STUDIES ON FURTHER PROPERTIES FOR AN ALKALINE AMYLASE IN THE DIGESTIVE JUICE OF THE SILKWORM, *BOMBYX MORI*

by

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INTRODUCTION

Silkworms with the genotype $+^{ae}/+^{ae}$ have a strong amylase activity in the digestive juice. This type of amylase, "silkworm digestive amylase" is specially characterized with its optimal pH in the stronger alkaline range. It has been pointed out and noticed by numerous workers for long (FUJII and KATO, 1930 ; YAMAFUJI, 1933 ; HORIE, 1959 ; ITO *et al.*, 1962 ; NISHIDA and HAYASHIYA, 1969), since common amylases from animals, plants, bacteria, fungi, insects other than silkworms, *etc.* are of the optimal pH in the nutral or slight acidic range. Recently it has been gradually revealed that amylases with the optimal pH in the weak to strong alkaline range are also produced by fungi (ROBYT and ACKERMAN, 1971), bacteria (HORIKOSHI, 1971 ; BOYER and INGLE, 1972) and althropods (SATHER, 1969). Comparative studies of silkworm digestive amylase with other normal and alkaline amylases are expected to serve valuable informations.

In addition, a discussion that the silkworm digestive amylase consists of two types of amylase, namely a saccharifying and liquefying amylases, has been proposed by many workers (KIKKAWA, 1950 ; MUKAIYAMA, 1958 ; NISHIDA and HAYASHIYA, 1969). But many of the experimental bases of their discussions are so flimsy that it is still uncertain whether the digestive amylase is uniform or varied.

To clarify those problems mentioned above, it is necessary to obtain pure amylase sample and characterize them. Partial purification of silkworm digestive amylase was first attempted by NISHIDA and HAYASHIYA (1969) using ammonium persulfate precipitation, buthanol extraction, acetone precipitation, gel-filtration and ion exchange chromatography. The author also attempted

the purification of silkworm digestive amylase using the amylopectin co-precipitation technique, mainly, and showed that this enzyme has a pH optimum at 9.3, heat stable below 55°, and has a sedimentation coefficient of 5.3. The activation energy of the enzyme are 17.0 kcal at 0–25° and 3.8 kcal at 25–40°, when amylose is used as the substrate. The *Km* value is 2.57×10^{-3} μmoles of glucosidic bond per liter (KANEKATSU, 1973).

The present study was initiated to determine further properties of the silkworm digestive amylase. Results of molecular weight estimation, amino acid analysis, estimation of the role of divalent cations and the mode of actions of the amylase are described.

Before going into the main subject, the author wishes to express his gratitude to Assistant Professor Tokio Watanabe, Shinshu University, for his help in the preparations of the manuscript. Thanks are also due to Dr. Narumi Yoshitake, Tokyo University, for his invaluable advice in performing experiments.

MATERIALS AND METHODS

Silkworm—In order to obtain silkworm digestive juice, stock strain "Daizo" was used. This is one of the "easiest-to-rear" silkworm strains with its genotype $+^{ae}/+^{ae}$. Silkworms reared on mulberry leaves to be 4-day old of the 5th instar, were treated with chloroform moisture after 4 hours' starvation. Digestive juice was easily collected by this method. Digestive juice thus obtained was centrifuged for 15 min at 10,000 rpm, and supernatant was served for further treatment.

Column chromatography—CM-Sephadex column chromatography and Bio-gel column chromatography were performed according to the previous report (KANEKATSU, 1973).

Amylases—Silkworm digestive amylase (SDA) was purified from the digestive juice of "Daizo" montioned above, following the purification procedure that was previously reported (KANEKATSU, 1973). The purity of this preparation was ascertained by disc-electrophoresis, analytical ultracentrifugation and immunological technique.

Commercial products of barley α- and β-amylases (Wako Pure Chemicals, Tokyo), *Apergillus oryzae* α-amylase (Tokyo Kasei Co., Tokyo) and porcine pancreatic α-amylase (Sigma) were used for the comparative study. When β-amylase was used in preparation of amylopectin β-limit dextrin, the enzyme was mildly acid-treated before use according to the method of TAKEDA and HIZUKURI (1969) to eliminate a possible contamination with α-amylase.

Substrates—Soluble starch ("for determination of amylase activity"—Wako Pure Chemicals), amylose from potato starch (BDH Chemicals, Poule), *p*-nitrophenyl-α-glucopyranoside and *p*-nitrophenyl-α-galactopyranoside (Senn Chemicals, Schweiz) and maltose (Wako Pure Chemicals) were used without further purification.

Amylopectin β-limit dextrin was prepared as follows. Five hundred mg of amylopectin (Calbiochem., Los Angeles) suspended in a small volume of distilled water as a slurry, was added dropwise into boiling water to obtain a clear solution of 0.5% amylopectin. It was further autoclaved at 110° for 2 hours and diluted with the addition of half volumes of 0.05M 2-(*N*-morpholino) ethanesulfonic acid buffer pH 6.5 (MES buffer, Dojin Laboratories, Kumamoto) and incubated with 2 mg of acid treated β-amylase for 24 hours at 30°. After the incubation, β-limit dextrins is formed as a reaction product. This was precipitated by the addition of ethanol to the final concentration of 50% and then it was dissolved in distilled water. The whole procees was repeated several times in order to purify the product (final recovery : 79%).

Buffers—Three kinds of buffer solution were applied. Borate buffer (M/10) and M/20 cyclohexylaminopropanesulfonic acid buffer (CAPS buffer, Dojin Laboratories) were used to hold the basic pH, 9.2. The borate buffer is commonly used for the assay of SDA (KANEKATSU, 1972). The slightly acidic pH, 6.5 was held with M/20 MES buffer.

