in the method. First, the specific modification of a single amino acid residue in protein is usually difficult, even though group-specificity of the modification is attained. It is necessary to choose conditions of the modification to minimize the excess modification or side reactions, and to separate the excess or unmodified enzyme from the mixture of modified enzyme. Second, the effect of modification, the introduction of bulky groups onto the enzyme protein is unavoidable, and it may cause a local conformation change of the enzyme due to steric hindrance. Thus, the modified and inactivated enzyme must be examined intensively as possible in order to elucidate the role of the modified amino acid residues for the activity of the enzyme.

Chemical modification of β-amylase has been investigated especially on the SH groups of the enzyme and it was found that its modification by various SH reagents such as iodoacetamide (*23*,*28*), N-ethylmaleimide (*28*,*80*), *p*-chloromercuribenzoate (PCMB) (*2*,*23*,*25*,*81*, *82*) and 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) (*28*) caused inactivation. On the basis of inactivation with these reagents, β-amylase has been known as an SH-enzyme. However, the role of the SH groups in the enzymatic action is not clearly understood. Gertler

and Birk (*25*), based on the results of modification of soybean β-amylase with PCMB, suggested that the SH group participated either in the catalytic step of the enzymatic action or in the stabilization of the active structure of the enzyme. On the other hand, Thoma *et al.* (*23*) and Spradlin and Thoma (*28*) observed the inactivation of sweet potato β-amylase by alkylating reagents, and they concluded that the SH group participates neither in catalysis nor in substrate binding but suggested that the inactivation might be caused by a conformation change around the active site. Since specific or selective modification of the SH group that participate in the inactivation of the enzyme was not attained in these studies, the role of the so-called essential SH group of β-amylase remained uncertain.

In this chapter, the author describes the selective modification of individual SH groups of soybean β-amylase, their properties and the effect of modification on the activity of the enzyme. Two SH groups were distinguished in terms of the modification rate; one was not related to the inactivation of the enzyme, while modification of the other resulted in inactivation. They were designated as SH1 and SH2 on the basis of their reactivity toward SH reagents (*83*).

1. Properties of Five SH Groups of Soybean β-Amylase and Selective Modification of SH1 and SH2

Soybean β-amylase, component 2, has been found to have five half-cystines, the same as in component 4 (*2*), but detailed knowledge

Table VI. Number of SH Groups in Soybean β-Amylase (Component 2).

Method	Condition	Number detected	Residual activity
Amino acid analysis	After performic acid oxidation	5.1	-
DTNB[1)]	in 3 M guanidine-HCl pH 8.0	5.1	-
PCMB[2)]	in 0.1 M phosphate buffer pH 7.0	5.0	0
DTNB[1)]	in 0.1 M Tris-HCl buffer pH 8.0	1.2	80

1) Determined by the method of Ellman (*67*).

2) Determined by the method of Boyer (*84*).

of their forms in component 2 has not yet been obtained. Table VI shows the number of SH groups of component 2 detected under various conditions. As in the case of component 4, all five cysteines could be titrated with PCMB. After the denaturation of the enzyme with guanidine hydrochloride, all the SH groups became readily accessible to DTNB, but only 1.2 mol of SH groups in the native enzyme was reactive with DTNB in 0.1 M Tris-HCl buffer, pH 8.0. In the latter case, the residual activity was 80%. The reactivity of SH groups with DTNB, however, was influenced by the ionic strength of the medium, as shown in Fig. 7. At low ionic strength, only one SH group (SH1) was accessible to DTNB without loss of activity. The second-order rate constant for the reaction was calculated to be 14 $M^{-1} \cdot s^{-1}$ from pseud first-order rate plot. On the other hand, two SH groups reacted with DTNB at high ionic strength, and the activity decreased to less than 1% of the initial activity. The reaction

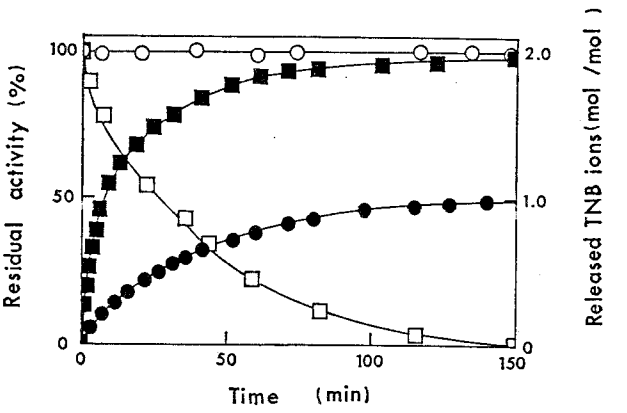


Fig. 7. Reactions of the SH Groups of Soybean β-Amylase with DTNB.

Soybean β-amylase (12.3 μM) was treated with 41 μM DTNB in 0.05 M Tris-HCl buffer containing 5 mM EDTA at pH 7.94 and 25°C for the reaction at low ionic strength (○,●). The enzyme (13.1 μM) was treated with 243 μM DTNB in 0.05 M Tris-HCl buffer containing 0.8 M KCl and 5 mM EDTA at pH 8.10 and 25°C for the reaction at high ionic strength (□,■). The amount of released TNB was determined using $\varepsilon = 13{,}600$ at 412 nm according to Ellman (*67*). —●— and —■—, amount of released TNB (mol/mol enzyme); —○— and —□—, residual activity.

apparently consisted of rapid and slow processed. The second-order rate constants were calculated by curve fitting based on Runge-Kutta method (*85*), and it was found to be 35 $M^{-1} \cdot s^{-1}$ for the fast reaction and 1.9 $M^{-1} \cdot s^{-1}$ for the slow reaction. The rate constant for inactivation was calculated to be 1.9 $M^{-1} \cdot s^{-1}$, indicating that the slowly DTNB-reactive SH group, SH2, at high ionic strength is related to the inactivation of the enzyme. Maltose, the product of the enzymatic process, and cyclohexadextrin, a competitive inhibitor (*11*), protected soybean β-amylase from inactivation caused by modification with DTNB, as shown in Fig. 8. However, no protective effect was

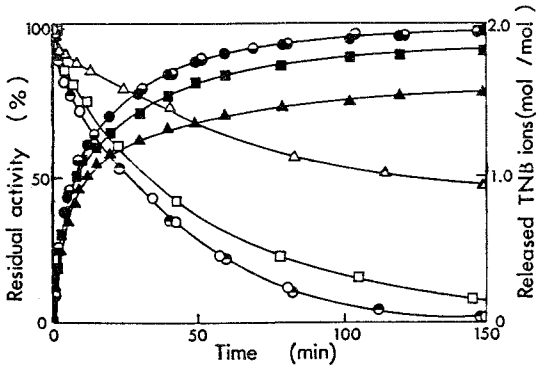


Fig. 8. Effect of Substrate Analogs on Modification of the SH Groups with DTNB.

Reactions were carried out at a protein concentration of 13.7 μM and a DTNB concentration of 250 μM in 0.05 M Tris-HCl buffer containing 0.8 M KCl and 5 mM EDTA at pH 8.1 and 25°C in the absence (○,●) or presence of 40 mg/ml sucrose (◓,◒), cyclohexadextrin (□,■) or maltose (△,▲). —●—, —◑—, —■— and —▲—, amount of released TNB ions (mol/mol enzyme); —○—, —◓—, —□— and —△—, residual activity.

seen with sucrose. Only SH2 was protected against the modification in the presence of maltose or cyclohexadextrin, while the initial release of TNB, the reaction of SH1, was not affected. The degree of modification of SH2 was compatible with that of inactivation of the enzyme, and the protective effect of maltose was much stronger than that of cyclohexadextrin. These results indicate that only SH2 out of the two reactive SH groups toward DTNB may be located near the active site of soybean β-amylase and that its modification causes inactivation of the enzyme.

Alkylation of SH groups of soybean β-amylase with iodoacetamide

causes a marked loss of activity, the final activity being only 2% of the initial activity. When the enzyme (20 μM) was incubated with 50 mM iodoacetamide in 0.1 M Tris-HCl buffer, pH 8.5, the activity decreased apparently with first-order kinetics, and 1.9 mol of carboxymethyl cysteine per mol of the enzyme was detected upon amino acid analysis. These two SH groups are compatible with SH1 and SH2, and the reaction of them with the alkylating reagent can be followed separately by DTNB titration at low ionic strength and by decrease of the enzymatic activity. As it is known that only mercaptide anions are available for alkylation by iodoacetamide (*86*), the observed second-order rate constant, k_{obs}, at a given pH can be expressed by the equation: $k_{obs} = \bar{k} - k_{obs} \cdot [H^+]/K$, where $\bar{k}$ is the second-order rate constant for the mercaptide anion, K is the dissociation constant of SH groups and $[H^+]$ is the hydrogen ion concentration calculated from the pH of the reaction mixture. Fig. 9 shows a plot of k_{obs} *vs.* $k_{obs} \cdot [H^+]$ obtained from the inactivation measurements, and Fig. 10 shows a similar plot obtained by titration of the remaining amount of SH1 with DTNB at low ionic strength according to the method described in the legend of Fig. 7. From these plots, pK and $\bar{k}$ for the individual SH groups were calculated by least-squares method and summarized in Table VII, together with the values for mercaptoacetate and essential SH group of papain. The $\bar{k}$ values of SH2 were influenced by the kind of buffer used, and the enzymatic activity decreased rapidly above pH 10.5 in NH_4OH-NH_4Cl buffer (Fig.

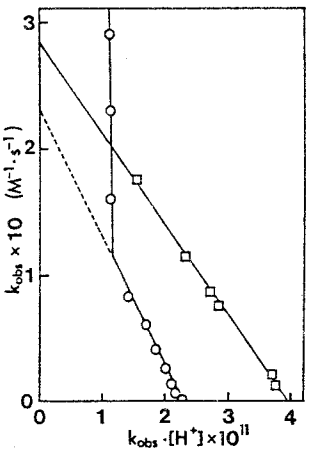


Fig. 9. k_{obs} *vs.* $k_{obs} \cdot [H^+]$ Plot for the Reaction of SH2 with Iodoacetamide. Soybean β-amylase (10 μM) was treated with 50 mM iodoacetamide at 25°C and the reaction was followed by taking aliquots from the reaction mixture into 0.2 M acetate buffer, pH 5.4, containing 50 mM 2-mercaptoethanol and 5 mM EDTA, and by measuring the decrease of enzymatic activity by the standard procedure. Buffers used were NH_4OH-NH_4Cl (○) and Na_2CO_3-$NaHCO_3$ (□) adjusted to ionic strength 0.1 by the addition of 1 M KCl

9), probably due to inactivation of the enzyme at alkaline pH. The $\bar{k}$ value of SH2 was 6 times smaller than that of SH1, but both $\bar{k}$ values were much smaller than that of mercaptoacetate or the essential SH group of papain. These results suggest the low reactivity of the two SH groups of soybean β-amylase, especially SH2, toward SH reagents. The pK value of SH2 is higher than that of SH1 and methyl mercaptoacetate, and it is close to the value of mercaptoacetate which has carboxyl group near its SH group. Though Gertler and Birk (*25*) and Nitta *et al.* (*26*) reported the possible participation of a pK 8 group

to the activity of soybean β-amylase, the group is presumably not SH2, which has a pK value of 9.9.

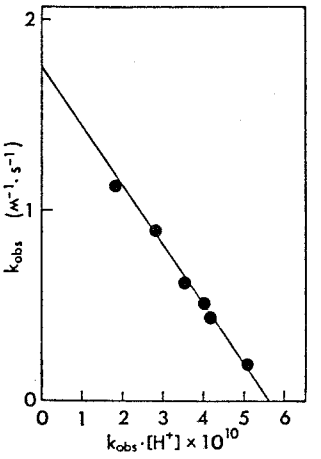


Fig. 10. k_{obs} *vs.* $k_{obs} \cdot [H^+]$ Plot for the Reaction of SH1 with Iodoacetamide. Soybean β-amylase was treated with 10-50 mM iodoacetamide at 25°C in Na_2CO_3-$NaHCO_3$ buffer adjusted to ionic strength 0.1. The reactions were followed by taking aliquots from the reaction mixture into 1 M acetate buffer, pH 5.0, containing 5 mM EDTA, then measuring the concentration of the SH groups, after gel filtration of the solution, with DTNB at low ionic strength as described in the legend of Fig. 7.

Table VII. pK and $\bar{k}$ of SH Groups Determined by Iodoacetamide.

SH group		pK	$\bar{k}$ ($M^{-1}.s^{-1}$)	Ref.
soybean β-amylase				
SH1	in carbonate buffer μ=0.1	9.4	1.7	
SH2	in carbonate buffer μ=0.1	9.9	0.21	
	in ammonium buffer μ=0.1	9.9	0.29	
papain		8.4	900	*87*
mercaptoacetate		10.25	63.2	*88*
methyl mercaptoacetate		7.9	-	*88*

Comparison of the reactivity of iodoacetamide, neutral reagent, toward these two SH groups with that of monoiodoacetate, negatively charged reagent, is of interest in connection with microenvironment of the SH groups in the folded enzyme protein. The alkylation of the SH groups by monoiodoacetate should be influenced by charged amino acid groups in the vicinity of the SH groups. The rate constants of the reactions of SH1 and SH2 with monoiodoacetate and iodoacetamide are summarized in Table VIII. The reaction of SH2 with iodoacetamide occured 64 times faster than that with monoiodoacetate, but the reactions of SH1 with the reagents differed only 8 fold. The reaction of SH2 with iodoacetamide was also influenced by the ionic strength of the reaction mixture. Inactivation of soybean β-amylase with iodoacetamide at high ionic strength (0.1 M Tris-HCl buffer containing 1 M KCl, pH 7.9) was found to proceed 7 times faster than at low

Table VIII. Rate Constants for the Reactions of the SH Groups of Soybean β-Amylase with Monoiodoacetate and Iodoacetamide.

