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Purification and characterization of digestive amylase from the tasar silkworm, *Antheraea mylitta* (Lepidoptera: Saturniidae)

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Digestive amylase was purified from larvae of Indian tasar silkworm, *Antheraea mylitta* using ammonium sulphate precipitation, glycogen complex precipitation and gel filtration chromatography. Specific activity increased from 0.673 AU/mg in the crude digestive juice to 94.80 AU/mg in the final purified sample. Activity of the purified enzyme was 15-fold less than that of the digestive amylase of silkworm. *Bombyx mori*. The zymogram pattern of the purified amylase was similar to that of crude digestive juice on 7.5% native PAGE. The purified enzyme exhibited five bands on native PAGE. IEF of the purified enzyme also revealed five bands with p*I*s of 6.5, 6.15, 5.9, 5.8 and 4.7, respectively. The purified enzyme is a single polypeptide chain with a M_r of 58 kDa. The amylase is most active at pH 9.5 and is a Ca^{2+} dependent endoenzyme which hydrolyses starch into maltose, maltotriose and maltotetrose and hence behaves as an α-amylase (EC 3.2.1.1). The enzyme was unaffected by the presence or absence of Cl^- , with K_m for soluble starch of 0.113%.

Key words: Tasar silkworm; *Antheraea mylitta*; Digestive juice; Amylase; Optimum pH; Isozymes; K_m; Effect of Ca^{2+}

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Introduction

Amylase is one of the key enzymes involved in carbohydrate digestion and metabolism in insects (Daone *et al.*, 1975; Buonocore *et al.*, 1976). α-Amylase is an endoenzyme which hydrolyses the α-1,4-glycosidic bonds of starch which is a polymer of linear amylose chains connected by α-1,6 bonds. Amylases from different origins have been purified and their physical and chemical properties characterized (Fisher and Stein, 1960; Podoler and Applebaum, 1971; Takagi *et al.*, 1971; Kanekatsu, 1972; Baker, 1983, 1987, 1991; Baker and Woo, 1985; Abraham *et al.*, 1992). In silkworm, *Bombyx mori*, and many other insect species successful adaptation depends on the level of digestive amylases (Hirata, 1974; Buonocore *et al.*, 1976). Silk content, cocoon weight and other quantitative traits of silkworm are negatively correlated with the higher level of digestive amylases (Hirata, 1974; Moon and Seol, 1983; Chatterjee and Datta, 1992). The tasar silkworm, *Antheraea mylitta*, an economically important sericigenous insect is widely distributed in India and it feeds on many diverse plant species (Jolly *et al.*, 1979).

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Cocoon and silk yield of larvae raised on different food plants depend on carbohydrate, protein fibre and moisture contents of the foliage (Jolly *et al.*, 1974). However, the enzymes that are involved in the digestion have not been studied in this commercially important insect. In the present study, amylase was purified from the digestive juice and characterized.

Materials and Methods

Insects and collection of samples

Larvae of *A. mylitta* were reared on *Terminalia tomentosa* using procedures described by Jolly *et al.* (1974). Digestive juice was collected from 6-day-old 5th stadium larvae, into precooled tubes. Larvae were starved for 6–8 hr, and vomiting was induced by brief exposure to chloroform vapours. Collected digestive juice sample was then centrifuged at 10,000 *g* for 10 min to remove undigested leaf particles and stored at −20°C until use.

Enzyme assay

Enzyme activity was measured with the dinitrosalicylic acid (DNS) procedure using soluble starch as substrate (Bernfeld, 1955; Abraham *et al.*, 1992). Samples were incubated in 1.5 ml 20 mM Tris–HCl (pH 9.5) buffer containing 0.2% soluble starch and 1 mM $CaCl_2$ at 37°C for 30 min. Maltose hydrate was used as standard. One amylase unit (AU) was defined as the amount of enzyme that produced 1 mg of maltose hydrate/min at 37°C. Samples were diluted prior to the assay to maintain linearity. The final purified sample was diluted 1:10 with buffer and 5 μl aliquots were assayed for the reaction. Protein was determined by the Folin phenol procedure with bovine serum albumin as standard (Lowry *et al.*, 1951).