Analytical methods—Amylase activities were estimated by the application of SOMOGYI-NELSON's colorimetric method, the details of which was described in the previous report (KANEKATSU, 1972). Blue values (intensities of amylose —iodine complex colour) were determined as follows ; 0.2ml of reaction mixture were added to 5 ml of N/3000 I_2-KI solution (acidified with N/10 acetic acid). Absorbance at 690 nm was measured and values were represented as per cent to the control. Glucosidase or maltase activities were assayed with "Glucostat", a prepared reagent kit of glucose oxidase system (Worshington, New Jersey). Galactosidase activities were assayed with "Galactostat", also a prepared reagent system.

The amount of proteins was measured by the FOLIN's phenol reagent method described by LOWRY *et al.* (1951).

SDS-polyacrylamide gel electrophoresis—The separation method of electrophoresis based on molecular weight, reported by SHAPIRO *et al.* (1967) was performed, according to the manual described by HAYASHI and OHBA (1972). Acrylamide (Wako Pure Chemicals), methylene bis acrylamide (Seikagaku Kogyo, Tokyo) and sodium dodesyl sulfate (Nakarai Chemicals, Kyoto) were

commercially obtained and applied without further purification. Marker proteins of already known molecular weights, cytochrome c from horse heart, myoglobin of sperm whale, egg white albumin and bovine serum albumin were purchased from Mann. Research Laboratories (New York). The separated bands of individual proteins were visualized by staining with Coomassie brilliant blue R (Nakarai Chemicals).

Guanidine hydrochloride gel filtration—The molecular weight determining method established by FISH *et al.* (1969) was applied with beaded agarose gel (Sepharose 6B, Pharmacia Fine Chemicals). Column equilibliation and elution was performed with the 6 M solution of analytical grade guanidine hydrochloride (Nakarai Chemicals). Emergence of proteins was determined by the UV optics of 280 nm.

TLC analyses of maltodextrins—TLC analyses of maltodextrins produced by the amylase action on amylose were performed as follows; samples for the TLC analyses were prepared by mixing each aliquot of the digest with the equal volume of ethanol to precipitate the enzyme and high molecular weight materials. The resulting precipitate was removed by centrifugation at 10,000 rpm and the supernatant was applied on cellulose TLC sheets (Merck, DC Alufolien Cellulose), which had been pre-developed twice with a developing solvent system composed of *n*-buthanol—pyridine—distilled water (6 : 4 : 3). Development in the same solvent system was performed according to the multiple ascending technique described by ROBYT and FRENCH (1963). The development was repeated 5 times (development distance was 10 cm each time). Spots of maltodextrins were visualized by alkaline silver nitrate method described by TRAVELYAN *et al.* (1950). The author modified their method slightly and used ethanol solution of KOH in place of NaOH. The result is that it is possible to obtain clearer background. Sheets were further immersed in a solution of photographic film fixer (Fuji Fix, Fuji Photo Ind., Tokyo) to eliminate silver ions and washed gently with running water.

Amino acid analysis—Purified SDA (1.13 mg) was hydrolyzed with 4 ml of 6N HCl for 24, 48 and 72 hours at 110°. The acid was completely removed from the hydrolysate, with rotary evaporator at 70°, through the repeated process of the dissolution of the residue followed by the evaporation of the solution. Finally each residue was dissolved with 10 ml of acetate buffer, pH 2.2, and served for amino acid analysis. Analyses were performed according to the method of SUMIOKA and YOSHITAKE (1974) with a Yanagimoto Model LC-5S amino acid analyzer. Sample of 0.2 to 0.5 ml was applied for each analysis.

Tryptophan was assayed spectrophotometrically. Enzyme preparation of 0.76 mg was dissolved with 5 ml of 0.1N NaOH, and dialyzed overnight against the same solution. This sample was used to calculate the molar ratio of tryptophan to tyrosine, according to EDELHOF (1967).

RESULTS

Purity of SDA preparation—Maltase, glucosidase and galactosidase activities were assayed on the SDA preparation. One ml of 0.1% substrate solutions were incubated for 0.5, 2, 5 and 10 hours after adding 1 unit of SDA. Even traces of these enzyme activities were hardly detected at the pH of both 9.2 and 6.5. So the enzyme preparation was decided free from the contamination of other carbohydrate related enzymes.

SDS-polyacrylamide gel electrophoresis—SDA preparation was subjected to the SDS-polyacrylamide gel electrophoretic analysis, a molecular weight based method of separation. As the SDA appeared as a single band, it was supported to be uniform in respect of molecular size. Four experiments were made and

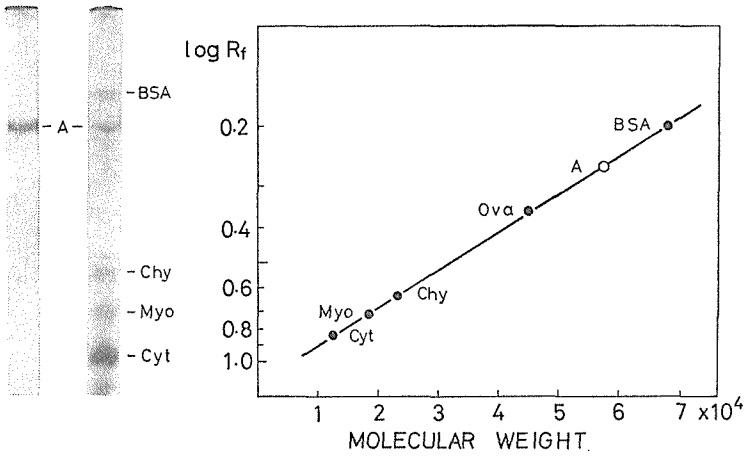

Fig. 1. Molecular weight estimation of silkworm digestive amylase with SDS-polyacrylamide gel electrophoresis.
A, silkworm digestive amylase ; Cyt, cytochrome c ; Myo, myoglobin ; Chy, chymotrypsinogen ; Ova, egg white albumin ; BSA, bovine serum albumin.

it was found that the mean of the migrating rate (R_f) of the SDA to bromophenol blue was 0.27. R_f values for several marker proteins of known molecular weights, namely, cytochrome c, chymotrypsinogen, egg white albumin and bovine serum albumin were further obtained and after comparison with

these values the assumed molecular weight of 57,000 to the SDA was given (Fig. 1).