SH group	Reagents	$k(M^{-1} \cdot s^{-1})$	Ratio	
SH2	monoiodoacetate	1.3×10^{-4}	1	
	iodoacetamide	8.3×10^{-3}	64	
SH1	monoiodoacetate	2.4×10^{-2}	185	1
	iodoacetamide	1.9×10^{-1}	1460	8

The reaction of the SH groups with the reagents were carried out in Tris-HCl buffer at pH 8.6, 25°C, μ=0.1. The reactions of SH2 were followed in terms of the decrease of enzymatic activity and those of SH1 were followed by titration with DTNB at low ionic strength, as described in the legend to Fig. 7.

ionic strength (0.1 M Tris-HCl buffer, pH 7.9). The high pK value of SH2, the effect of ionic strength on its reactivity with the modifiers, and the difference in reactivity of iodoacetamide and monoiodoacetate with this SH group suggest that an electrostatic interaction exists between SH2 and a polar side chain around it; a negatively charged group near the SH group should reduce the rate of the reaction with monoiodoacetate anion both by electrostatic repulsion and by raising the pK of the SH group. In addition, the electrostatic interaction between the SH group and a hypothetical carboxylate anion, or between the anion and negatively charged reagents such as DTNB and monoiodoacetate, shoud be reduced by raising the ionic strength of the reaction mixture.

2. The Role of SH2 in the Action of Soybean β-Amylase

In order to investigate the behavior of SH2 involved in the inactivation of soybean β-amylase, SH1 was first blocked with monoiodoacetate, which reacted with SH1 185 times faster than with SH2 (Table VIII). The modified enzyme retained 95% of the initial activity, and was purified on a column of CM-Sephadex C-50. Figure 11 shows the elution profile of the reaction products. Three fractions having the enzyme activity were defined as Fraction I, II and III. Fraction III was identical with the native soybean β-amylase, judging from its mobility on disc electrophoresis. Disc electrophoresis also showed that Fraction II was homogeneous, and this was confirmed

by polyacrylamide gel isoelectric focusing, as shown in insersion of Fig. 11. Fraction II had an activity equal to that of the native enzyme. Upon amino acid analysis, 1.0 mol of carboxymethyl cysteine was found per mol of Fraction II, and a trace amount of carboxymethyl histidine was also detected. On the other hand, Fraction I was not

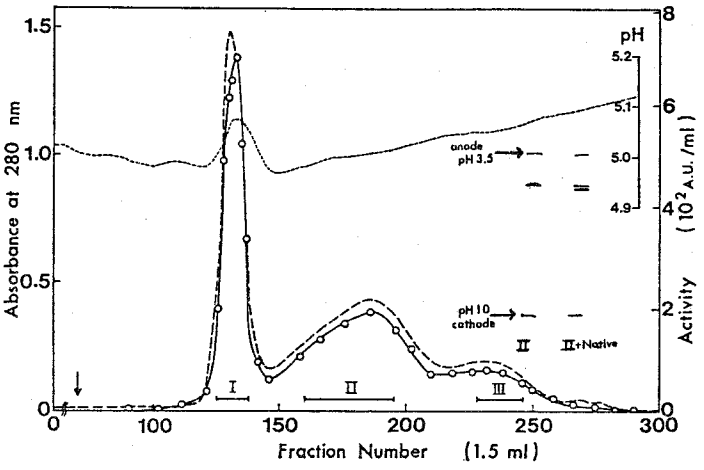


Fig. 11. Isolation of the Monoiodoacetate-treated Soybean β-Amylase on a Column of CM-Sephadex C-50.

In order to block only SH1 of soybean β-amylase, the enzyme (175 μM, total 50 mg) was exposed to 50 mM monoiodoacetate in 0.1 M Tris-HCl buffer, pH 8.6, at 25°C for 1 h. The modified enzyme was purified on a column (1.5 × 25 cm) of CM-Sephadex C-50, equilibrated with 50 mM acetate buffer containing 18 mM 2-mercaptoethanol and 1 mM EDTA, pH 5.0, then 15 ml of the sample solution was applied to the column, which was washed with 350 ml of the buffer solution. Elution was performed by changing the pH of the 50 mM buffer system at the vertical arrow to a linear gradient of pH 5.0 to 5.2 (150 ml each). Horizontal bars and Roman number indicate the fractions pooled. ------, absorbance at 280 nm; —○— activity of eluate; ·····, pH of eluate; insert, polyacrylamide gel isoelectric focusing of the monoiodoacetate-treated soybean β-amylase (Fraction II) and the native enzyme.

homogeneous in electrophoresis. It exhibited 85% of the activity of the native enzyme and contained 1.1 mol of carboxymethyl cysteine and a trace amount of carboxymethyl histidine. Therefore, Fraction I was considered to be a mixture of further modified enzymes, in which SH2 and some other amino acid residues were partly carboxymethylated. The selective modification of SH1 in Fraction II was confirmed by the reactions of DTNB and iodoacetamide with Fraction II. No SH group was reactive to DTNB at low ionic strength, while only one SH group was detected by DTNB at high ionic strength, accompanied by a decrease of the activity, as shown in Fig. 12. The

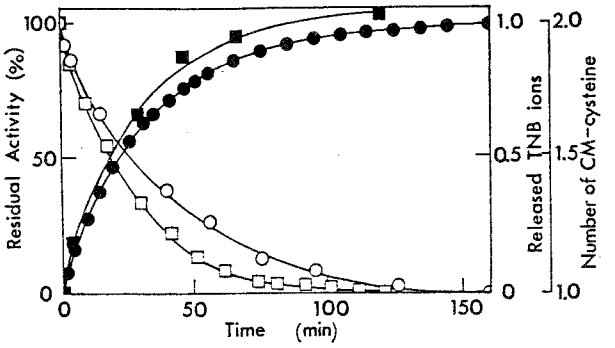


Fig. 12. Reaction of SH2 of SH1-Modified Soybean β-Amylase with DTNB and Iodoacetamide at High Ionic Strength.

Fraction II (15.5 μM) was treated with 262 μM DTNB in 0.05 M Tris-HCl buffer containing 0.8 M KCl and 5 mM EDTA at pH 8.15 and 25°C (○,●). Fraction II (32 μM) was also treated with 50 mM iodoacetamide in 0.1 M Tris-HCl buffer containing 1 M KCl and 5 mM EDTA at pH 7.92 and 25°C (□,■). —●—, amount of released TNB ions (mol/mol enzyme); —■—, number of detected carboxymethyl cysteine (mol/mol enzyme) by amino acid analysis; —○— and —□—, residual activity.

reaction with DTNB proceeded as a single second-order reaction, and the rate constant was found to be 1.8 $M^{-1} \cdot s^{-1}$, which is consistent with that of SH2 as mentioned before (Fig. 7). Figure 12 also shows the reaction of Fraction II with iodoacetamide. The enzyme activity decreased to 2% of the initial activity, with the formation of one residue of carboxymethyl cysteine.

Addition of 160 mM 2-mercaptoethanol to Fraction II modified at SH2 with DTNB resulted in full recovery of the initial activity with the release of 1 equivalent of TNB within 15 min at pH 8.0. The DTNB-modified Fraction II was also reactivated upon exposure to cyanide or sulfite. As shown in Fig. 13, both cyanide and sulfite released 0.9 equivalent of TNB; 65% of the initial activity was recovered on cyanide treatment, whereas 7% was recovered with sulfite.

In order to make sure the effect of substituent groups at SH2 on the activity of soybean β-amylase, the author used methyl 2,4-dinitrophenyldisulfide (MDPS) as another modifier of the SH groups of soybean β-amylase. This reagent was synthesized and kindly supplied by Dr. Y. Nagao, Institute of Chemistry, Kyoto University (*89*). Modification with MDPS converts SH group to $SSCH_3$ *via* thiol-disulfide exchange reaction and the absorption change is caused by scission of the disufide bond of the reagent. The substituent group, SCH_3, is much smaller than the substituent group introduced by DTNB, PCMB, N-ethylmaleimide and iodoacetamide, but is slightly larger than that introduced by cyanide. Thus, MDPS is useful to examin the

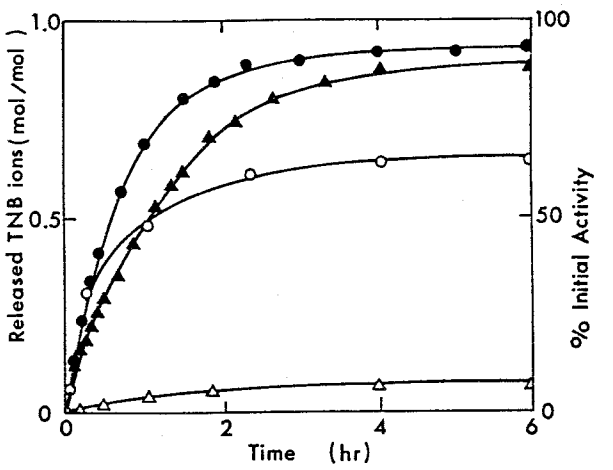


Fig. 13. Reactivation of DTNB-Modified Fraction II by Displacing TNB with Cyanide and Sulfite.

Fraction II modified at SH2 with DTNB (6.5 μM) was incubated at 25°C with 10 mM cyanide in 0.1 M Tris-HCl buffer, pH 8.0, containing 10 mM EDTA (○,●). The DTNB-modified Fraction II (5.9 μM) was also incubated at 25°C with 2 mM sulfite under the same conditions (△,▲). Enzymatic activity was followed by the standard procedure immediately after taking aliquots from the reaction mixture into a 10-fold excess volume of 0.2 M acetate buffer, pH 5.4. —●— and —▲—, amount of released TNB ions (mol/mol enzyme); —○— and —△—, per cent of the initial activity of Fraction II.

effect of substituent group at SH2 of soybean β-amylase on the enzymatic activity. Figure 14 shows the absorption spectra of MDPS and reduced MDPS by 2-mercaptoethanol. The spectrum of MDPS has a peak at 330 nm, but the reduced form, 2,4-dinitrothiophenol, has a peak at 408 nm. The molar extinction coefficient of 2,4-dinitrothiophenol is reported as 13,800 at 408 nm in aqueous media (90). In order to correct the remaining absorption of MDPS at 408 nm, the apparent

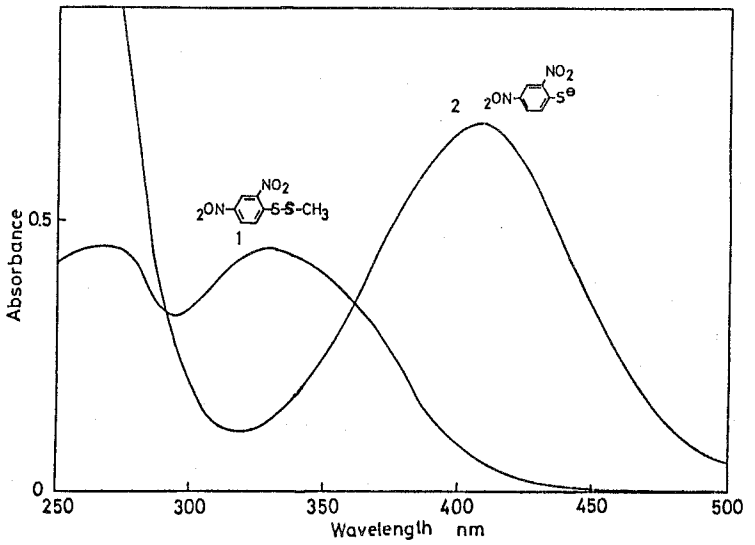


Fig. 14. Absorption Spectrum of MDPS and Reduced MDPS.

1, Absorption spectrum of 49.5 μM MDPS in 0.1 M Tris-HCl buffer, pH 8.0, containing 5% ethanol. MDPS was initially dissolved in 50% ethanol solution; 2, Absorption spectrum of the same solution in the presence of 1 mM 2-mercaptoethanol.

molar absorption coefficient, 12,900 was used for the determination of formed 2,4-dinitrothiophenol during modification. The MDPS reaction was tested with a variety of thiol-containing compounds. As shown in Table IX, in all cases the release of 2,4-dinitrothiophenol was found to be specific for thiols and essentially quantitative. The lineality of the MDPS reaction with cysteine was demonstrated at a cysteine concentration from 0 to 150 μM and at pH 6 to 9. The product of the MDPS reaction with 2-mercaptoethanol was identified with $HOCH_2CH_2SSCH_3$ by GC-MS. These results demonstrated the effectiveness of MDPS as a methane-thiolating reagent of SH groups in protein.

Table. IX. Methanethiolation of Thiol-containing Compounds by MDPS.

Compound	Concentration (μM)	Molar excess of reagent	Time (min)	Yield[1)] (%)
2-Mercaptoethanol	20	4.5	3	98
Cysteine	20	4.5	4	97
Glutathione	20	4.5	3.5	97
Bovine serum albumin	29	3.1	1	98
β-Amylase (in 6M Gu-HCl)	36	2.8	3	99

The reactions were carried out in 0.1 M Tris-HCl buffer, pH 8.0, containing 2 mM EDTA and 5% acetone.

1) Yields are based on the initial thiol content, as separately determined with DTNB.

Figure 15 shows the reaction of soybean β-amylase with MDPS in 0.1 M Tris-HCl buffer, pH 8. The reaction was followed by measuring the release of 2,4-dinitrothiophenol or by measuring the decrease of

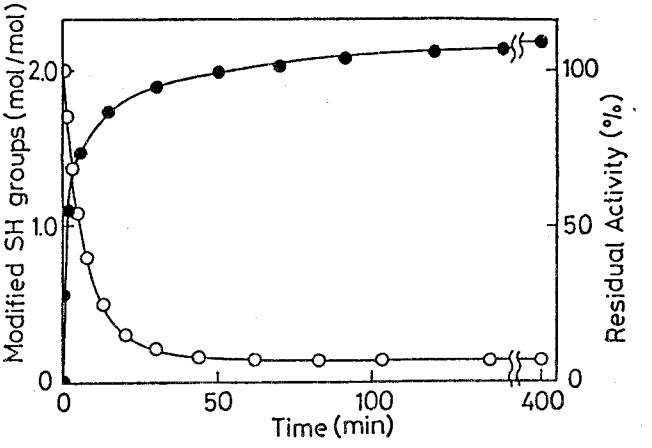


Fig. 15. Reaction of the SH Groups of Soybean β-Amylase with MDPS.