Purification of amylases

Amylase was purified using the procedure of Loyter and Schramm (1962). Digestive juice (35 ml) was collected from 25 6-day-old 5th stadium larvae. Digestive juice proteins were precipitated with different concentrations of ammonium sulphate at 4°C for 30 min: (1) 0–0. 5 M, (2) 0.5–1.5 M, (3) 1.5–2.5 M and (4) 2.5–3.5 M. The precipitates were centrifuged at 10,000 *g* for 20 min. Pellets were dissolved in Tris–HCl, pH 8.0 (Tris buffer), and dialysed against the same buffer overnight at 4°C. Fractions 2 and 3 were pooled together and the enzyme was further purified by amylase–glycogen complex precipitation followed by autodigestion of the glycogen as described by Loyter and Schramm (1962) and Baker and Woo (1985). Final purification of the amylase was carried out by size exclusion column chromatography on Sephadex G-100 (55 × 1.5 cm) equilibrated in Tris buffer. Flow rate was maintained at 15 ml/hr and 4 ml fractions were collected. Protein was monitored by measuring the absorbance at 280 nm and 5 μl aliquots from each fraction were assayed for amylase activity. Column void volume (V_o) and elution volume (V_e) of standard protein were determined using Blue Dextran and protein molecular weight standards (Sigma), respectively.

Enzyme properties

The pH optima of amylase was determined by incubating 5 μl of 10-fold diluted crude enzyme solution in 1.5 ml of 0.2% soluble starch in 0.1 M buffer containing 20 mM NaCl and 0.1 mM $CaCl_2$. Tris–HCl (pH 6.5–10), glycine–NaOH (pH 8.5–11) and Tris–borate (pH 8.5–11) buffers were used. Results obtained are mean values of two independent assays with duplicates (corrected with starch + DNS blank) at each pH.

Thermal stability of the purified digestive amylase in the presence of calcium chloride was determined as follows: three samples, one dialysed overnight against 1 mM EDTA, another dialysed against 1 mM $CaCl_2$ and the control dialysed against buffer without EDTA or $CaCl_2$, were used. Each sample was incubated at 45°C and aliquots removed at 20, 40 and 60 min of incubation, diluted 10 times with the dialysis buffer and 5 μl of diluted sample was assayed. A similar experiment was carried out at 60°C. The results are the mean of three independent determinations of each sample at each temperature with duplicates (corrected for absorbance due to starch).

K_m values for starch were determined using the purified amylase. Aliquots (5 μl) of 10-fold diluted enzyme were incubated with 11 substrate concentrations from 0.025 to 0.5% in 100 mM Tris–HCl, pH 9.5

containing 20 mM NaCl and 0.1 mM $CaCl_2$. Results are mean values of two sets of experiments with duplicates at each starch concentration.

Electrophoresis

Crude and purified enzyme samples were analysed by native polyacrylamide gel electrophoresis (PAGE) and isoelectric focusing (IEF).

Crude digestive juice and purified amylase samples were analysed on 1 mm thick slab gel using 7.5% polyacrylamide at pH 8.3 (Gabriel, 1971). After the run, gels were washed in Tris buffer for 15 min and incubated in the same buffer containing 0.5% soluble starch and 1 mM $CaCl_2$, at 37°C for 45 min and stained with KI–I_2 solution to detect the enzyme activity. A parallel electrophoresis of the purified sample was stained with Coomassie Brilliant Blue.

IEF was performed on an Ampholine PAG plate 240 × 110 × 1 mm (precast gel from Pharmacia, Sweden). Purified protein sample (5 μg) was applied to the gel in duplicate. Isoelectric markers (Broad p*I* kit, Pharmacia) were also applied to determine the p*I* of the purified protein. Electrofocusing was carried out using a Multiphor system (Pharmacia, Sweden) and the gel was run according to the manufacturer's instructions. After electrofocusing, the portion of the gel containing purified amylase and isoelectric markers was stained in Coomassie Brilliant Blue R-250. The remaining portion of the gel with purified amylase was developed for amylase activity as described earlier.