Guanidine hydrochloride gel filtration—A descending type chromatography column with a demension 26 mm×450 mm, packed with Sepharose 6B was equilibrated with 6M guanidine hydrochloride solution. Protein sample of 0.5 mg in each analysis was denatured with dithiothreitol after TAKAGI (1973) and applied onto the column. The K_d value for each protein was calculated as :

$$K_d=(W_e-W_o)/(W_t-W_o)$$

where W_e, the weight of eluent needed till the emergence of the protein : W_o, the void volume of the column, determination of which was made by the emergence of blue dextran 2000 : and W_t, the end of elution determined by the emergence of DNP-alanine. Because of the caused shrinkage of the Sepharose gel bed as the repeated use of the column, void volume of the column (W_o) varied from 78g to 74g. So, development was performed several times for each protein, and K_d values were calculated at each analysis. As for the standard, myoglobin, chymotrypsinogen, egg white albumin and bovine serum albumin were used, and molecular weight of SDA was estimated to be approximately 47,000 with this method (Fig. 2).

Amino acid analysis—To calculate amino acid quantities in each analysis,

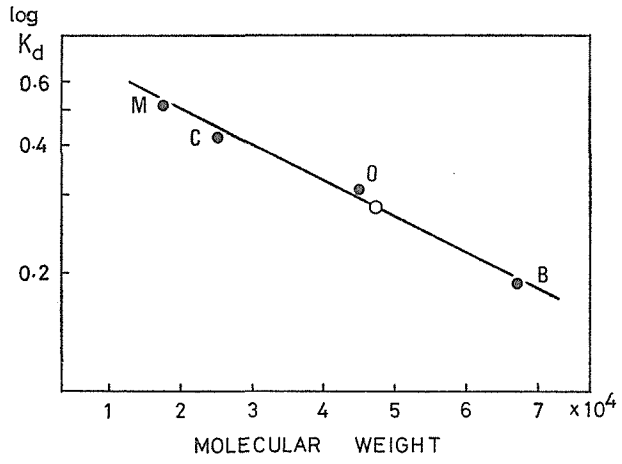

Fig. 2. Molecular weight estimation of silkworm digestive amylase with 6M guanidine hydrochloride gel filtration (Sepharose 6B). M, myoglobin ; C, chymotrypsinogen ; O, egg white albumin ; B, bovine serum albumin. Open circle, silkworm digestive amylase.

three to four replications for each sample were performed and each result was compared with that of the analysis of standard sample mixture. In the case of the type of amino acids the quantity of which decreased with the progress of hydrolysis time, the extrapolated values to the time 0 were taken. On the other hand, in the case of the type of amino acids which increase in greater quantity in proportion to the time of hydrolysis, the values at 72 hours were taken. If tendency of such kinds was not noticed, the average value of three was used. The relative amount of tryptophan to tyrosine was estimated from UV optics to be 0.554 (molar ratio). The result of amino acid analysis is displayed in Table I. In the column A of the table, moles of each amino acid per 100 g protein are shown, while in the column B, grams of each amino acid per 100 g protein are mentioned, for the convenience to the comparison with other amylases. The most abundant amino acid residue is aspartic acid,

Table I. Amino acid composition of silkworm digestive amylase

Amino acid	A moles/100g protein	B g/100g protein
Aspartic acid	15.2×10^{-2}	17.5
Threonine*	5.3	5.4
Serine*	4.0	5.4
Glutamic acid	8.5	11.0
Proline	4.2	4.1
Glycine	10.3	5.9
Alanine	8.0	5.7
Cysteine	0.0	0.0
Valine**	6.5	6.4
Methionine	2.7	3.5
Isoleucine**	5.2	5.9
Leucine	6.2	7.0
Tyrosine	3.9	6.3
Phenylalanine	4.3	6.3
Tryptophan***	2.2	4.0
Lysine	1.5	2.0
Histidine	2.7	3.7
Arginine	7.8	12.1
Ammonia*	40.7	6.9

All values are the average of 24-, 48- and 72-hr hydrolyses in 6M HCl, except where indicated.

* Extrapolated to zero time of hydrolysis.

** Values from 72-hr hydrolysis.

*** Value obtained spectrophotometrically.

followed by glycine, glutamic acid and alanine. Cystein was not detected in this amylase.

Effect of divalent ions on the stability of SDA—The following experiment was done in order to clarify the role of divalent ions on the stability of SDA. The enzyme preparation dissolved in M/100 borate buffer, pH 8.5, to be 80U/ml, in the condition of which the SDA is usually stable (KANEKATSU, 1973), was divided into two fractions. An aliquot was dialyzed against the buffer containing 1 mM of ethylenediamine tetraacetic acid (EDTA) for a week by changing the outer solution several times during the experiment, while another aliquot was used for the control experiments.

First, the heat stability property of the two was investigated. The enzyme solutions were incubated at 50° for 30 min, followed by the incubation at 55° for 30 min, and so on……, and the remaining activities were assayed with the progress of the incubation time. As shown in Fig. 3, denaturation alredy occurred at 50° in the case of dialyzed enzyme, while in the case of nondialyzed one, denaturation did occur only if the treatment at 65° or above was carried out. This evidence suggests the existence of any divalent ion which afford the stability for SDA.