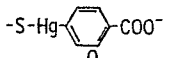
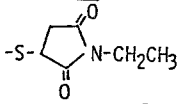
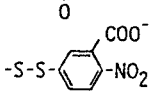
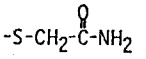
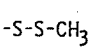
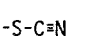
Soybean β-amylase (30.4 μM) was treated with 100 μM MDPS in 0.1 M Tris-HCl buffer containing 2 mM EDTA and 5% ethanol at pH 8.0 and 25°C. —●—, amount of modified SH groups (mol/mol enzyme) determined from the amount of released 2,4-dinitrophenol; —○—, residual activity.

the enzymatic activity. Two and two tenth of SH groups per mol of the enzyme reacted with the reagent and 7.2% of the initial activity was remained. The residual activity was constant after 50 min, while 0.2 mols of SH groups were further modified after 50 min. After the reaction, the MDPS-modified enzyme was treated with iodoacetamide, but no loss of the enzymatic activity was found. The two MDPS-modified SH groups seem to be SH1 and SH2 by considering the reaction of soybean β-amylase with DTNB (Fig. 7), and the remaining 7.2% activity surely represents the effect of the size of the substituent at SH2 on the enzymatic activity.

Table X summarizes the effect of the size of the substituent at SH2 of soybean β-amylase examined with a series of different derivatives. The enzyme contained a bulky substituent, PCMB and DTNB, exhibited less than 0.5% of the initial activity: other values were iodoacetamide 2%, sulfite 7%, MDPS 7.2% and cyanide 65%. Apparently, only the small substituent, cyanide, is compatible with high catalytic activity. These results exclude the possibility that SH2 is directly involved in catalysis or maintenance of an active enzyme conformation. The inactivation of soybean β-amylase with the bulky substituent at SH2 is caused by a local conformation change at the active site due to influence of the substituent groups on the position and reactivity of neighboring amino acid residues.

The possibility that the modification of SH2 of soybean β-amylase reduces the binding of substrate is also excluded, as conclud-

Table X. Effect of Modification at SH2 on Enzymatic Activity.

Reagent	Structure of modified thiol group	Extent of modification (mol /mol)	Enzymatic activity (% initial value)
PCMB		N. D.*	0.5
NEM		N. D.*	0.5
DTNB		1.0	0.5
Iodoacet-amide		1.0	2.0
Sulfite		0.90	7
MDPS		N. D.*	7
Cyanide		0.94	65
Control	-SH (regenerated)	1.0	102

* Not determined.

ed for sweet potato β-amylase (*23,28*). In addition, the author found that the Km values of soybean β-amylase blocked by iodoacetamide and cyanide at SH2 were 2.6 and 2.3 mg amylopectin/ml, respectively, which are close to or consistent with that for the native enzyme (2.3 mg amylopectin/ml). The effect of modification of SH2 on the binding of maltose or cyclohexadextrin to the enzyme is further discussed in the next chapter.

Since the significance of the size of the reagent molecule is demonstrated in the result of Vanaman and Stark (*91*) with aspartate transcarbamylase by cyanylation of it's single SH group, some of the so-called SH enzyme, as well as soybean β-amylase, have been found

to have nonessential SH groups for their activities. Isocitrate dehydrogenase (*92*), cytoplasmic aspartate aminotransferase (*93*) and creatine kinase (*94*) were demonstrated to restore their activities by cyanylation of their so-called essential SH groups. Creatine kinase (*95*) and aspartate aminotransferase (*91*) were also found to retain part of their activities after methanethiolation of their SH groups. However, cyanylation of the essential SH groups of papain (*96*), glyceraldehyde-3-phosphate dehydrogenase (*97*) and chloramphenicol acetyltransferase (*98*) completely inhibited their activities.

The nonessential SH groups of the enzymes whose blocking with bulky reagents causes a loss of the enzymatic activity are interesting in a view of *in vivo* regulation of their enzymatic activities. Oxidation of the nonessential SH groups with disulfides enables the reversible inactivation of the enzymes. It was reported that β-amylase in barley (*46*) and wheat (*47*) partly exists in the latant form. Treatment of the extract with 2-mercaptoethanol or papain activated the activity of β-amylase. In this connection, the author investigated the inactivation of soybean β-amylase with some naturally occuring disulfides. Figures 16 and 17 show the time course of inactivation of soybean β-amylase by disulfides in the presence of EDTA or Cu^{2+} ion. The inactivated soybean β-amylase by these disulfides was fully reactivated by the treatment with 100 mM 2-mercaptoethanol and no polymeric protein was observed on a SDS-gel electrophoresis without reducing reagents. Table XI summarizes the appar-

ent first-order rate constants of these reactions. Obviously, positively charged disulfides, cystamine and cystinedimethylester, inactivated soybean β-amylase faster than negatively charged disulfides. The significant effect of the positive charge on the disulfides may suggest the effect of the proposed negative charge on the

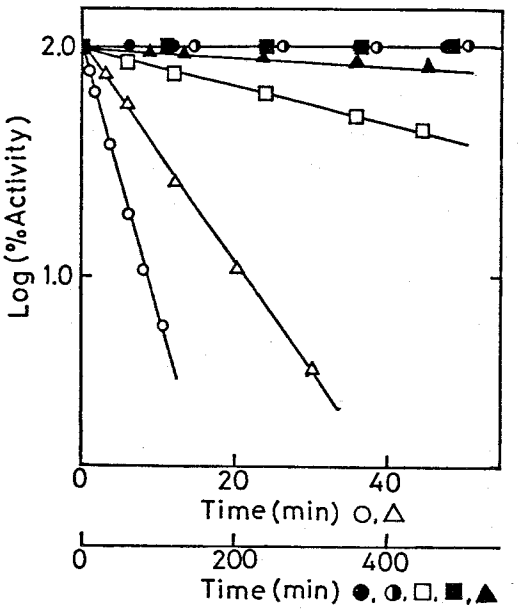


Fig. 16. Inactivation of Soybean β-Amylase by Disulfides in the Presence of EDTA.

Soybean β-amylase (9.08 μM) was incubated with the following disulfides in Tris-HCl buffer containing 5 mM EDTA at pH 8.5 and 25°C, adjusted to ionic strength 0.1, and the residual activity was measured in the standard procedure after the reaction was terminated by dilution of aliquots of the mixture with 0.5 M acetate buffer, pH 5.0. ○, 2 mM cystamine; △, 10 mM cystinedimethylester; □, 2.5 mM cystine; ▲, 1.25 mM homocystine; ◑, 7.5 mM glutathione; ■, 12.5 mM β,β'-dithiodipropionic acid; ●, without disulfides.

neighboring amino acid residue of SH2. The inactivation of soybean β-amylase with cystamine was further examined in different concentrations of cystamine as shown in Fig. 18. The inactivation of the enzyme did not proceed completely, but reached a constant value, corresponding to the concentration of the disulfide. This result indicates the equilibrium state as the following equation.

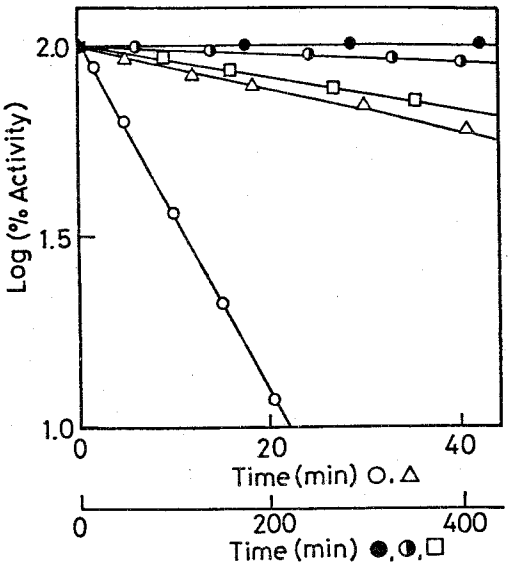


Fig. 17. Inactivation of Soybean β-Amylase by Disulfides in the presence of Cu^{2+}.

Soybean β-amylase was incubated with the following disulfides in Tris-HCl buffer, pH 8.5, containing 1 mM cupric acetate adjusted to ionic strength 0,1, and the residual activity was measured in the standard procedure after the reaction was terminated by dilution of aliquots of the mixture with 0.5 M acetate buffer, pH 5.0. ○, 3.44 μM β-amylase and 1.33 mM cystamine; △, 5.09 μM β-amylase and 2 mM cystinedimethylester; □, 5.09 μM β-amylase and 12.5 mM β,β'-dithiodipropionic acid; ◑, 5.09 μM β-amylase and 7.5 mM glutathione; ●, 5.09 μM β-amylase as a control.

Table XI. Inactivation of Soybean β-Amylase by Disulfides

Reagent	Formula	k_{app} ($M^{-1} \cdot s^{-1}$)	
		+EDTA	+Cu
Glutathione	$(_2HN(COOH)CHCH_2CONHCH(CONHCH_2COOH)CH_2S-)_2$	N.D.(5mM for 12h)	5.7×10^{-4}
β,β′-dithio-dipropionic acid	$(HOOCCH_2CH_2S-)_2$	N.D.(10mM for 12h)	1.4×10^{-3}
Dithiodiglicolic acid	$(HOOCCH_2S-)_2$	N.D.(10mM for 12h)	——
Homocystine	$(_2HNCH(COOH)CH_2CH_2S-)_2$	3.3×10^{-3}	——
Cystine	$(_2HNCH(COOH)CH_2S-)_2$	7.7×10^{-3}	——
Cystinedimethyl ester	$(_2HNCH(COCH_3)CH_2S-)_2$	8.8×10^{-2}	1.2×10^{-1}
Cystamine	$(_2HNCH_2CH_2S-)_2$	1.2	1.3

Reactions were carried out in Tris-HCl buffer, μ=0.1 at pH 8.5 and 25°C.
N.D., not detected; ——, not examined owing to precipitation.

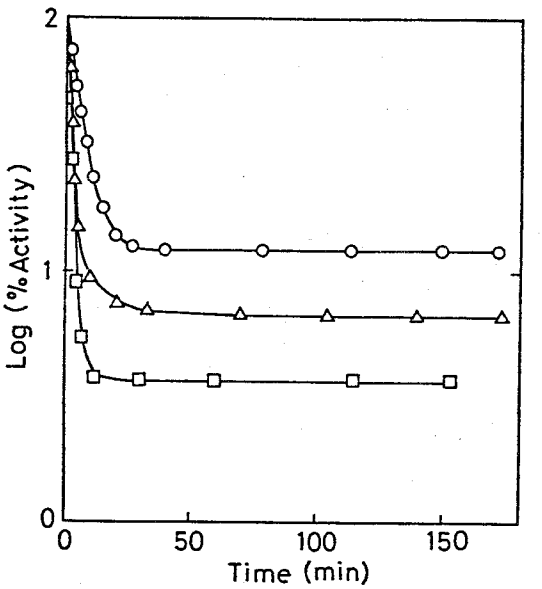


Fig. 18. Time Course of Inactivation of Soybean β-Amylase by Cystamine.

Soybean β-amylase was incubated with cystamine in Tris-HCl buffer, μ=0.1, pH 8.5 at 25°C in the presence of 5 mM EDTA. 1, 9.08 μM β-amylase was incubated with 2 mM cystamine (○); 2, 12.4 μM β-amylase was incubated with 5 mM cystamine (△); 3, 12.4 μM β-amylase was incubated with 10 mM cystamine (□). The residual activity reached 13.0, 6.5 and 3.6% of the initial activity for 1, 2 and 3, respectively.

$$R\text{-}S\text{-}S\text{-}R + E\text{-}SH2 \underset{}{\overset{K}{\rightleftharpoons}} R\text{-}S\text{-}S2\text{-}E + R\text{-}S\text{-}H$$

Where R-S-S-R and E-SH2 represent cystamine and SH2 of soybean β-amylase. The approximate equilibrium constant, K, was calculated from the finally residual activity of β-amylase, and it was found to be about 2.5×10^{-2} in Tris-HCl buffer, μ=0.1, at pH 8.5 and 25°C. The equilibrium lies so far to the left and it explaine the reduced reactivity of SH2 toward the disulfide. Thus the physiological significance of the *in vivo* inactivation system of soybean β-amylase *via* thiol-disulfide exchange reaction requires further investigation.

3. Effect of Modification of SH Groups on the Structure of Soybean β-Amylase

In order to clarify the mechanism of inactivation of soybean β-amylase by SH reagents, it is necessary to examine the effect of the modification on the structure or conformation of the enzyme. Figure 19 shows the circular dichroic spectra of the native and the SH1,SH2-modified soybean β-amylase with iodoacetamide. The content of α-helix and β-pleated sheet conformation of the enzyme was estimated to be 30% and 5-10%, respectively, on the basis of the analysis of the CD-spectrum in the far ultraviolet range (99). No difference was observed between the native and modified enzyme in the far ultraviolet range, but a little difference was detected in the near ultraviolet region due to tryptophan and tyrosine residues. Figure 20 shows the difference spectra induced by alkylation of the SH groups.