SDS–PAGE was carried out on 1 mm thick 10% acrylamide slab gels (Sambrook *et al.*, 1989). The molecular weight of the purified protein was determined from the relative mobility of the standard proteins such as bovine serum albumin (66 kDa), ovalbumin (45 kDa), glyceraldehyde 3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), trypsinogen (24 kDa), soyabean trypsin inhibitor (20 kDa) and α-lactalbumin (13 kDa).

Paper chromatography

Purified amylase (1 μg) was incubated with 0.5 ml of 1% starch solution in Tris–HCl pH 8.3 at 37°C. At intervals of 5, 10 and 120 min aliquots were removed, enzyme was inactivated at 90°C for 3 min and product was chromatographed on Whatman No. 1 sheets using butanol–ethanol–water (10:1:2) as solvent system (Spiro, 1966). A mixture of glucose, maltose and partial acid hydrolysis product of starch was used as standards. After 180 hr run, the samples were stained with alcoholic silver nitrate and sodium hydroxide (Spiro, 1966).

Results

Purification of amylase

Following ammonium sulphate precipitation, ethanol precipitation and glycogen complex formation the specific activity of the amylase increased 141.6-fold from 0.673

Table 1. Purification of amylase from larval digestive juice of tasar silkworm, *Antheraea mylitta*

Fractions	Protein* (mg)	Amylase units†	Amylase Sp. act‡	Recovery§ (%)	Fold purification‖
Crude	170.5	114.00	0.67	100.00	1.00
0.0–0.5 M	8.4	5.40	0.64	4.70	0.95
0.5–1.5 M	13.9	60.68	4.36	53.20	6.46
1.5–2.5 M	20.8	27.50	1.32	24.00	1.97
2.5–3.5 M	80.5	17.85	0.21	15.66	0.31
Ethanol ppt. of 0.5–2.5 M¶	29.5	85.67	3.11	75.14	4.62
Glycogen ppt.	1.4	50.83	35.79	44.58	53.17
Sephadex G 100	0.36	34.13	94.80	29.90	141.60

*Total recovered protein.
†Amylase units/aliquot × total sample volume. One amylase unit is the amount of enzyme that produces 1 mg maltose hydrate/min at 37°C.
‡Specific activity = activity units/mg protein.
§Based on amylase units in crude extract.
‖Based on specific activity in crude extract.
¶Fractions 0.5–1.5 and 1.5–2.5 M were combined prior to ethanol precipitation.

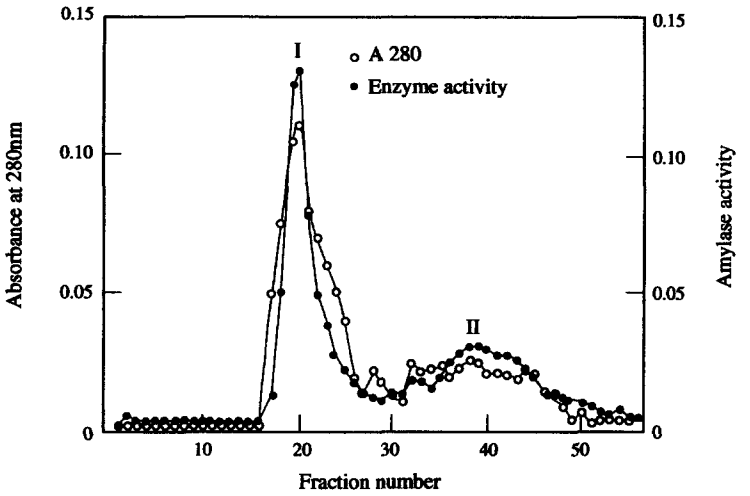

Fig. 1. Purification of amylases by gel filtration column chromatography. Amylase–glycogen complex after autodigestion was loaded on to a Sephadex G-100 column (50 × 1.5 cm) equilibrated in Tris–HCl buffer. Four millilitre fractions were collected and the fractions were followed by absorption at 280 nm (–△–) and by enzyme assay (–□–).