Inactivation of dialyzed SDA at 55° was interrupted by the re-addition of

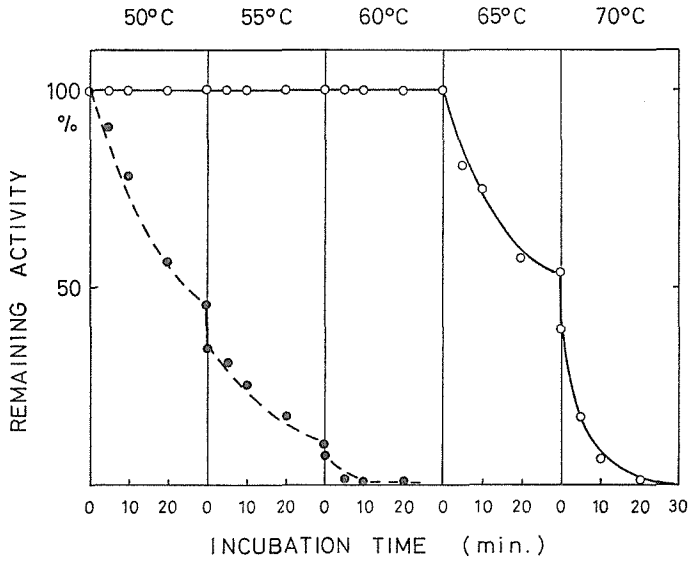

Fig. 3. Heat inactivation of silkworm digestive amylase which had been dialyzed against EDTA solution (pH 8.4).
●, dialyzed amylase ; ○, non-dialyzed amylase.

Ca^{++}. After 45 min incubation of dialyzed SDA at 55°, 100 mM solution of $CaCl_2$ was added to a final concentration of 10 mM and continued the incubation. In this case if addition of $CaCl_2$ was performed in the form of water solution, amylase would be rapidly inactivated with the extreme pH decline of the solution by an excess Cl^- ion as the result that a part of Ca^{++} would be chelated by ready existing EDTA. The author used a M/10 borate buffer pH 9.2 to make the stock solution of $CaCl_2$, to avoid such apprehension. As seen in Fig. 4,

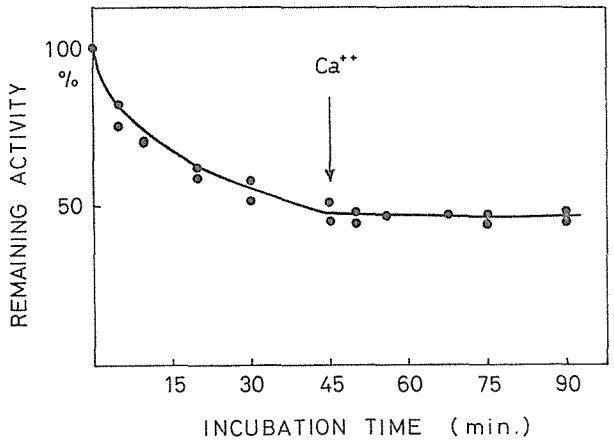

Fig. 4. Role of Ca^{++} on heat inactivation of apo-digestive amylase.

enzyme inactivation was interrupted at the point of Ca^{++} addition, although the recovery of enzyme activity was not observed. Activity was not recovered, either, by the storage of this solution at 4° for a considerable period. So the heat inactivation of SDA in the absence of Ca^{++} was decided to be irreversible.

Next, several divalent cations, Mg^{++}, Ba^{++} and Zn^{++} were also tested for the ability of protection from the heat denaturation of SDA. All of them were used as chloride form, and 20 mM buffered solutions same as in the case of $CaCl_2$ were used. They were added to the dialyzed SDA preparation to be a final concentration of 8 mM and incubated at 55°, with periodical test of remaining activity. Pure dialyzed SDA preparation, and dialyzed preparation with $CaCl_2$ added were also served for the respective control experiment. Figure 5 shows that the Ca^{++}, Mg^{++} and Ba^{++} possess protecting ability against heat denaturation of SDA, to some extent. Although Ca^{++} was most effective, it was impossible for apo-SDA to restore all of its stability even with Ca^{++}. In the case of Zn^{++} added preparation, pH of the solution was reduced to be 6.3, although the pH of preparations with other cations changed only slightly (pH

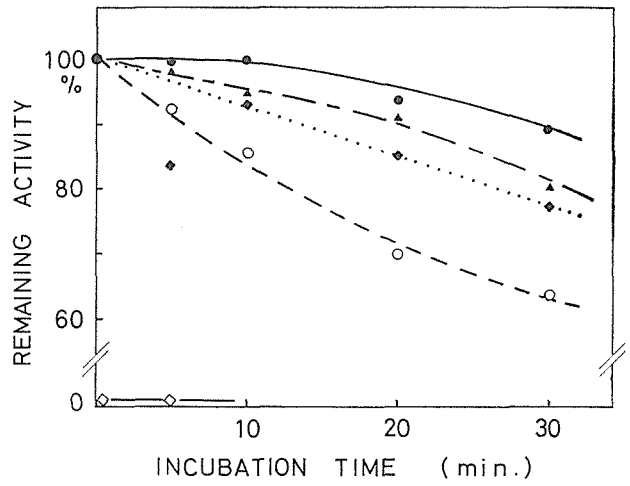

Fig. 5. Effect of divalent cations on the heat stability of apo-digestive amylase (pH 8.4, 55°).
●, Ca^{++}; ▲, Mg^{++}; ◆, Ba^{++}; ◇, Zn^{++}; ○, null.

8.1–8.5). But the inactivation of SDA in this case was not due to the reduction of pH value. As the enzyme was completely inactivated before incubation, this inactivation seemed to be due to the inhibiting nature of Zn^{++} itself.