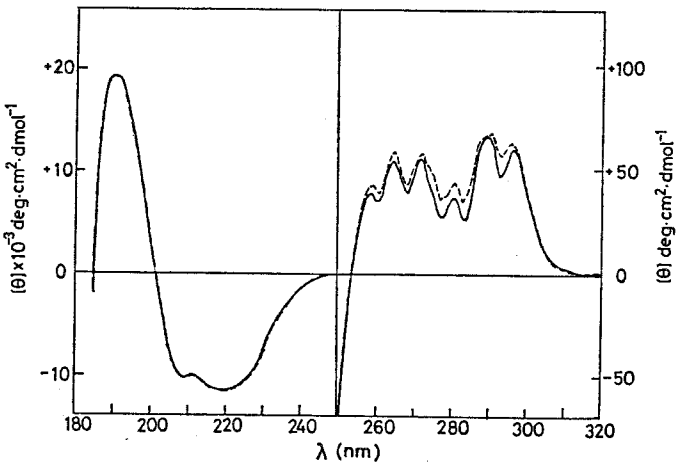


Fig. 19. CD Spectra of Native and Modified Soybean β-Amylase.

The spectra were measured in 0.1 M acetate buffer, pH 5.4, containing 2 mM EDTA at a protein concentration of 11 μM. ———, native enzyme; - - - -, SH1,SH2-modified β-amylase with iodoacetamide. CD spectra were measured at room temperature using a JASCO J-500 spectropolarimeter. Cells having a light path of 0.1 mm and 10 mm were used for the measurements in far and near ultraviolet wavelength region, respectively.

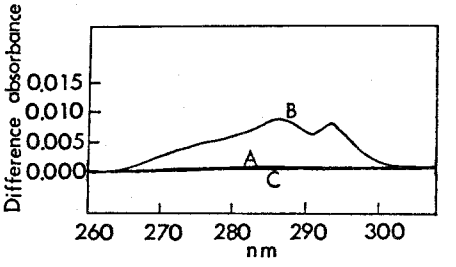


Fig. 20. Difference Spectra of Alkylated Soybean β-Amylase Against the Native Enzyme in 0.1 M Acetate Buffer, pH 5.4.

The spectra shown are those of: A, monoiodoacetate-treated soybean β-amylase (Fraction II) against the native enzyme at a protein concentration of 12.4 μM; B, iodoacetamide-treated Fraction II against the native enzyme at a protein concentration of 14.1 μM; C, iodoacetamide-treated soybean β-amylase, in which SH1 and SH2 were previously protected by DTNB then exposed by treatment with 2-mercaptoethanol, against the native enzyme at a protein conentration of 10.3 μM.

The carboxymethylation of SH1 with monoiodoacetate induced no difference spectrum against the native enzyme and no loss of the enzymatic activity was found. However, the carboxyamidemethylation of SH2 of SH1-carboxymethylated β-amylase with iodoacetamide produced a difference spectrum mainly due to tryptophan residues. In order to confirm that the difference spectrum was specific for the modification of SH2, the following experiment was performed. Soybean β-amylase was first treated with DTNB at high ionic strength to block the two SH groups, and the DTNB-modified enzyme (35 μM) was then treated with 50 mM iodoacetamide in 0.1 M Tris-HCl buffer, pH 8.6, at 25°C for 100 min. After subjecting the reaction mixture to gel filtration, bound TNB was released by the addition of 2-mercaptoethanol, which was then removed by gel filtration, and the difference spectrum of the enzyme was recorded against the native soybean β-amylase. No difference spectrum was observed as shown in Fig. 20 (curve C). Therefore, it was concluded that the previous difference spectrum was specific for the modification of SH2. These finding indicate that the modification of SH2 does not alter secondary structure of the enzyme but does affect the environment of tryptophan and tyrosine residues near SH2.

IV. INTERACTION OF NATIVE AND SH-MODIFIED β-AMYLASE OF SOYBEAN WITH CYCLOHEXADEXTRIN AND MALTOSE

Studies of the interaction of various amylase with their substrates, products or inhibitors by the difference spectroscopic technique have suggested that α-amylase from porcine pancreas (*100*) and β-amylase from sweet potato (*82*) have a tryptophan residue near the binding sites of their product, maltose, and that α-amylase from *Bacillus subtilis* (*101*, *102*), Taka-amylase A (*103*) and glucoamylase from *Rizopus niveus* (*104*) have a tryptophan residue near the binding sites of their substrates, products and inhibitors. The technique is also useful to investigate the effects of modification of amino acid residues on the catalytic activity or the binding of substrate, product or inhibitors.

In the previous chapter, the author demonstrated that SH2 of soybean β-amylase does not concern the catalytic step of the enzyme action. The degree of inactivation of the enzyme was dependent on the bulkiness of the substituent at SH2, but the Km value for amylopectin was little affected by the modification of SH2 with iodoacetamide. However, the modification itself was prevented by maltose or by cyclohexadextrin. Thus the possibility remains that SH2 might be involved in the binding of smaller sugars, such as cyclohexadextrin and maltose. And the different protective effect of cyclohexadextrin and maltose may reflect the different binding site of these sugars.

In this chapter, the author describes the interaction of native

and modified soybean β-amylase with cyclohexadextrin or with maltose by using ultraviolet difference spectra of the enzyme produced by these sugars. In order to investigate the effect of different substituent at SH2, modification was carried out with iodoacetamide or DTNB (*105*).

1. Interaction of Native and Modified Soybean β-Amylase with Cyclohexadextrin and Maltose

Figure 21 shows the difference spectra of native and SH1,SH2-modified β-amylase produced by cyclohexadextrin and maltose. The environment of tryptophan and tyrosine residues of soybean β-amylase were affected by the binding of cyclohexadextrin or maltose to the enzyme. The difference spectrum of the native enzyme produced by cyclohexadextrin has a characteristic peak at 299 nm (Fig. 21-A), whereas that produced by maltose has a small trough at around 300 nm (Fig. 21-B). The different effects of binding of cycloheptadextrin and maltose have been demonstrated by circular dichroic study of soybean β-amylase (*99*). In the CD-spectra, cycloheptadextrin reduced the peak at 297 nm, but maltose did very slightly. These observations suggest that the local conformation change induced by the binding of cyclohexa(hepta)dextrin is different from that induced by maltose. As shown in Fig. 21, the difference spectrum of the native enzyme produced by cyclohexadextrin was affected little by the modification of SH1 and SH2 with iodoacetamide or DTNB. The difference

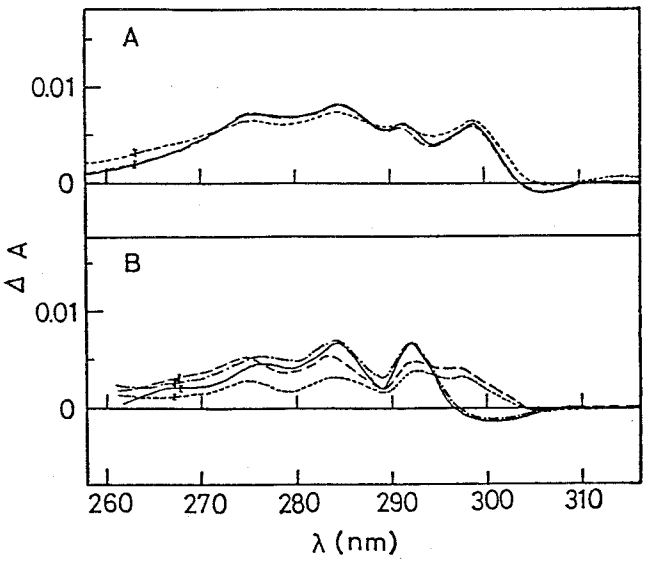


Fig. 21. Difference Spectra of Native and Modified Soybean β-Amylase Produced by Cyclohexadextrin and Maltose.

Difference spectra were measured with enzyme solutions in 0.1 M acetate buffer, pH 5.4, containing 2 mM EDTA by means of a Shimadzu MPS-5000 spectrophotometer at 25°C. A: Difference spectra produced by cyclohexadextrin. The spectra of 9.83 μM β-amylase (———), 9.76 μM SH1,SH2-modified β-amylase with iodoacetamide (-----) and 10.7 μM SH1,SH2-modified β-amylase with DTNB (·····) were recorded in the presence of 3.23 mM cyclohexadextrin against the reference solution without cyclohexadextrin. B: Difference spectra produced by maltose. The spectra of 9.69 μM β-amylase and 17.0 mM maltose (———), 10.3 μM SH1-carboxymethylated β-amylase and 15.8 mM maltose (— · —), 9.95 μM SH1,SH2-modified β-amylase with iodoacetamide and 16.4 mM maltose (-----) and 9.80 μM SH1,SH2-modified β-amylase with DTNB and 17.1 mM maltose (·····) were recorded against the reference solution without maltose.

spectra have peaks at 285, 292 and 299 nm. On the other hand, the difference spectrum of the enzyme produced by maltose was strongly affected by the modification of SH1 and SH2 with iodoacetamide or DTNB, but it was little affected by the modification of only SH1 as

shown in Fig. 21-B. The difference spectra of native and SH1-carboxymethylated soybean β-amylase have peaks at 285 and 292 nm, and a trough at around 300 nm, whereas that of SH1,SH2-modified enzymes have diminished peaks at 285 and 292 nm and a new peak at 297 nm instead of the trough at around 300 nm in the case of native enzyme. The modification of the SH groups with iodoacetamide or with DTNB showed almost the same effect on the difference spectrum produced by maltose. These observations are compatible with the result obtained by the difference spectra induced by the modification of SH2 against the native enzyme as shown in Fig. 20. The tryptophan residue that is affected by the modification of SH2 seems to correspond to that affected by the binding of maltose to the enzyme. As the peaks at 299 nm produced by cyclohexadextrin and the peaks at 292 and 297 nm produced by maltose are caused by tryptophan residues (*106*,*107*), the author examined the behavior of the spectra in these wavelength regions. The apparent molar absorption change, $\Delta\varepsilon_{obs}$, at 299 nm of the native and the modified enzyme produced by cyclohexadextrin is shown in Fig. 22-A. $\Delta\varepsilon_{obs}$ at 292 nm of the native and $\Delta\varepsilon_{obs}$ at 292 and 297 nm of the iodoacetamide-modified enzyme produced by maltose are shown in 23-A. $\Delta\varepsilon_{obs}$ at 299 nm in Fig. 22-A and $\Delta\varepsilon_{obs}$ at 297 nm in Fig. 23-A showed typical saturation curves without subtraction of the nonspecific solvent perturbation effects of cyclohexadextrin and maltose, respectively. These results are consistent with the fact that the difference spectrum of a tryptophan residue near 300 nm is insensitive to the effect of nonpolar nature

of the medium (*104*,*107*,*108*). On the other hand, $\Delta\varepsilon_{obs}$ at 292 nm of the native and the modified enzymes produced by maltose gradually increased even at high maltose concentration, owing to nonspecific solvent perturbation of tryptophan residues, as shown in Fig. 23-A. Thus the molar difference absorption coefficient, $\Delta\varepsilon$, at 292 nm of the native and the modified enzymes produced by the specific binding of maltose was estimated by sabtracting the solvent perturbation effect of sucrose as shown in Fig. 23-A. These saturation curves are analyzed by the following Michaelis-Menten type equations, (1) and (2), on the assumption that the magnitudes of $\Delta\varepsilon_{obs}$ at 299 and 297 nm and $\Delta\varepsilon$ at 292 nm are proportional to the concentration of the enzyme-cyclohexadextrin and the enzyme-maltose complexes, res-

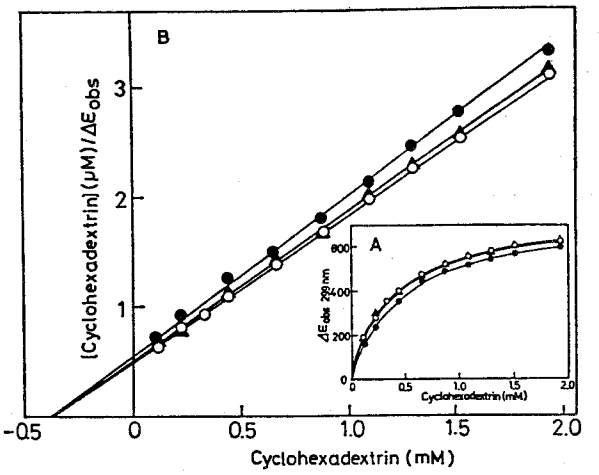


Fig. 22. A: Effects of Concentration of Cyclohexadextrin on the Absorption Increments of Native and Modified β-Amylase Observed at 299 nm. —○—, β-amylase; —●—, SH1,SH2-modified β-amylase with iodoacetamide; —▲—, SH1,SH2-modified β-amylase with DTNB. B: Plots of [cyclohexadextrin]/$\Delta\varepsilon_{obs,299\ nm}$ *versus* [cyclohexadextrin]. The symbols are the same as in Fig. 22 A.

pectively (*109*).

$$[\text{cyclohexadextrin}]/\Delta\varepsilon_{\text{obs, 299 nm}} = [\text{cyclohexadextrin}]/\Delta\varepsilon_{\text{max}} + Kd_{\text{cyc}}/\Delta\varepsilon_{\text{max}} \quad (1)$$

$$[\text{maltose}]/\Delta\varepsilon_{292\text{ nm}}([\text{maltose}]/\Delta\varepsilon_{\text{obs, 297 nm}}) = [\text{maltose}]/\Delta\varepsilon_{\text{max}} + Kd_{\text{mal}}/\Delta\varepsilon_{\text{max}} \quad (2)$$

Here Kd_{cyc} and Kd_{mal} represent the dissociation constants of the enzyme-cyclohexadextrin complex and the enzyme-maltose complex, respectively, and $\Delta\varepsilon_{\text{max}}$ represents the maximum molar coefficient.