units in the crude digestive juice to 94.80 AU/mg (Table 1). Total recovery was 29.9% of the initial activity found in the crude digestive juice. After ammonium sulphate precipitation most of the activity was found in fractions 2 and 3. Native PAGE analysis of these two fractions showed similar enzyme banding pattern (data not shown). Thus, these two fractions were combined for further purification. Amylase released after autodigestion of the amylase–glycogen complex at ambient temperature was chromatographed on a Sephadex G-100 column. Most of the amylase activity was associated with a sharp protein peak that was eluted between fractions 17

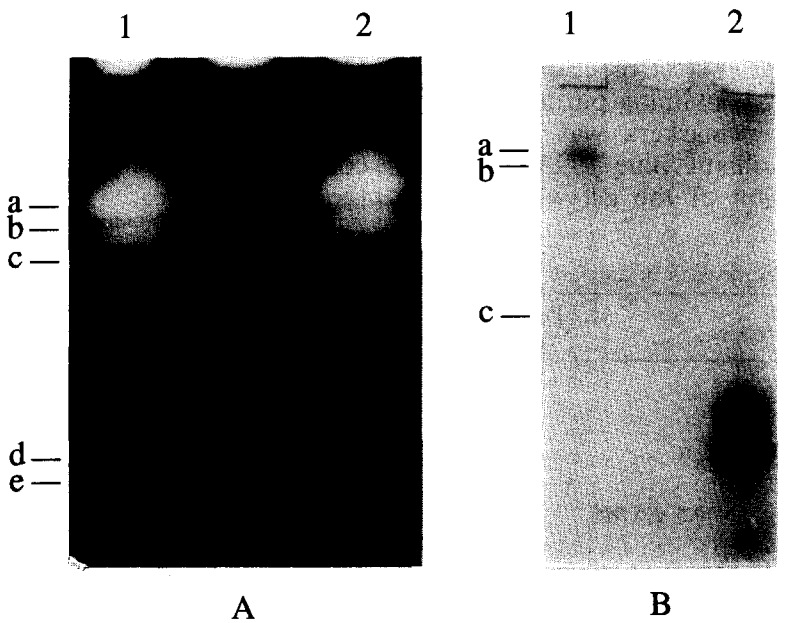

Fig. 2. Native PAGE (pH 8.3) analysis of the crude digestive fluid and purified enzyme. (A) KI–I_2 staining for amylase. (B) Coomassie Blue Staining. Lane 1: 5 μg of purified amylase; lane 2: 10 μl of 10 × diluted crude digestive juice. Bands a–e indicate amylase bands detected at R_m 18, 22, 26, 54 and 59 mm, respectively. Both A and B were from the same PAGE but photographs are not to scale.

and 26 (peak I). A small and wide activity peak (peak II), (Fig. 1) was eluted between fractions 35 and 50. Peak I and II were collected and separately lyophilized. Native PAGE analysis of peak I and II showed similar activity profiles but specific activity of peak I was many folds higher than peak II. Therefore, only peak I was used for further studies.

Electrophoresis and isoelectric point determination

Amylase in the crude digestive juice was separated into five bands on native PAGE. The major band occurred at R_m 18 mm (band a) while minor bands were visible at R_m 22 mm (band b), R_m 26 mm (band c) R_m 54 mm (band d) and R_m 59 mm (band e) (Fig. 2A, lane 2). The purified enzyme also showed similar banding pattern on native PAGE (Fig. 2A, lane 1) indicating that the purified enzyme represents all amylase isozymes present in the crude digestive juice. The bands a, b and e were apparent in the Coomassie Blue stained parallely run purified sample (Fig. 2B, lane 1). The intensity of the bands was consistent with the enzyme activity of the respective bands (Fig. 2A, lane 1). Purified enzyme was resolved into five active bands on IEF (Fig. 3). The activity pattern of the purified amylase was similar for both native PAGE and IEF (Figs 2A and 3A). Coomassie Blue staining of a parallel IEF gel developed two protein bands and the intensity of the bands was consistent with the enzyme. The IEF profile of the purified sample was identical to that of crude digestive sample (Figs 2 and 3). The isoelectric points of the five bands were determined to be 6.5 (band i), 6.15 (band ii), 5.9 (band iii), 5.8 (band iv) and 4.7 (band v).