The inactivation of SDA by Zn^{++} ion was further investigated quantitatively. Substrate solution of 0.2% soluble starch in pH 9.2 borate buffer (M/10),

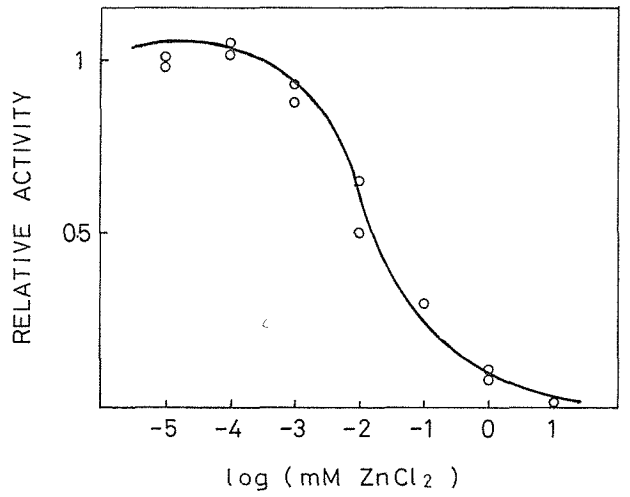

Fig. 6. Effect of Zn^{++} on holo-digestive amylase activity.

containing 1 mM $CaCl_2$ was added 10^{-5}M to 10^{0}M of $ZnCl_2$, and enzyme reaction was performed, namely with 25μl enzyme preparation, 30° 1 min. The SDA preparation used for this experiment was "non-dialyzed" SDA sample. As shown in Fig. 6, Zn^{++} ion of concentration above 10^{-3} to 10^{-2}M appeared to work as the inhibitor for SDA. The author would like to add a little more precaution that the colour intensity of SOMOGYI-NELSON's method was reduced by about 10 % when the concentration of $ZnCl_2$ in a sample was elevated to 10^{0}M.

Action on β-limit dextrin—A 0.4% solution of β-limit dextrin was prepared with 0.05M CAPS buffer pH 9.2. The solution was incubated with 3.2 mg of the SDA at 30°, with a drop of liquid paraffin on the surface of the reaction mixture to prevent a bacterial activity. Soon after the reaction was started,

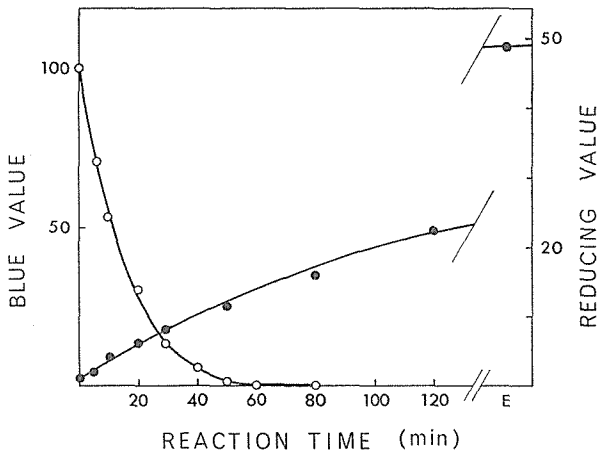

Fig. 7. Hydrolysis of amylopectin β-limit dextrin by silkworm digestive amylase.
○, blue value ; ●, reducing value ; E, one week incubation.

the blue value rapidly decreased and became null after the reaction time of 50 min. The reducing value, in contrast with the blue value, gradually increased as the reaction time was prolonged and finally reached as high as 50%, after one week incubation (Fig. 7). Here, the value of percentage, used to express the amount of appeared reducing value, is the relative value on the assumption that the substrate molecules would be completely hydrolyzed into maltose. The reducing value never showed any further increase with more incubation time. These results indicate that β-limit dextrin is hydrolyzed by the SDA into dextrins with a mean degree of polymerization of about 4. As it is usually said that the branched α-limit dextrin which is produced from amylopectin or glyco-

gen by the action of α-amylase has a degree of polymerization of 3–5 (GREENWOOD and MILINE, 1968), SDA can be considered to be able to hydrolyze the β-limit dextrin, completely.

Mode of action on amylose—Amylose (0.1%) was subjected to the action of five amylases : SDA, barley α-amylase, *Aspergillus oryzae* α-amylase, porcine pancreatic α-amylase and barley β-amylase. All the enzyme action was performed at pH 6.5, only the SDA reaction both at pH 6.5 and 9.2. Samples of reaction mixture were withdrawn after various period of incubation from 5 min to 120 min, and their blue value and reducing value were determined. The effect of using different buffer solutions to dissolve the substrate was compensated, using the internal standard by adding maltose of constant amount. The curves obtained by plotting the blue values against the reducing values (Fig. 8) suggest that the SDA may be classified into α-amylases, with a clear distinction to β-type amylase in a respect of its action pattern. But once a quantitative

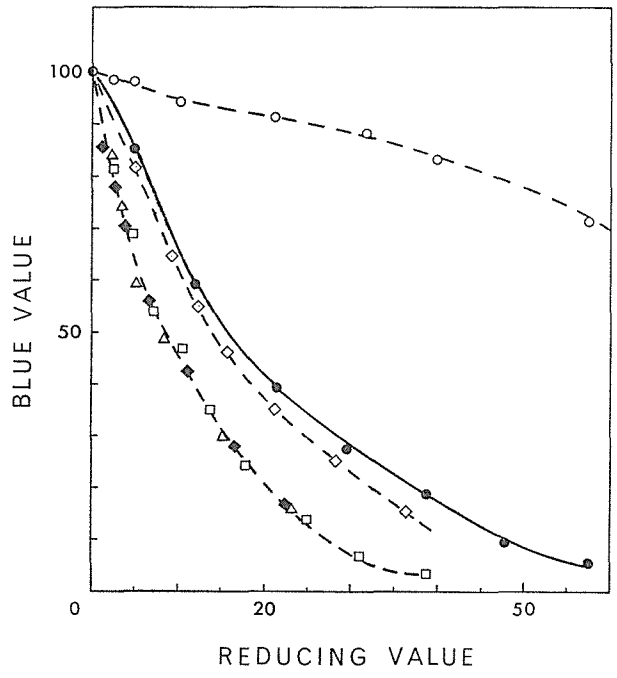

Fig. 8. Comparison of the drop in blue value *vs.* increase in reducing value, for the hydrolysis of amylose by various amylases. ●, silkworm digestive amylase at pH 9.2 ; ◆, silkworm digestive amylase at pH 6.5 ; □, barley α-amylase at pH 6.5 ; △, *Aspergillus oryzae* α-amylase at pH 6.5 ; ◇, porcine pancreatic α- amylase at pH 6.5 ; ○, barley β-amylase at pH 6.5.