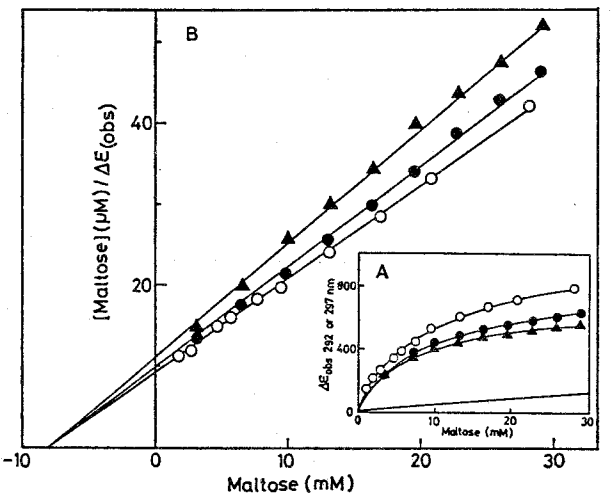


Fig. 23. A: Effects of Concentration of Maltose on the Absorption Increments of Native and Modified β-Amylase Observed at 292 and 297 nm. —○—, β-amylase observed at 292 nm; —●—, SH1,SH2-modified β-amylase with iodoacetamide observed at 292 nm; —▲—, SH1,SH2-modified β-amylase with iodoacetamide observed at 297 nm. B: Plots of $[\text{maltose}]/\Delta\varepsilon_{292\text{ nm}}$ and $[\text{maltose}]/\Delta\varepsilon_{\text{obs, 297 nm}}$ *versus* [maltose]. The symbols are the same as in Fig. 23 A. The values of $\Delta\varepsilon_{292\text{ nm}}$ were obtained from $\Delta\varepsilon_{\text{obs, 292 nm}}$ by subtracting the nonspecific solvent perturbation effect of sucrose.

Table XII. Kd and $\Delta\varepsilon_{max}$ for Soybean β-Amylase-cyclohexadextrin and Soybean β-Amylase-maltose Complexes

Soybean β-amylase derivatives	Cyclohexadextrin		Maltose		
	Kd (mM)	$\Delta\varepsilon_{max,\ 299\ nm}$	Kd (mM)	$\Delta\varepsilon_{max,\ 292\ nm}$	$\Delta\varepsilon_{max,\ 297\ nm}$
Native	0.35 ± 0.01	740 ± 5	8.1 ± 0.1	880 ± 20	-
Iodoacetamide-modified	0.36 ± 0.02	710 ± 20	8.0 ± 0.1	655 ± 20	720 ± 20
DTNB-modified	0.34 ± 0.02	735 ± 20	-	-	-

Figure 22-B shows plots of [cyclohexadextrin]/$\Delta\varepsilon_{obs,\ 299\ nm}$ *versus* [cyclohexadextrin] for the native and the modified enzymes. Figure 23-B shows plots of [maltose]/$\Delta\varepsilon_{292\ nm}$ *versus* [maltose] for the native and the modified enzymes, and the plots of [maltose]/$\Delta\varepsilon_{obs,\ 297\ nm}$ *versus* [maltose] for the modified enzyme. From these plots, the dissociation constants, Kd_{cyc} and Kd_{mal}, and the maximum molar coefficients, $\Delta\varepsilon_{max}$, were obtained from the intercepts on the [cyclohexadextrin] and [maltose] axes and the reciprocal of the slopes, respectively. The values determined by the least square method are summarized in Table XII. The determined Kd values for native β-amylase-cyclohexadextrin and β-amylase-maltose complexes were 0.35 mM and 8.1 mM, respectively. The obtained Kd value for the enzyme-cyclohexadextrin complex was consistent with the Ki value for cyclohexadextrin measured by the effect of this inhibitor on the enzymatic activity. Thus, cyclohexadextrin showed competitive inhibition with amylopectin as substrate, and its Ki value was determined to be 0.34 mM. The obtained Kd value for soybean β-amylase-maltose complex is

smaller than that reported by Uehara and Mannen (82) for sweet potato β-amylase-maltose complex (24 mM at pH 3.8). As shown in Table XII, the Kd values for soybean β-amylase-cyclohexadextrin and the enzyme-maltose complexes are not changed by the modification of SH1 and SH2 with iodoacetamide or DTNB. This clearly demonstrates that SH2 of soybean β-amylase does not concern the binding of these sugars, though its modification alters the absorption spectrum of the enzyme produd by maltose, especially affecting the tryptophan residues.

2. Competitive Binding of Cyclohexadextrin and Maltose to Soybean β-Amylase

In order to elucidate the relationship between the binding of cyclohexadextrin and that of maltose, the absorption change of β-amylase induced by cyclohexadextrin was observed in the presence of maltose. Figure 24 shows the difference spectra of the native and DTNB-modified β-amylase produced by 6.54 mM cyclohexadextrin in the

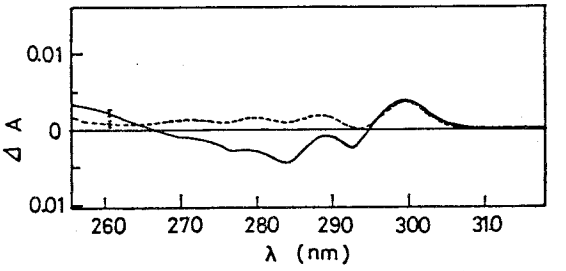


Fig. 24. Difference Spectra of Native and Modified β-Amylase Produced by Cyclohexadextrin in the Presence of Maltose.

The spectra of 9.35 μM β-amylase (———) and 9.31 μM SH1,SH2-modified β-amylase with DTNB (-----) were recorded in the presence of 125 mM maltose and 6.54 mM cyclohexadextrin against the solution without cyclohexadextrin.

presence of 125 mM maltose. The spectrum of the native enzyme has peaks at 299 nm, and troughs at 285 and 292 nm. By using $\Delta\varepsilon_{obs}$ at 299 nm, the apparent dissociation constant of the enzyme-cyclohexadextrin complex in the presence of maltose, Kd_{app}, was determined from the plots of [cyclohexadextrin]/$\Delta\varepsilon_{obs,\ 299\ nm}$ *versus* [cyclohexadextrin], on the assumption that $\Delta\varepsilon_{obs,\ 299\ nm}$ corresponds to the enzyme-cyclohexadextrin complex even in the presence of maltose. Figure 25 shows the plots for the native enzyme in the presence or absence of 125 mM maltose. The Kd_{app} and $\Delta\varepsilon_{max}$ in the presence of 125 mM maltose were determined to be 3.2 mM and 670 from the intercept on the [cyclohexadextrin] axis and the reciprocal of the slope, respectively. The presence of 125 mM maltose caused about 10-fold

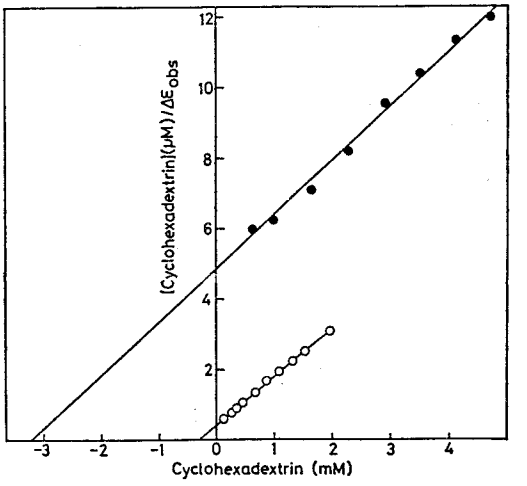


Fig. 25. Plots of [cyclohexadextrin]/$\Delta\varepsilon_{obs,\ 299\ nm}$ *versus* [cyclohexadextrin] for the Specific Difference Spectra of β-Amylase Produced by Cyclohexadextrin in the Presence of Maltose.

—●—, in the presence of 125 mM maltose; —○—, in the absence of maltose.

increase in the apparent dissociation constant of the enzyme-cyclohexadextrin complex, but the change of the $\Delta\varepsilon_{max}$ was small (Table XII). If cyclohexadextrin and maltose bind to β-amylase independently, Kd_{app} obtained from Fig. 25 should be consistent with Kd_{cyc} obtained from Fig. 22. To elucidate the interaction between the binding of cyclohexadextrin and that of maltose, the effect of concentration of maltose on the Kd_{app} was further examined, and the obtained Kd_{app} for the native and DTNB-modified enzyme was plotted against the concentration of maltose, as shown in Fig. 26. The data follow straight lines and no difference was observed between the native and the modified enzyme. When cyclohexadextrin and maltose bind to β-amylase competitively, equation (1) can be rewritten as the following equation (3) by analogy with the competitively inhibited enzyme

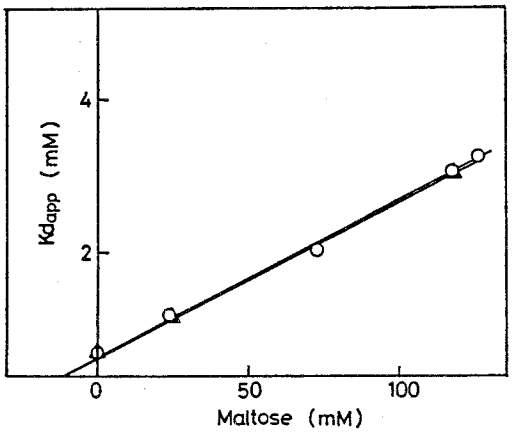


Fig. 26. Effects of Concentration of Maltose on Kd_{app} for Native and Modified β-Amylase-cyclohexadextrin Complexes.

—○—, β-amylase; —▲—, SH1,SH2-modified β-amylase with DTNB.

kinetics (*110*).

$$[\text{cyclohexadextrin}]/\Delta\varepsilon_{\text{obs, 299 nm}} = [\text{cyclohexadextrin}]/\Delta\varepsilon_{\text{max}} + (Kd_{\text{cyc}} + [\text{maltose}] \cdot Kd_{\text{cyc}}/Kd_{\text{mal}})/\Delta\varepsilon_{\text{max}} \quad (3)$$

Combination of equation (3) with equation (1) allows the apparent dissociation constant of the enzyme-cyclohexadextrin complex in the presence of maltose, Kd_{app}, to be expressed by equation (4).

$$Kd_{\text{app}} = Kd_{\text{cyc}} + [\text{maltose}] \cdot Kd_{\text{cyc}}/Kd_{\text{mal}} \quad (4)$$

From equation (4), the dissociation constant of maltose, Kd_{mal}, and the dissociation constant of cyclohexadextrin, Kd_{cyc}, were determined to be 9 mM and 0.32 mM from the intercept on the [maltose] axis and the reciprocal of the slope of the straight line in Fig. 26. These values are in good agreement with the values in Table XII. This result demonstrates that cyclohexadextrin and maltose bind to soybean β-amylase competitively and that the modification of SH2 does not affect the relationship.

Misra and French (*111*) reported that maltose was a competitive inhibitor of sweet potato β-amylase, and that the Ki value of maltose was 6 mM. In contrast with the result of Misra and French, Thoma *et al.* reported that maltose was a noncompetitive inhibitor of sweet potato enzyme (*11*). In this study, however, it was found that cyclohexadextrin and maltose bind to soybean β-amylase competitively. Therefore, the binding site of cyclohexadextrin and that of maltose are very close to the substrate binding site of β-amylase. Moreover, the effect of modification of SH2 on the difference spectrum

induced by maltose indicates that SH2 is located near the binding site of maltose and cyclohexadextrin. In the previous chapter, the author described that SH2 is protected against DTNB by maltose or by cyclohexadextrin and that the protective effect of maltose is much stronger than that of cyclohexadextrin. The different effect of maltose and cyclohexadextrin is compatible with the different effect of modification of SH2 between on the difference spectra produced by maltose and cyclohexadextrin. The protective effect of maltose and cyclohexadextrin seems to be caused not by the direct screening of SH2 by these sugars located on the binding sites but by a local conformation change around the active site due to their binding. The results of this chapter show that the local conformation change around the active site of soybean β-amylase caused by maltose is more favorable to the protection of SH2 against its modifier than that caused by cyclohexadextrin. In summary of Chapter III and IV, the mechanism of inactivation of soybean β-amylase by modification of SH2 may as follows. SH2 is situated close to the substrate binding site, and bulky substituent groups at SH2 may cause a conformation change that prevents the substrate from binding productively at the active site. Alternatively, SH2 may be situated close to both the binding site of substrate and the catalytic site, and the bulky substituent groups may inhibit the catalytic action directly by local conformation change due to steric hindrance.

V. LOCATION OF SH GROUPS ALONG THE POLYPEPTIDE CHAIN OF SOYBEAN β-AMYLSAE

In the previous chapters, the author suggested that SH2 is located close to the binding site of substrate in the active region of soybean β-amylase. It is interesting to identify the SH groups on the primary structure of the enzyme to know the relationship between the structure and function of the enzyme. The location of SH2 on the polypeptide chain of soybean β-amylase will serve to elucidate the active region of the enzyme by X-ray crystallographic determination of its three dimentional structure. If the cleavage of the polypeptide chain is attained selectively at the SH groups and the resulting peptides are characterized, it is possible to identify the positions of the SH groups.

Specific cleavage of the polypeptide chain at cysteine residues can be achieved after modifying the SH groups to SCN according to the method of Degani and Patchornik (*112*) and Jacobson *et al.* (*113*). Two methods are known for the preparation of S-cyanocysteine residues in protein (*113*). One is the one step method using 2-nitro-5-thiocyanobenzoic acid (NTCB) for cyanylation of cysteine residues, and it has been used for cleavage studies of enzyme and proteins (*114-117*). The other is a two step method using cyanide after preparation of mixed disulfides at the cysteine residues to be cleaved (*113*). In order to locate the five SH groups on the polypeptide chain of soybean β-amylase, the author used the latter method for

limited cleavage at the selectively modified SH groups with 2,2'-dithiopyridine (2-PDS). The fragments formed were identified by the estimation of molecular weights by SDS-polyacrylamide gel electrophoresis as well as by using radioactive cyanide (*118*).