Molecular weight

The purified amylase ran as a single protein band on SDS–PAGE (Fig. 4). From a plot of log molecular weight against relative mobility in 10% acrylamide gel, molecular weight was estimated to be 58 kDa. On a Sephadex G-100 column the amylase was slightly retarded because of non-specific interaction with the carbohydrate matrix and the native molecular weight was determined as 50 kDa.

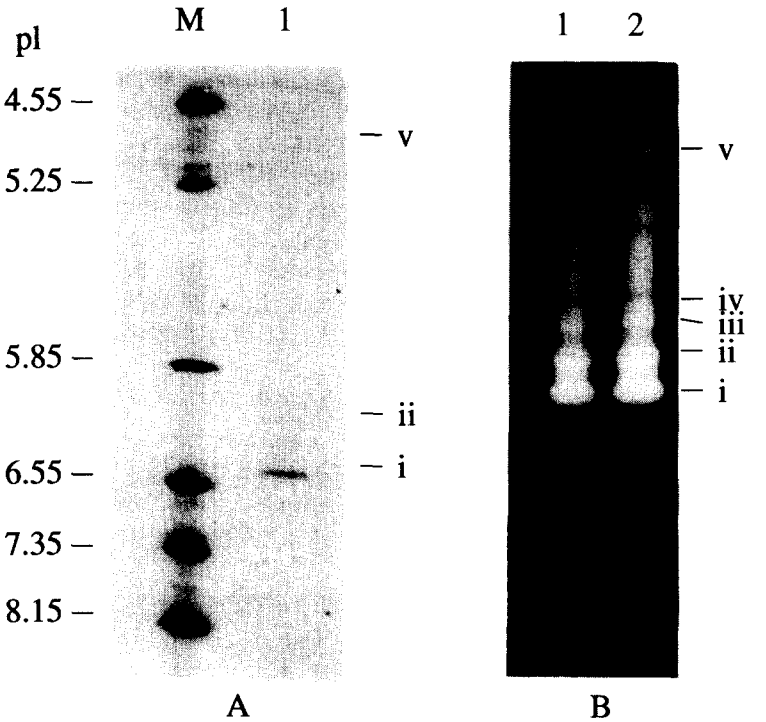

Fig. 3. Isoelectric point determination of the purified amylase. (A) Coomassie Blue staining. (B) KI–I_2 staining for amylase. Bands i–v indicate amylase bands. Lanes: M, 1, and 2 are isoelectric point calibration markers, 5 μg of purified protein and 10 μl of 10 × diluted crude digestive fluid. Both A and B were from the same PAGE but photographs are not to scale.

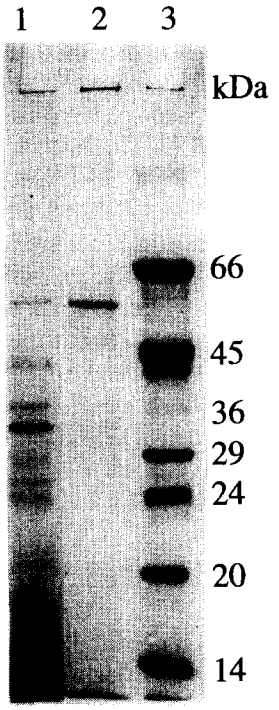

Fig. 4. SDS–PAGE analysis of the purified enzyme. Lanes 1–3 are 10 μl crude digestive fluid, 5 μg of purified protein and molecular weight standards, respectively.