comparison was made, many singularities may be pointed out. The reducing value corresponding to the 50% blue value is 8% for the barley and *Aspergillus* α-amylases in good agreement with the values reported by previous workers (KUNG *et al.*, 1953 ; ROBYT and FRENCH, 1967), whlie that of SDA at pH 9.2 (optimal condition) is about 16%, which is still superior to the data for porcine pancreatic α-amylase. So, the drop of blue value by SDA is slower than any other α-amylases, while if the SDA was worked at less optimal condition, pH 6.5, its action pattern on amylose became quite similar to that of barley and *Aspergillus* α-amylase, in which almost all α-amylases are included.

Next, the constitution of the digestion products was determined by the time sequence TLC analysis. Amylose solution (0.4%, pH 9.2) was incubated with the SDA and aliquots of the reaction mixture were withdrawn after various length of incubation time from 10 min to 24 hr. An aliquot of withdrawn samples was divided into two and one was treated as described in "MATERIALS AND METHODS" to prepare TLC sample, while the other was subjected for the iodine colour reaction. This experiment revealed that the digests became "achroic" (not to show the amylose—iodine colour) at 160 min. In the chromatogram (Fig. 9), small maltodextrins, G_1—G_4, were detected from the very early stage of reaction (10 min), continuously increased in amount until 24 hr except for G_4 whose amount declined slightly at 24 hr. Larger maltodextrins, G_5–G_8,

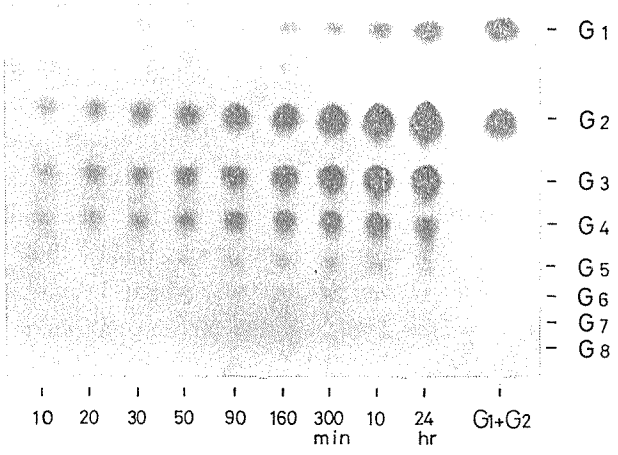

Fig. 9. Time sequence TLC analysis of silkworm digestive amylase action on amylose at pH 9.2.
Achroic point of the reaction was at 160 minutes. G_1, G_2, G_3,…… represents glucose, maltose, maltotriose, *etc.*

appeared later than the smaller ones and they were most conspicuously detected near the achroic point (160 min). This pattern of early appearence of smaller maltodextrins resembles that obtained with the porcine pancreatic α-amylase (ROBYT and FRENCH, 1967) but is in contrast to that obtained with other α-amylases which give rise to the production of larger maltodextrins which gradually change into smaller fractions.

DISCUSSION

Up to nowadays, reports for the mode of action of silkworm digestive amylase (SDA) were very few and only the discussion for dissimilarities from the "amylase of animal origin" a call used almost synonymously with "α-amylase" was made on the respect of its inhibitor, activator and other properties (ITO *et al.*, 1962). NISHIDA and HAYASHIYA (1969), who have first attempted a partial purification of SDA, discussed that their preparation of SDA has both saccharifying and liquefying properties and that the mode of activation or inactivation by inorganic ions also differed from typical examples for animal α-amylases. Probably because of these results, "α-" was not adopted in their report, either.

In this report, as the author observed the ability for SDA to hydrolyze amylopection β-limit dextrin efficiently, the amylase should be classified as α-amylase [EC 3. 2. 1. 1.] in the respect of its action pattern. So the author proposes to call the SDA as "silkworm digestive α-amylase" and the discussion of this paper may be focused upon the comparison with other α-amylases.

Although the purity of the SDA preparation was ascertained by ultracentrifugation (KANEKATSU, 1973) and electrophoresis, the estimated values proposed for its molecular weight are so varied. The presumed value of 14,800, obtained from the gel filtration with Bio-gel P-60 column chromatography (KANEKATSU, 1973) is extremely small as compared to the molecular weight of 45,000 generally known for several α-amylases ; beside that, it is even as small as the value of ribonuclease A (13,900), which is one of the smallest among enzyme proteins. The smallest value for the molecular weight of α-amylase was reported by MANNING and his co-workers (MANNING and CAMPBELL, 1961 ; MANNING *et al.*, 1961) to be 15,600, for α-amylase from *B. stearothermophilus*. But α-amylase from *B. stearothermophilus* dealed by them is now, in itself, doubted for its existence (PFUELLER and ELLIOTT, 1969). Although on the performance of gel chromatography to estimate the molecular weight of α-amylase, the author used the beaded acrylamide gel, Bio-gel, in place of the crosslinked dextran, Sephadex, to avoid possible retardation of the sample