1. General Scheme for Selective Cyanylation of SH Groups in Soybean β-Amylase

The five SH groups of soybean β-amylase were designated as SH1, SH2, SH3, SH4 and SH5, in order of their reactivity toward SH reagents. As shown below, SH1, SH2 and SH3 are distinguished by the order of their reactivity toward 2-PDS, while SH4 and SH5 are buried. The author tried to identify these five SH groups along the polypeptide chain of the enzyme by cyanylation and cleavage at the partic-

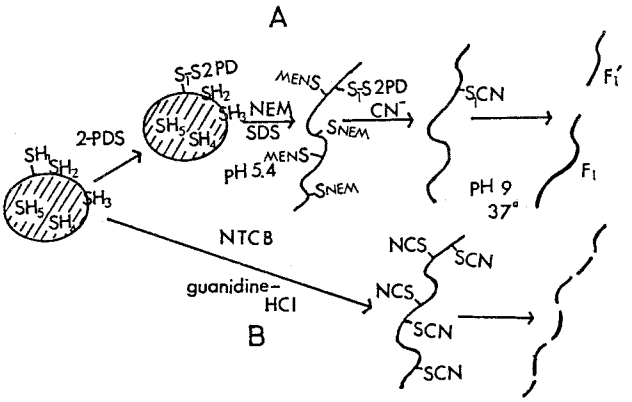


Fig. 27. Schematic Diagram of the Reactions for Cysteine-Specific Cleavage of Soybean β-Amylase.

A, Reactions for the cleavage at the selected SH group (SH1); B, Reactions for cleavage at all SH groups. The fragments formed from soybean β-amylase were designated such as F_1 and F_1' for the fragments cleaved at SH1; F1 represents the larger fragment and F_1', the smaller fragment.

ular SH groups. Typical procedures for cyanylation and cleavage at a selected SH group (*e.g.* SH1) and that for at all the five SH groups are shown schematically in Fig. 27. The author used 2-PDS for the selective modification because the reactivity of NTCB was too low to cyanylate the SH groups of soybean β-amylase in the native state. The bound 2-PD is easily replaced by cyanide to form cyanocysteine residues. Moreover, in contrast to DTNB, the release of 2-PD can be followed even in slightly acidic conditions, as described by Grassetti and Murray (*119*). For the selective cyanylation at the 2-PD bound SH group, it is important to prevent the decomposition of the modified SH groups during following denaturation and irreversible blocking of the remaining SH groups with NEM. Denaturation causes the exposure of internal, free SH groups and it releases the bound 2-PD at the modified SH groups *via* the thiol-disulfied exchange reaction. Fig. 28 shows the release of 2-PD from β-amylase modified at SH1 when

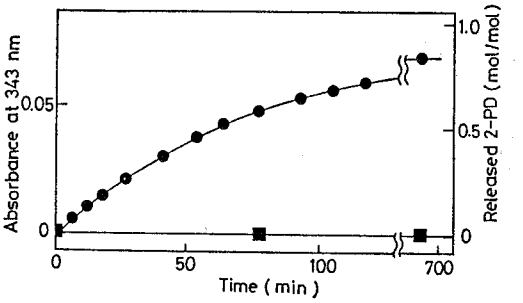


Fig. 28. Kinetics of the release of 2-PD from Soybean β-Amylase Modified at SH1 with 2-PDS by Denaturation with 1% SDS.

Denaturation of SH1-modified β-amylase (9.8 μM) was carried out by the addition of SDS in 0.1 M acetate buffer containing 5 mM EDTA, at pH 5.4 and 25°C, and the release of 2-PD was followed spectrophotometrically using $\varepsilon_{343\ nm} = 8.08 \times 10^3\ M^{-1}.cm^{-1}$ (*120*). —●—, in the absence of NEM; —■—, in the presence of 10 mM NEM.

it was denatured with 1% SDS at pH 5.4. The reaction was followed by first-order kinetics, and the rate constant was determined to be 1.8×10^{-4} s^{-1}. Under the same conditions, NEM reacted with the five SH groups more rapidly than the release of 2-PD from the modified enzyme. The reaction of NEM with the five SH groups was followed spectrophotometrically in 0.1 M acetate buffer, pH 5.4, containing 1% SDS and 5 mM EDTA at the protein concentration of 83 μM according to the method of Glazer *et al.* (*121*). Though the reaction did not follow simply second-order kinetics, the apparent second-order rate constant of the slowest phase (about 1 SH group) was estimated to be greater than 0.4 $M^{-1} \cdot s^{-1}$. Thus prior addition of NEM at a concentration of more than 10 mM in the denaturation step can protect

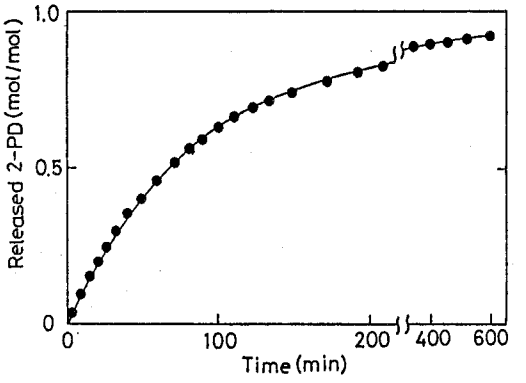


Fig. 29. Release of 2-PD from Soybean β-Amylase Modified at SH1 with 2-PDS by Cyanide.

After denaturation and NEM-treatment, the derivative of soybean β-amylase modified at SH1 with 2-PDS was cyanylated with 10 mM KCN at a protein concentration of 20 μM in 0.1 M Tris-HCl buffer, pH 7.6, containing 5 mM EDTA and 2% SDS at 25°C, and the release of 2-PD was followed spectrophotometrically.

the release of 2-PD from the SH1-modified enzyme as indicated in Fig. 28. Next the bound 2-PD at SH1 of the denatured enzyme was replaced with cyanide as shown in Fig. 29, and about 90% of the previously incorporated 2-PD was replaced with cyanide. These procedures were used for the selective cyanylation of SH1-, SH1 & SH2-, and SH2 & SH3-modified β-amylase with 2-PDS, and the cleavage of the enzyme at the modified SH groups could be attained.

2. Cleavage of Soybean β-Amylase at the Selectively Modified SH Groups

Figure 30 shows the reaction of SH groups in soybean β-amylase with 2-PDS at low and high ionic strength. Only one SH group, SH1,

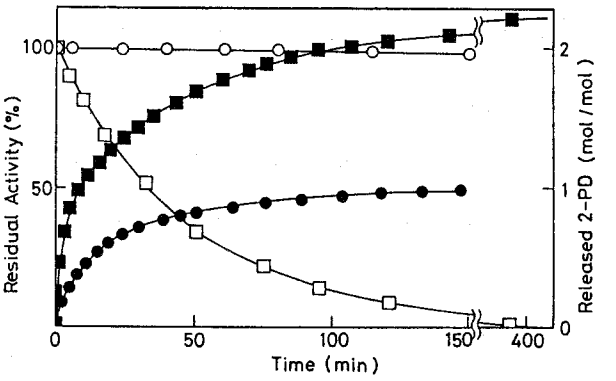


Fig. 30. Modification of SH1 and SH2 of Soybean β-Amylase with 2-PDS.

β-Amylase (19 μM) was treated with 116 μM 2-PDS in 0.01 M Na-phosphate buffer containing 5 mM EDTA, at pH 7.6 and 25°C (○,●). The enzyme (16 μM) was also treated with 202 μM 2-PDS in 0.1 M Tris-HCl buffer containing 1 M NaCl and 5 mM EDTA, at pH 8.0 and 25°C (□,■). —●— and —■—, amount of released 2-PD (mol/mol enzyme); —○— and —□—, residual activity.

reacted with 2-PDS at low ionic strength and no loss of enzymatic activity was found. On the other hand, at high ionic strength, 2.2 mols SH groups per mol of the enzyme reacted with 2-PDS, and the enzymatic activity was decreased by modification of SH2. These β-amylases with 2-PD bound at SH1 or at SH1 and SH2 were denatured with 2% SDS in the presence of 20 mM NEM to block the internal, free SH groups. Then they were cyanylated with cyanide and cleaved at SH groups previously bound with 2-PD. Figure 31-A and -B show the resulting fragments detected by SDS-polyacrylamide gel electrophoresis. Only one fragment, F_1 (Mw. 50,000), was found for the β-amylase cleaved at SH1, while F_1, F_2 (47,000) and F_{1-2} (39,000) were detected for the β-amylase cleaved at both SH1 and SH2. A relatively large amount of F_2 was detected together with the larger fragment cleaved at SH1. A smaller quantity of F_{1-2} shows that it was formed by cleavage at both SH1 and SH2. Besides these, a smaller quantity of a pair of fragments (Mw. 31,000 and 25,000) corresponded to the modification of SH3 (0.2 mol/mol enzyme) as a side reaction.

For the purpose of cleavage of β-amylase at SH2 and SH3, the reaction with 2-PDS of the SH groups in the SH1-carboxymethylated β-amylase was followed in the presence or absence of maltose as shown in Fig. 32. Two mols of SH groups per mol of the enzyme reacted with 2-PDS in accord with the decrease of enzymatic activity in the absence of maltose. On the other hand, in the presence of

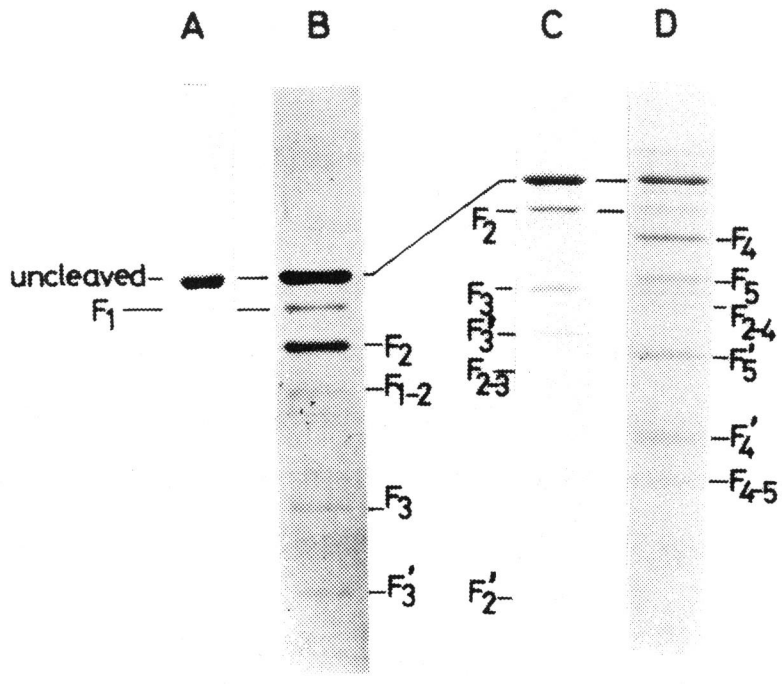


Fig. 31. Polypeptide Fragments of Soybean β-Amylase Produced by Cleavage at the Selectively Modified SH Groups.

The fragments were detected by SDS-polyacrylamide gel electrophoresis in slabs, and the protein bands were detected with Coomassie Brilliant Blue R-250 (59). Polyacrylamide concentrations were 10% (A and B) and 15% (C and D). A, Fragments produced by cleavage at SH1; B, Fragments produced by cleavage at both SH1 and SH2 ; C, Fragments produced by cleavage at both SH2 and SH3 (Modification with 2-PDS was carried out in the presence of maltose); D, Fragments produced by cleavage at both SH4 and SH5. In each case, the derivatives of β-amylase modified with 2-PDS were cyanylated at the protein concentration of 15-30 μM in 0.1 M Tris-HCl buffer, pH 7.6-8.0, containing 5 mM EDTA and 2% SDS at 25°C until the release of 2-PD reached about 90% of the previously incorporated 2-PD. Then the pH of the reaction mixture was adjusted to 9.0 with 2 M Tris, and it was incubated at 37°C for 12 h. The cleavage reaction was terminated by adding 200 mM 2-mercaptoethanol, and aliquots of the reaction mixture were analyzed by SDS-gel electrophoresis.

maltose, the number of modified SH groups decreased to about one residue and the decrease in enzymatic activity was very small. These results demonstrate that in the case of SH1-carboxymethylated soybean β-amylase, SH3 as well as SH2 tends to be accessible to 2-PDS and that SH2 is protected by maltose against this modifier as described in Chapter III. These two derivatives of soybean β-amylase were denatured, the remaining SH groups were blocked with NEM, and then the products were cleaved at the 2-PDS-modified SH groups. The formed fragments were analyzed by SDS-gel electrophoresis as

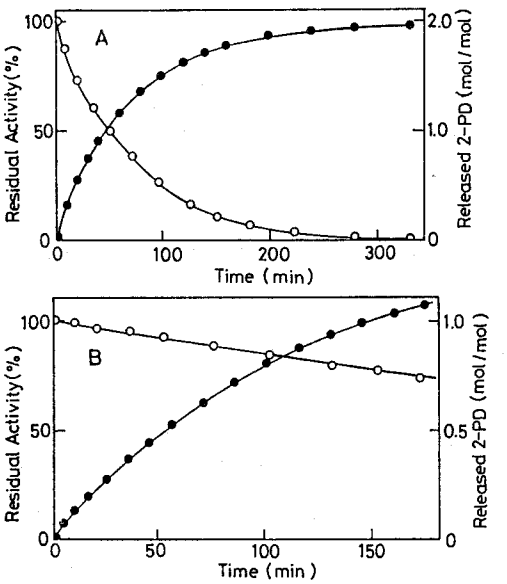


Fig. 32. Modification of SH2 and SH3 with 2-PDS in the Presence or Absence of Maltose.