Properties

The enzyme sample dialysed against EDTA lost 49% of its activity when it was incubated at 45°C for 60 min (Fig. 5), and 58% when the temperature was raised to 60°C. On the other hand 78 and 70% of the initial enzymatic activity was retained in samples dialysed against buffer containing $CaCl_2$ incubated at 45 and 60°C, respectively (Fig. 5). The activity of the undialysed controls did not differ from that of $CaCl_2$ treated sample incubated at 45°C for up to 40 min. However, when the sample was incubated at 60°C, 38.2% of the activity was lost as compared with 30.4% loss of the $CaCl_2$ treated enzyme (Fig. 5). Initial results obtained with amylase incubated with or without NaCl did not show any difference in enzyme activity under normal-experimental conditions (data not shown).

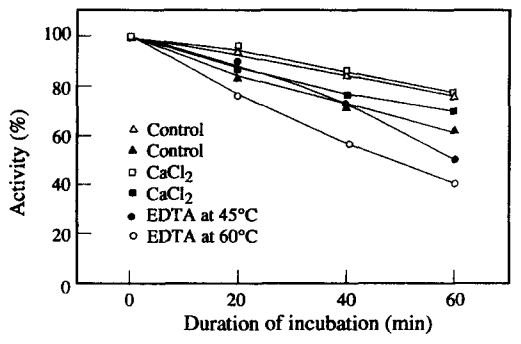

Fig. 5. Effect of $CaCl_2$ on the thermal stability of the amylase purified from the digestive fluid of *A. mylitta*. Samples were held for 60 min at 4 or 60°C. Relative activities determined are means of three sets of assays with duplicate at each temperature.

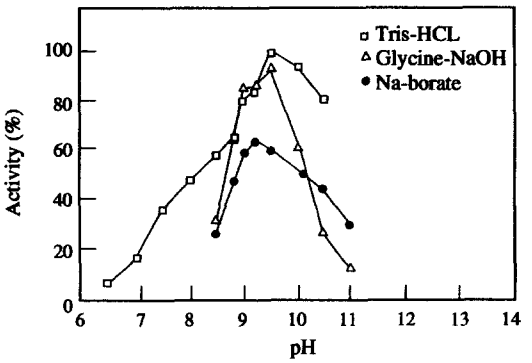

Fig. 6. Effect of pH on the activity of amylase purified from the digestive fluid of *A. mylitta*. Values are means of two sets with duplicate at each pH.

Effect of pH

The purified amylase was most active at the alkaline pH range (Fig. 6). Maximum activity occurred between pH 9 and 10 with optimum activity at pH 9.5 in both Tris–HCl and Tris–glycine buffers. Activity was slightly higher in Tris–HCl buffer as compared to Tris–glycine buffer, whereas activity in the borate–NaOH buffer was lower than the other two with the maximum activity shifting towards pH 9.2.

Effect of substrate concentration on activity

This was determined as soluble starch in the range of 0.025 to 0.5%. Michaelis constant ($K_m = 0.113\%$) was determined from a linear plot of $1/V$ vs. $1/S$ (Fig. 7).

Starch hydrolysis

Paper chromatography of the aliquots removed during the reaction revealed that maltose, maltotriose and maltotetrose along with higher maltodextrins were released after 5 min and 10 min of the initiation of reaction (data not shown). After 120 min of hydrolysis products were

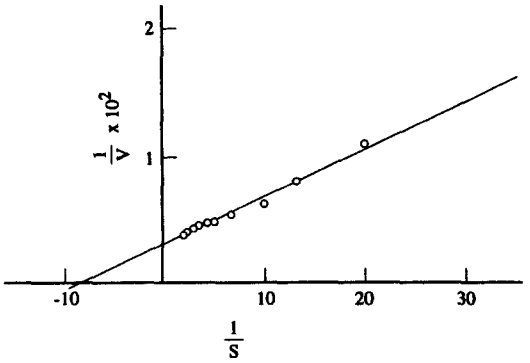

Fig. 7. Double reciprocal plot of concentration of soluble starch against rate of hydrolysis by amylase preparation from the digestive juice of *A. mylitta*. Each point is the mean of duplicate analysis of each starch concentration for two sets of assays.

predominantly maltose, maltotriose and maltotetrose with very low amount of higher maltodextrins.