that may be caused by the enzyme—substrate interaction between α-amylase and gel matrix, the molecular weight was also estimated too small. Similar examples have been also reported by KAUFFMAN *et al.* (1970), JACOBSEN and SÖNJU (1971), VANDERMEERS *et* CHRISTOPHE (1968) and ALLAN *et al.* (1970). KAUFFMAN, JACOBSEN, and their co-workers discussed this phenomenon that as the α-amylases have such a great amount of amino acid residue with OH or COOH group, ie., aspartic acid and glutamic acid, that they form considerable amount of hydrogen bond against the carboxyl groups of Sephadex gel or Bio-gel, and so the rate of migration within the column may be retarded. In fact, over 20% of structural amino acids of α-amylases are occupied by Asp+Glu ; moreover, if Ser and Thr are added, as much as 35% of amino acids are occupied (GREENWOOD and MILINE, 1968 ; PFUELLER and ELLIOTT, 1969). Alkaline α-amylase produced by the microorganism grown on the strong alkaline medium also has the tendency (HORIKOSHI, 1971). In Table II, the relative amount of Asx+Glx and Asx+Glx+Ser+Thr of α-amylases from silkworm, human saliva, rat pancreas, *Aspergillus oryzae*, *B. subtilis* liquefying, *B. subtilis* saccharifying and *B. stearothermophilus*, were reviewed. In respect of the amino acid composition of SDA, a very close resemblance between other

Table II. Contents of OH-amino acid residues in α-amylases from various sources

Source	Asx+Glx	Asx+Glx+Thr+Ser
Silkworm digestive juice	23.7%	33.0%
Human saliva*	22.9	35.0
Rat Pancreas*	20.1	31.2
*Aspergillus oryzae**	20.8	36.2
*B. subtilis L**	23.6	35.2
*S**	24.8	40.6
*B. stearothermophilus***	20.6	35.1

Values are represented as per cent in moles of amino acid residues.
* GREENWOOD and MILINE (1968)
** PFUELLER and ELLIOTT (1969)

α-amylases is observed. From the above reason, the author concluded that the value of molecular weight obtained on the SDA from the technique of Bio-gel P-60 column chromatography is the under-estimated value mainly caused by the elecrostatic interaction between the enzyme and the gel matrix.

Next, the value of 57,000 obtained by the analytical SDS-polyacrylamide gel electrophoresis may be discussed in the same manner. In this case, as the

gel matrix used for the elctrophoresis was polyacrylamide, the same as that of Bio-gel, it seems to be very reasonable to consider that the obtained value might be the over-estimated value as the result from the slower migration caused by the very same electrostatic interaction mechanism, if the abnormal emergence of enzyme from the column of gel chromatography was the fact. In fact, MITCHELL *et al.* (1973) observed the over-estimation for the molecular weight of *B. subtilis* α-amylase by the SDS-polyacrylamide gel electrophoresis. Provided that in their paper they discussed to attribute this phenomenon to the reduced conbinability of SDS to the protein as the result of its specificity of amino acid composition.

The author further attempted the molecular weight assumption by 6 M guanidine hydrochloride gel chromatography. It seems quite necessary to perform gel chromatography of the denatured enzyme to estimate its molecular weight, especially if the enzyme is known or thought to interact against the gel material as α-amylases. For example, COZZONE *et al.* (1970) obtained a reasonable value for the molecular weight of porcine pancreatic α-amylase by the gel chromatography with a buffer containing mercaptoethanol. The author introduced the experimental condition normalized by FISH *et al.* (1969), to enable direct comparison with other workers' results. As the method seems to have no particular defects, the author thought that the obtained value of estimation, 47,000, should be considered as the most actual one. This theme should be brought to be perfect with further studies of ultracentrifugation and many other experiments.

Amino acid composition of SDA very well resembles those of many other α-amylases, as mentioned already. In SDA, cystein or half cystine was not detected. Although, -SH group of cystein is known to have a considerable role on activity or stability of the enzyme in many cases of α-amylases, one which possesses no cystein residue also exists, for example, *B. subtilis* liquefying α-amylase (GREENWOOD and MILINE, 1968). Next apparent characteristic of amino acid composition of SDA may be the large amount of arginine residues. It seems to have much contribution upon the very high pH of the isoelectric point of SDA previously mentioned (KANEKATSU, 1973).

It is generally said that α-amylases are all metalloenzymes having Ca^{++} ions within each molecule. Numbers of Ca^{++} within a enzyme molecule varies with the origin of the α-amylase from 1 to 5. Their intensities of binding to the enzymes also vary, from as weak as to be released merely by dilution, to so firm as is impossible to be released even with chelating reagent or electrodialysis (STEIN *et al.*, 1964). In general, role of Ca^{++} ion is regarded as the

stabilizing agent for the α-amylases (PFUELLER and ELLIOTT, 1969 ; HORIKOSHI, 1971), though some workers reported that Ca^{++} ion acts as an activator for α-amylases from silkworm digestive juice and any other insects (ITO *et al.*, 1962 ; PODOLER and APPLEBAUM, 1971). On the other hand, some type of α-amylase also exists, that the enzyme activity quickly reduces if Ca^{++} is removed by dialyzing against the chelating reagent or any other methods (TODA and NARITA, 1968 ; TODA *et al.*, 1968 ; MITCHELL et *al.*, 1973). Usually, it is known that almost the whole enzyme activity is recovered by the re-addition of Ca^{++} ion, in this case. In the case of SDA, the author reported previously that the treatment of the enzyme merely by dialyzing against a buffer solution without Ca^{++} seemed to have no effect on the stability (KANEKATSU, 1973). On the contrary, however, after the experiment of Ca^{++} removal by dialyzing against EDTA solution, it was revealed that the range of heat stability decreases by 15°, although the quick reduction of enzyme activity was not observed. The Ca^{++} ion(s) that play(s) a part for the enzyme stability seem(s) to bind so firmly to the enzyme that it is hard to release it (them) from the holo-SDA by the condition only to remove Ca^{++} ion from the enzyme solution or to dialyze against the buffer solution without Ca^{++} ion. The apo-SDA obtained by dialyzing against EDTA-containing buffer, which has a reduced stability than the holo-SDA, easily retains its stability by the re-addition of Ca^{++} ion, although it was not effective to add Ca^{++} ion onto the heat denatured apo-SDA.