SH1-carboxymethylated soybean β-amylase (13 μM) was treated with 78 μM 2-PDS in 0.1 M Tris-HCl buffer containing 5 mM EDTA and 0.8 M KCl, at pH 8.0 and 25°C in the absence (A) or presence (B) of 50 mg/ml maltose. —●—, amount of released 2-PD (mol/mol of the enzyme); —○—, residual activity.

shown in Fig. 31-C. Five fragments were found and four of them were identified as F_2, F_2', F_3 and F_3' by considering the results of cleavage at SH2. The smaller quantity of another fragment (Mw. 21,000) was assigned to be the fragment formed by cleavage at both SH2 and SH3 (F_{2-3}). Effects of maltose on the quantity of the fragments were analyzed by the densitogram as shown in Fig. 33. If we assume that the cleavage yield is proportional to the number of 2-PDS-modified SH groups, the quantity of the fragments is given by the formula, $F_{i\pm mal} = \alpha_i \times [SHi]_{\pm mal}$, where $F_{i\pm mal}$ is the quantity of

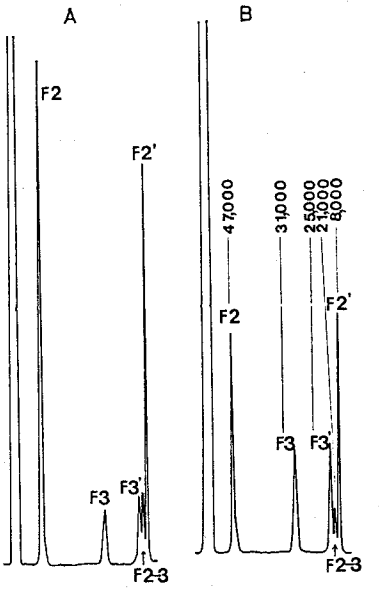


Fig. 33. Electrophoretograms of Soybean β-Amylase after Cleavage at both SH2 and SH3.

Modification of SH2 and SH3 with 2-PDS was carried out in the absence (A) or presence (B) of maltose as shown in Fig. 32. After cleavage for 24 h, fragments were detected by SDS-gel electrophoresis (10% acrylamide gel), using a densitometer, Shimadzu dual-wavelength TLC scanner CS-910.

the fragments formed by the cleavage at SHi modified by 2-PDS in the presence or absence of maltose, α_i is a constant for the cleavage at SHi, and $[SHi]_{\pm mal}$ is the concentration of the SHi modified with 2-PDS (mol/mol enzyme) in the presence or absence of maltose. Thus the product of the ratio, F_{3-mal}/F_{2-mal} and F_{2+mal}/F_{3+mal} can be described by the ratios of the number of 2-PDS-modified SH groups as follows:

$$(F_{3-mal}/F_{2-mal}) \times (F_{2+mal}/F_{3+mal}) = ([SH3]_{-mal}/[SH2]_{-mal}) \times ([SH2]_{+mal}/[SH3]_{+mal}).$$

The value for the left side of the equation was calculated to be 0.34 from the area of the peaks in Fig. 33, and the right side was calculated to be 0.35 from the number of modified SH groups as shown in Fig. 32. The agreement of the values reveals that the cleavage yield is proportional to the number of 2-PDS-modified SH groups, and it confirms that F_2 and F_3 are formed by cleavage at SH2 and SH3, respectively.

For the cleavage of β-amylase at the two buried SH groups, the reactive SH groups in the native state were blocked with NEM before cleaving, as shown in Fig. 34. The rapid decrease of enzymatic activity corresponded to the modification of SH2, and three mols of SH groups per mol of enzyme were blocked with NEM. The remaining SH groups were cyanylated after they were modified with 2-PDS in the presence of 2% SDS. After cleavage, the fragments were analyzed by SDS-gel electrophoresis as shown in Fig. 31-D. Two pairs of pre-

dominantly formed fragments, Mw. 38,000 and 18,000, and 35,000 and 23,000, were designated as F_4, F_4', F_5 and F_5', respectively. Other fragments were assigned to be F_2, F_2', F_3 and F_3'. There were two new fragments, Mw. 28,000 and 15,000 which seem to have been formed by cleavage at two SH groups. By considering the estimated molecular weights of the predominant fragments, F_4, F_4', F_5 and F_5', the one fragment (Mw. 15,000) was identified with that

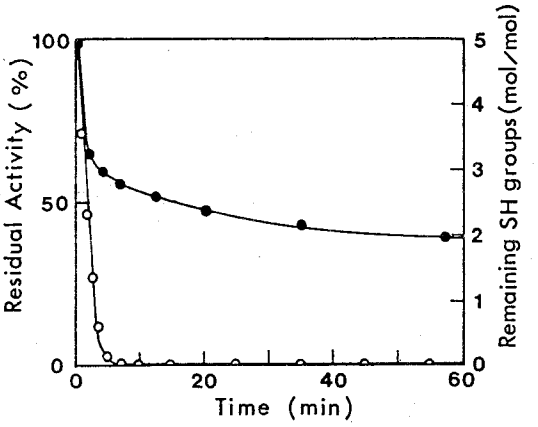


Fig. 34. Irreversible Blocking of SH1, SH2 and SH3 with NEM.

Soybean β-amylase (99 μM) was treated with 20 mM NEM in 0.1 M Na-phosphate buffer containing 0.8 M KCl and 5 mM EDTA, at pH 7.5 and 25°C. For the titration of the remaining SH groups, aliquots of the reaction mixture were withdrown. The reaction was terminated by lowering the pH to 5.0 with 0.5 M acetate buffer, followed by gel filtration on a Sephadex G-25 coloumn equilibrated with 0.1 M Tris-HCl buffer containing 5 mM EDTA, pH 8.0. Immediately after the elution, protein fractions were collected and the protein concentration was calculated from the absorption at 280 nm. Then 20% SDS solution was added to make 2% concentration. One hour after the addition of SDS, the number of SH groups was determined with DTNB according to the method of Ellman (*67*). —○—, residual activity; —●—, amount of remaining free SH groups (mol/mol of the enzyme) determined by DTNB.

formed by cleavage at both SH4 and SH5 (designated as F_{4-5}). The other fragment (Mw. 28,000) seems to be that formed by cleavage at both SH4 and SH2 (designated as F_{2-4}).

3. Cleavage of Soybean β-Amylase at Its Five SH Groups.

Cleavage of soybean β-amylase at all five SH groups using NTCB as cyanylating reagent resulted in the formation of 10 fragments at first and a total of 20 fragments through the cleavage reaction, as shown in Fig. 35. The ten fragments at the first stage were formed by cleavage at one of the five SH groups in the enzyme molecule, and they make up to five pairs of fragments identified by the previous experiments (the left side of Fig. 35). The other fragments

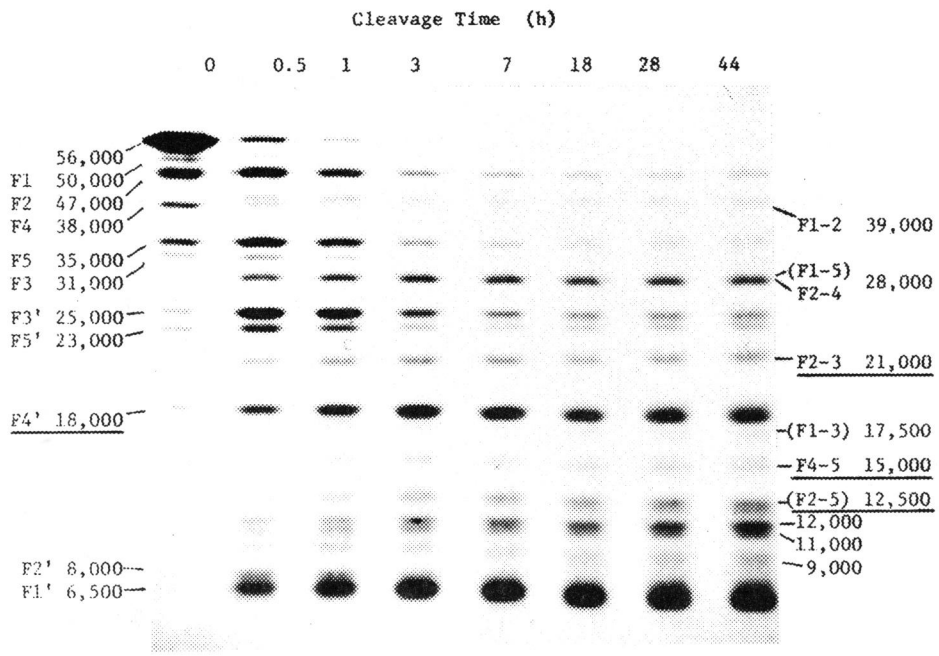


Fig. 35. Polypeptide Fragments of Soybean β-Amylase Produced by Cleavage at Five SH Groups with NTCB.

Cyanylation of all five SH groups was carried out by using 5 mM NTCB at a protein concentration of 65 μM in 0.1 M Tris-HCl buffer, pH 8.0, containing 5 mM EDTA and 6 M guanidine-HCl at 25°C for 1 h. Then cleavage reaction was started by raising the pH of the reaction mixture to 9.0 with 2 M Tris, and the reaction mixture was incubated at 37°C. At a appropriate time intervals, aliquots were withdrown, and the cleavage reaction was terminated by adding 200 mM 2-mercapto-ethanol. The fragments were analyzed by SDS-polyacrylamide gel (15% acrylamide) electrophoresis after the medium of the aliquots was replaced with 0.1 M Tris-HCl buffer, pH 7.6, containing 4 M urea and 2% SDS by passing through a Sephadex G-25 column. Left side, fragments formed by cleavage at one of the five SH groups and their estimated molecular weights. Right side, fragments formed by cleavage at two of the five SH groups and their estimated molecular weights. Underlined molecular weights represent the abnormal fragments examined by a Furgason plot (Fig. 36). The fragments in parentheses show the SH groups estimated to be the cleavage sites. In order to estimate molecular weights, bovine serum albumin (67k daltons), egg white albumin (45k daltons), yeast alcohol dehydrogenase (36k daltons), equine myoglobin (17.2k daltons) and CNBr-treated fragments of whale myoglobin (14.9 + 8.3 + 6.4 + 2.55k daltons) were used as satandards. CNBr-treated myoglobin was prepared according to the method of Gross and Witkop (*122*).

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were formed by cleavage at two of the five SH groups, and their estimated molecular weights, together with the identified fragments number, are shown on the right side of Fig. 35. In order to make sure of the estimated molecular weights of these 20 fragments, their behavior in the electrophoresis was examined by a Furgason plot as

shown in Fig. 36. As the plot showed that a few of them (underlined fragments in Fig. 35) behaved abnormally compared with the standard proteins (except for CNBr-treated myoglobin), their estimated molecular weights were not used in deducing the alignment of the fragments.

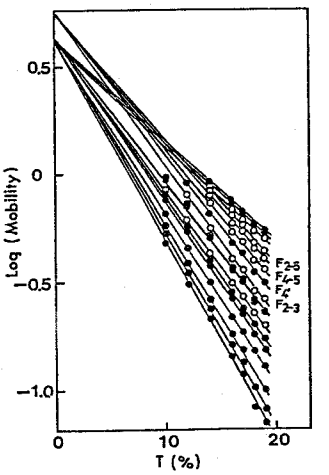


Fig. 36. Furgason Plots of the Fragments Formed by Cleavage at the Five SH Groups. The fragments of soybean β-amylase produced by the cleavage at five SH groups were analyzed by SDS-gel electrophoresis in a different concentration of polyacrylamide, and the mobility of the fragments were plotted against the concentration of polyacrylamide as described by Hayashi *et al.* (*123*). —●—, fragments formed by cleavage at one of the five SH groups; —○—, fragments formed by cleavage at two SH groups.

Cyanylation of the five SH groups by using ^{14}C-labeled cyanide allowed us to follow the radioactive distribution over the fragments after cleavage. During cleavage reaction, the radioactive carbon of the cyanocysteine residue fixed into the five-menbered ring to form the iminothiazolidine derivatives (*113*) as shown in Fig. 37.

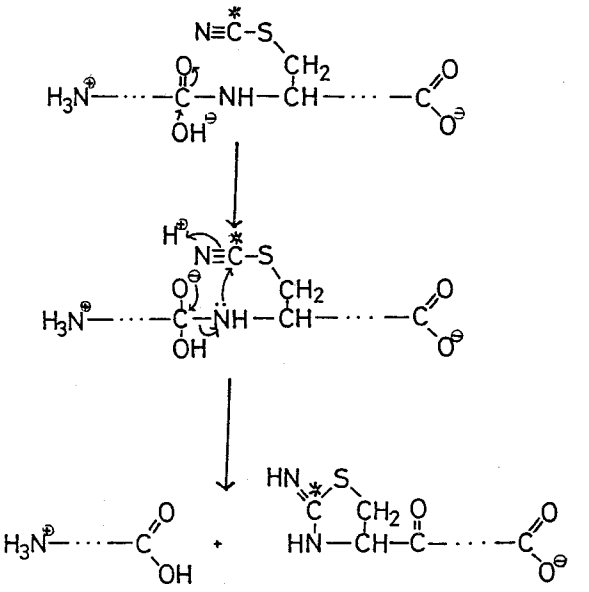
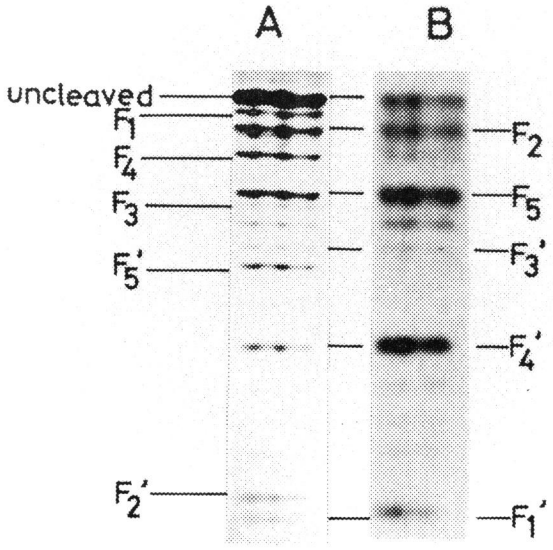


Fig. 37. A Mechanism for the Cleavage Reaction at Cyanocysteine Residue.