Discussion

Among sericigenous lepidopteran insects digestive amylases have been studied in detail only in the monophagous silkworm, *B. mori* (Kanakatsu, 1978; Abraham *et al.*, 1992). In the polyphagous tasar silkworm *A. mylitta* amylase activity was detected in both haemolymph (data not shown) and digestive juice. In the present study, we purified and characterized digestive amylase. After four ammonium sulphate fractionations higher enzymatic activity was found in fractions 2 and 3. Enzyme recovered from these fractions was complexed with glycogen and then precipitated. During Sephadex G-100 chromatography the majority of the amylase was eluted as a single peak. Activity of the purified amylase was 94.80 AU/mg which is 15-fold less than the digestive amylase activity of *B. mori* (1479 AU/mg, Kanakatsu, 1972) and was comparable with that of amylase purified from *Anagasta kuehniella* (94.4 AU/mg) (Baker, 1989).

The amylase of *A. mylitta* liberates maltodextrins which are predominantly maltose, maltotriose and maltotetrose similar to the hydrolysis pattern of α-amylase of *B. mori* (Kanakatsu, 1978). The other physicochemical properties of the purified enzyme such as Ca^{2+} dependence and a single polypeptide of 58 kDa are similar to most of the α-amylases described in the literature. Hence, we suggest that the amylase purified in the present study is α-amylase.

The identical nature of the amylase zymograms of the purified enzyme sample and crude digestive juice show that the former represents the full spectrum of amylase isozymes. Although the banding pattern of the purified amylase was similar for both IEF and native PAGE, sufficient evidence was not available to assign the same band designation for the activity in IEF analysis. Purified enzyme revealed a single protein band of 58 kDa that contributed to more than 50% of total enzyme activity (band a on the native PAGE; Fig. 2A) suggesting that isozymes detected in the present study may be 'pseudo-isozymes' resulting from the deamidation of the major amylase (band a) as suggested by Daone *et al.* (1975). Deamidation results in a more anodic protein and is favored under alkaline conditions (Keller *et al.*, 1971). Since the zymogram patterns are similar in purified as well as in crude digestive juice it is probable that deamidation process would occur under the alkaline conditions of the larval gut. Similar observations have also been reported for digestive amylase in *B. mori* (Kanakatsu, 1972; Abraham *et al.*, 1992), *Drosophila hydei* (Daone *et al.*, 1975), chicken (Lerner and Malacinski, 1975) and human (Karn and Rosenblum, 1975). However, the possibility of proteolytic cleavage of amylase during or after collection cannot be ruled out. Only further studies can reveal whether the amylases are products of different genes, gene duplications, or post-translational modifications.

Optimum activity between pH 9 and 9.5 of the amylase purified in the present study was similar to the reported optimum activity for amylases of many insect species including *B. mori* (pH 9.2) (Abraham *et al.*, 1992), *Spodoptera littoralis* (9.5) (Ishaaya *et al.*, 1971), *Mammestra brassicae* L (pH 9.5) (Kusano and Tanabe, 1986), *Erinnyis ello* (pH 9.8) (Santos *et al.*, 1986), *A. kuehniella* (Baker, 1989), in contrast to many coleopteran larval amylases which are known to be active in the neutral to slightly acidic pH range (Applebaum and Konijn, 1965; Podoler and Applebaum, 1971; Buonocore *et al.*, 1976).

Although all α-amylases possess the same catalytic function, their amino acid sequences vary (Janecek, 1992) which could explain the differences in their isoelectric point. The p*I* of amylases purified (see Results) in the present study differs from that of the major amylases of *B. mori* which is above 9.5 (Nagaraju and Abraham, unpublished results). It is interesting to note that the p*I* of band v was different from the rest of the amylase bands and similar to the amylase detected in *B. mori* (Abraham *et al.*, 1992). Hence, we would like to suggest that it may belong to evolutionarily conserved acidic amylases of silkworm. Further studies, however, on amylases in different silkworm species are necessary to support this argument.