Instead of Ca^{++}, it was known that Mg^{++} or Ba^{++} partially contribute to the stability of apo-SDA. Whether Ca^{++} ion is able to re-substitute or not, to the Mg^{++} or Ba^{++} bound to the apo-SDA, will be necessary to observe.

As to the role of Zn^{++} ion, though it is known that it takes an important role for the formation of quartary structure of *B. subtilis* α-amylase (KAKIUCHI *et al.*, 1965 ; ROBYT and ACKERMAN, 1973), the inhibitory effect for α-amylases has not been known. After the result obtained by the author, the two metal ions, namely Cu^{++} (NISHIDA and HAYASHIYA, 1969) and Zn^{++} should be admitted as the inhibitors for the SDA.

Last of all, the author would discuss on the action pattern analysis for the SDA. As the discussion about the action on amylopectin β-limit dextrin was already mentioned at the beginning, here, the action on amylose will be discussed.

If the SDA action on amylose took place, the number of enzyme actions that are needed to reduce the amylose—iodine colour (blue value) to the given degree is far more than that needed by the action of *Aspergillus* and barley α-amylases. So SDA is thought to have higher reactivity on the end of sub-

strate molecules. Types of amylases, with the reaction specificity strictly limited on the end of the substrate, are the exo-type amylases such as β-amylase, glucoamylase, G_4-producing amylase produced by *B. stutzeri* (ROBYT and ACKERMAN, 1971), G_6-producing amylase from *Aerobacter aerogenes* or *cloacae* (KAINUMA *et al.*, 1972). In the case of SDA, however, it is apparent that the enzyme is, persistently, endo-type amylase, as apparent from the result of Fig. 7. The fact that the drop of plotted curve for SDA is apparently very fast, as compared to that of β-amylase, also supports it. But it should be said that among endo-type amylases, SDA actually has high reactivity on the near end of amylose molecules. In the respect of the amount of reducing value produced at the point of 50% blue value, the value of 16% for the action of SDA is higher than any other α-amylases, even than that of porcine pancreatic α-amylase, which is known as the one to have a saccharifying tendency most markedly because of its "multiple attack nature", namely, the nature of exo-type action to hydrolyze adjacent several glucosidic bonds just after a hydrolysis in endo-type enzyme action (BANKS *et al.*, 1971 ; ROBYT and FRENCH, 1967, 1970). In SDA, though it is apparent that it has a rather saccharifying nature, much more experiments will be necessary to conclude whether it takes the action of multiple attack as porcine pancreatic α-amylase, or not. But the author considers that it is possible to say that, so far as the results of Fig. 8 and 9 are concerned, mode of action of SDA closely resembles that of porcine pancreatic α-amylase. The author also considers that the reason that SDA has been thought to have twofold characteristics of both liquefying and saccharifying nature might be derived from this singular pattern of action. The fact that at the slightly acidic condition, singularity of the pattern of SDA action on amylose disappears and comes to resemble many other general α-amylases, the similar phenomenon is also reported on porcine pancreatic α-amylase by ROBYT and FRENCH (1970), may perhaps serve any hints to the data reported by NISHIDA and HAYASHIYA (1969) that their preparation of SDA shows a reversed ratio of liquefying to saccharifying activity at two different pH conditions.

In any case it is apparent that the SDA produces maltodextrins of low degree of polymerization such as G_2, G_3 and G_4 from the very early stage of reaction, and so imagined to serve the convenience to the silkworm to digest and absorb starch molecules ingested as a food material. Though the author guesses that from the result of Fig. 9, maltodextrins larger than G_4 can be digested by SDA, further investigation may be definitely required ; for example, the examitation of SDA activity on maltodextrins in accordance with different

degrees of polymerization (NITTA *et al.*, 1971 a, b).

SUMMARY

Silkworm digestive amylase (SDA) preparation, free from any glycosidase or galactosidase activities, was investigated for its further properties. The enzyme is an α-amylase [EC 3. 2. 1. 1.] which can well hydrolyze β-limit dextrin into maltodextrins having a mean degree of polymerization of 4.

The molecular weight of the enzyme was estimated to be about 47,000, by the method of 6 M guanidine hydrochloride gel chromatography. In addition, the estimated values, of about 14,800 by Bio-gel P-60 column chromatography and about 57,000 by SDS-polyacrylamide gel electrophoresis were discussed as under- and over-estimated values, respectively, derived from the same mechanism caused by the electrostatic interaction between the enzyme and the gel matrix.

Amino acid analysis of the SDA revealed that aspartic acid (or asparagine), glycine, glutamic acid (or glutamine) and alanine are abundant in this order, whereas the enzyme has no cystein in its amino acid composition.

The enzyme is generally stable below 60° at pH 8. 4. But by dialyzing against EDTA solution, the maximum range of heat stability was lowered by 15°. The heat stability of apo-SDA was restored by the re-addition of Ca^{++}, although it has no use to add Ca^{++} upon the ready heat denatured apo-SDA. Instead for Ca^{++}, both Mg^{++} or Ba^{++} partially played an effective role.

The enzyme was inhibited by 10^{-2} M of Zn^{++}, whether in the presence or absence of Ca^{++}.

The action of SDA on amylose was investigated and revealed that it is an α-amylase with rather apparent saccharifying nature and that it can produce low molecular weight maltodextrins, G_2, G_3 and G_4 in high frequency. The saccharifying nature of the enzyme was lost if the enzyme reaction was performed at a less optimal condition of SDA, namely pH 6. 5.

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