In this form, the radioactive carbon is stable at the modified amino-terminus of the nascent fragment which was located at the carboxyl-terminal side of the cyanocysteine residue to be cleaved. Thus, the five SH groups of soybean β-amylase was modified with 2-PDS in the denatured condition, and the enzyme was cleaved at the five SH groups after cyanylation with [^{14}C]cyanide. The radioactive fragments were detected by fluorography after SDS-polyacrylamide gel electrophoresis, as shown in Fig. 38. Out of the five pairs of fragments formed by the cleavage, the radioactivity was predominantly incorporated into one menber of each pair: F_1', F_2, F_3', F_4' and F_5.



Fug. 38. Distribution of Fragments Incorporating ^{14}C over the Fragments Formed by Cleavage at Each One of the Five SH groups.

All five SH groups of β-amylase (12 mg in 3.5 ml buffer) were modified by 434 μM 2-PDS under the denaturation conditions (0.1 M Tris-HCl buffer, pH 8.0, containing 5 mM EDTA, 2% SDS and 6 M urea). After the excess amount of the reagent was removed by passing through a Sephadex G-25 column, the modified enzyme was treated with 10 mM Na[^{14}C]CN (obtained from the Radiochemical center, England) at a protein concentration of 210 μM at 25°C for 6 h. Then the cleavage reaction was carried out in the presence of 2% SDS and 6 M urea at pH 9.0 and 37°C for 24 h, and the fragments incorporating ^{14}C were detected by fluorography (*124*) after SDS-polyacrylamide gel electrophoresis. A, SDS-polyacrylamide gel (15% acrylamide gel) stained with Coomassie Brilliant Blue R-250; B, The fragments of A shown by fluorography , thus detecting the fragments incorporating ^{14}C.

The yield of the cleavage at all five SH groups with NTCB (Fig. 35) was high, compared with cleavage at the selectively cyanylated sites (Fig. 31). This is not due to a decrease in the yield of cya-

nylation due to the prior modifications but is mainly due to the difference in the denaturation conditions. The yield depended upon the denaturation conditions: 6 M guanidine-HCl > 6 M urea + 2% SDS > 2% SDS, as shown in Figs. 35, 38 and 31, respectively. The low yield of cleavage in 2% SDS, which was used for convenience, suggests the incomplete unfolding of the enzyme in SDS solution. A circular dichroic study of β-amylase supports this view (*99*). The yield of the cleavage were also different between the cyanylated SH groups even under the same conditions of denaturation as shown in Figs. 31, 35 and 38: the yields of cleavage at SH2, SH4 and SH5 were higher than that at SH1 and SH3. This fact suggests that the neighboring amino acid residues of SH1 and SH3 disturb the cleavage reaction.

4. Location of the Five SH Groups along the Polypeptide Chain of Soybean β-Amylase

The alignment of the cleaved fragments was obtained as follows. The results of Figs. 31-A, -B and -C enable us to align the fragments formed by cleavage at SH1, SH2 and SH3 as shown in Fig. 39-A. Independently, the alignment of the fragments formed by cleavage at SH4 and SH5 was obtained from the results in Fig. 31-D as shown in Fig. 39-B. Taking into account the fragments incorporating ^{14}C formed by cleavage at each SH group (Fig. 39-C), location of the five SH groups along the polypeptide chain of soybean β-amylase

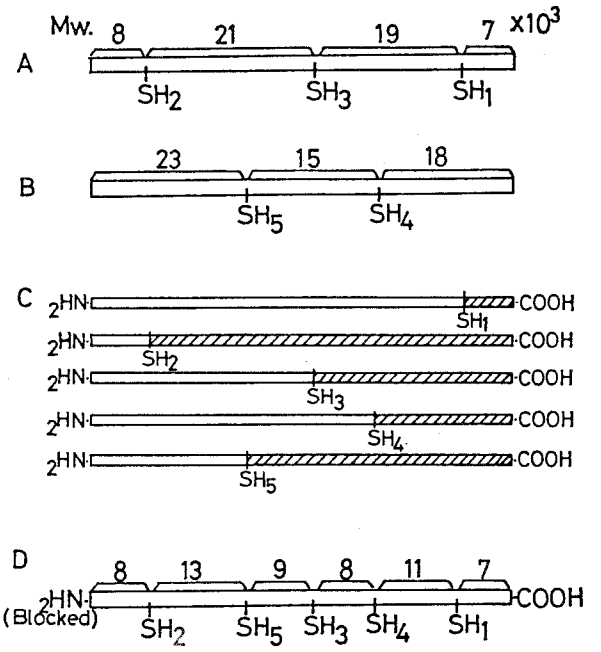


Fig. 39. Schematic Representation of the Polypeptide Chain of Soybean β-Amylase and the Positions of the SH groups.

A: Relative positions of SH1, SH2, and SH3 deduced from Fig. 31 (A-C). B: Relative positions of SH4 and SH5 deduced from Fig. 31-D. C: Alignments of fragments incorporating ^{14}C and the positions of the five SH groups. Fragments incorporating ^{14}C are represented as the shaded parts. D: Determined positions of the five SH groups along the polypeptide chain of soybean β-amylase.

was determined as shown in Fig. 39-D. The size of the fragments formed by cleavage at two SH groups observed in Fig. 35 is almost compatible with that of the fragments deduced from Fig, 39-D, though there are still three fragments, F_{1-4}, F_{3-4} and F_{3-5}, which couldn't be identified on SDS-gel owing to their similar molecular weights.

In order to confirm the alignment of the cleaved fragments,

F_2 was purified from the mixture of fragments formed by cleavage at all five SH groups, using preparative SDS-polyacrylamide-gel electrophoresis, and its carboxyl-terminal amino acid was examined by hydrazinolysis. Figure 40 shows purified F_2 on SDS-gel. Only glycine (0.7 mol/mol of the enzyme) was detected and it was consistent with the carboxyl-terminal amino acid of intact soybean β-amylase component 2 as described in Chapter I (Table V). This result

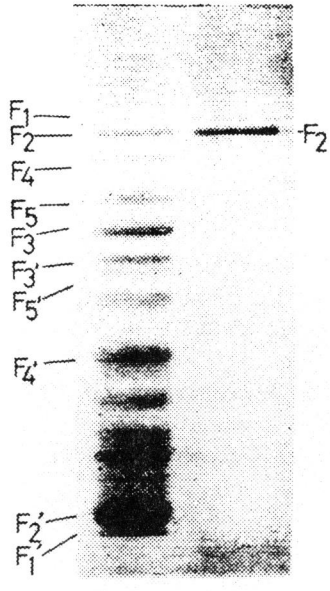


Fig. 40. Fragments Formed by Cleavage Using NTCB (left) and the Purified F_2-Fragment (right).

Soybean β-amylase (100 mg) was cyanylated and cleaved using NTCB in the presence of 6 M guanidine-HCl and F_2 was purified from the resulting mixture of fragments using preparative SDS-gel electrophoresis (0.5 × 25 × 14 cm slab). The peptide was cut off and extracted from the gel using an electrophoretic extraction apparatus (*125*). SDS and the dye were removed with an acetone-trimethylamine solution according to the method of Henderson *et al.* (*126*). Four mg of purified F_2 was obtained.

also supports the result of Fig. 39-D.

Figure 39-D shows that the five SH groups are located almost regularly along the polypeptide chain and that SH2, which is at the active site of the enzyme, is located on the amino-terminal side of the enzyme. This result is very important to elucidate the active region of soybean β-amylase by X-ray crystallographic determination of its three dimensional structure, because the analysis is now undertaken using isomorphous derivative with *p*-chloromercuribenzene sulfonate, which reacts with the SH groups of soybean β-amylase (*2*). Moreover, cleavage of soybean β-amylase at the five SH groups gives six large polypeptides, which are suitable for the determination of the primary structure of this enzyme protein because the positions of the five SH groups have been determined in this study, and a method of opening the blocked N-terminus of the nascent peptides formed by the cleavage is now available (*127*).

VI. SUMMARY

The object of this thesis is to elucidate the relationship between structure and function of soybean β-amylase. For this purpose, the author intensively investigated the role of SH groups on the action of the enzyme by using chemical modification, and presented their positions along the polypeptide chain of the enzyme. Prior to the modification study, soybean β-amylase isozymes are separated and their enzymatic properties are characterized to know their structural differences. The results are summarized as follows:

1. The distribution of multiple forms of β-amylase in some varieties or species of soybean seeds was examined by the gel isoelectric focusing method, and seven components (1', 1, 2, 3, 4, 5 and 6) were found. Their respective isoelectric points were 5.07, 5.15, 5.25, 5.40, 5.55, 5.70 and 5.93 ± 0.04. The varieties or species of soybean seeds were separated into two types by their zymograph: the low pI type and high pI type. Component 6 was purified from commercial defatted soybean meal containing all seven components by ion-exchange column chromatography and gel filtration, and it was compared with previously purified components 2 and 4. Components 2, 4 and 6 had the same molecular weight and immunological properties but some differences were found in their amino acid compositions and enzymatic properties. The C-terminal amino acid of component 2 and 6 was glycine but that of component 4 was alanine. It was concluded from these results that differences between components

2, 4 and 6 were caused by charged amino acid substitution.

2. The behavior of SH groups of soybean β-amylase component 2 was investigated by chemical modification. Two SH groups out of five in the native enzyme reacted with 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), iodoacetamide or monoiodoacetate at high ionic strength, accompanied by inactivation of the enzyme. At low ionic strength, only one SH group was accessible to DTNB without inactivation. By using the reaction with iodoacetamide, the dissociation constants of these two SH groups and rate constants for the reaction were determined. The most reactive SH group, SH1, which was independent of the inactivation reacted with monoiodoacetate 185 times faster than the second reactive SH group, SH2. Thus, selective modification of SH1 with monoiodoacetate was carried out. The modified enzyme, having activity equal to that of the native enzyme, was purified by ion exchange column chromatography. It was successively treated with DTNB or iodoacetamide in order to achieve selective modification of SH2. The enzyme modified further with DTNB was fully reactivated by 2-mercaptoethanol treatment. It also recovered 65% of the original enzymatic activity with cyanide but 7% with sulfite. Methanethiolation of SH1 and SH2 with methyl 2, 4-dinitrophenyldisulfide also reduced the enzymatic activity to 7.2% of the original activity. The modification of SH2 with DTNB was prevented by substrate analogs such as maltose and cyclohexadextrin, the former being more effective. The ultraviolet absorption spectrum of the enzyme was changed by modification of SH2, but the

circular dichroic spectrum indicated that the modification of SH2 does not affect the secondary structure of the enzyme. It was concluded from these results that SH2 does not participate in the catalysis of soybean β-amylase, but the modification of SH2 with bulky substituent caused conformation change around its active region to prevent the enzymatic action.

3. Cyclohexadextrin and maltose bound to soybean β-amylase and affected the environments of tryptophan and tyrosine residues, producing characteristic difference spectra in the ultraviolet region. The difference spectrum produced by cyclohexadextrin, a competitive inhibitor, had peaks at 285, 292 and 299 nm, while that by maltose, a reaction product, had peaks at 285, 292 nm and a small trough at around 300 nm. By using peaks at 299 and 292 nm, the dissociation constants of the enzyme-cyclohexadextrin and the enzyme-maltose complexes were determined to be 0.35 and 8.1 mM, respectively. The dissociation constants of the enzyme-cyclohexadextrin and the enzyme-maltose complexes were not changed by the modification of SH1 and SH2 with iodoacetamide or DTNB, but the modification of SH2 strongly affected the difference spectrum produced by maltose. The spectrophotometric titration of β-amylase by cyclohexadextrin in the presence of maltose showed that cyclohexadextrin and maltose bind to the enzyme competitively, regardless of the modification of SH2. These results indicated that SH2 is located near the binding site of cyclohexadextrin and maltose, but it is not involved in the

binding of these sugars.

4. The location of the five SH groups along the polypeptide chain of soybean β-amylase was determined by specific cleavage at the amino side of their cyanocysteine residues which were formed by converting SH to SCN groups by cyanide after modifying the SH groups with 2,2'-dithiopyridine (2-PDS). The selective modification of SH groups was achieved as follows: SH1 reacted with 2-PDS at low and high ionic strength, while SH2 reacted only at high ionic strength. SH2 and SH3 were also modified with 2-PDS using SH1-carboxymethylated soybean β-amylase. The buried SH groups, SH4 and SH5, were modified with 2-PDS under the denaturation conditions after the reactive SH groups, SH1, SH2 and SH3, were irreversibly blocked with N-ethylmaleimide. On the other hand, the five SH groups were cyanylated with [^{14}C]cyanide or with 2-nitro-5-thiocyanobenzoic acid (NTCB) for the cleavage at all five SH groups. The molecular weight estimation of derivatives of cleaved soybean β-amylase by SDS-gel electrophoresis showed that the five pairs of fragments (Mw. 50,000 & 6,500, 47,000 & 8,000, 38,000 & 18,000, 35,000 & 23,000 and 31,000 & 25,000) were identified with the fragments formed by cleavage at SH1, SH2, SH3, SH4 and SH5, respectively. By considering fragments incorporating ^{14}C (Mw. 47,000, 35,000, 25,000, 18,000 and 6,500), the fragments were aligned along the polypeptide chain of soybean β-amylase, in order from N-terminus as SH2, SH5, SH3, SH4 and SH1. This order was supported by estimating the molecular

weights of fragments formed by high-yield cleavage using NTCB and by analyzing the COOH-terminal amino acid residue of the fragment cleaved at SH2.

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