K_m values of 0.113% which is an average value for the total amylase preparation against starch for *A. mylitta* amylase compares favourably with those reported for other insect species (Buonocore *et al.*, 1977; Terra *et al.*, 1977; Baker, 1991). This is an average value for all the isozymes and it is not known whether the affinity of each of the isozymes to starch varies.

α-Amylases of insects (Daone, 1969; Horie, 1971, 1972, Buonocore *et al.*, 1976; Terra *et al.*, 1977; Baker, 1983; Baker and Woo, 1985), as well as, mammalian and bacterial amylases (Robyt and Whelan, 1968) are reported to be activated in the presence of chloride ions. The only reported exception is amylase from *Callosobruchus chinensis* which is known to be inhibited by Cl^- (Podoler and Applebaum, 1971). The amylase purified from *A. mylitta* showed relatively higher activity in Tris–HCl buffer but dialysis of the enzyme against buffer without Cl^- did not contribute to any marked change in the activity suggesting that the tasar amylase activity is unaffected by chloride ions. These results are similar to the reports of Baker (1989) on *A. kuehniella* and Kusano and Tanabe (1986) on *M. brassicae* in which amylases were not affected by Cl^- ions.

The α-amylases are metalloenzymes that require calcium for maximum activity (Robyt and Whelan, 1968). Calcium affords stability to the amylases from both pH and temperature extremes. In *Tenebrio molitor* removal of Ca^{2+} by exhaustive dialysis against water causes irreversible inactivation of the enzyme (Buonocore *et al.*, 1976). Also, Ca^{2+} has been reported to activate and stabilize amylase activity in *C. chinensis* (Podoler and Applebaum, 1971). Activity of the purified digestive amylase from *B. mori* dialysed against the chelating agent EDTA declined by 55% when it was incubated at 50°C for 30 min while the non-dialysed amylase remained unaffected up to 60°C and started losing its activity only when it was held at 65°C (Kanakatsu, 1978). We also showed that Ca^{2+} stabilized our purified amylase against temperature extremes (Fig. 5). At 60°C the enzyme was found to be more stable than that of *B. mori* since only 50% activity was lost even after 60 min incubation. At above 75°C the enzyme lost its activity after 10 min even in the presence of Ca^{2+}.

References

Abraham E. G., Nagaraju J. and Datta R. K. (1992) Biochemical studies of amylases in the silkworm, *Bombyx mori* L.: comparative analysis in diapausing and nondiapausing strains. *Insect Biochem. molec. Biol.* **22,** 867–873.

Applebaum S. W. and Konijn A. M. (1965) The utilization of starch by larvae of the flour beetle, *Tribolium castaneum*. *J. Nutr.* **85,** 275–282.

Baker J. E. (1983) Properties of amylases from midguts of larvae of *Sitophilus granarius*. *Insect Biochem*, **13,** 421–428.

Baker J. E. (1987) Purification of isoamylases from the rice weevil *Sitophilus oryzae* (L.) (Coleoptera: Curculionidae), by high-performance liquid chromatography and their interaction with partially purified amylase inhibitors from wheat. *Insect Biochem.* **17,** 37–44.

Baker J. E. (1989) Interaction of partially purified amylases from larvae *Anagasta kuehniella* (Lepidoptera: Pyralidae) with amylase inhibitors from wheat. *Comp. Biochem. Physiol.* **93B,** 239–246.

Baker J. E. (1991) Purification and partial characterisation of amylase allozymes from the lesser grain borer, *Rhyzopertha dominica*. *Insect Biochem.* **21,** 303–311.

Baker J. E. and Woo S. M. (1985) Purification, partial charactacterisation and postembryonic levels of amylases from *Sitophilus oryzae* and *Sitophilus granarius*. *Arch. Insect Biochem. Physiol.* **2,** 415–428.

Bernfeld P. (1955) Amylases, α and β. In *Methods in Enzymology*, Vol. 1 (Edited by Colowick S. P. and Kaplan N. O.), pp. 149–158. Academic Press, New York.

Buonocore V., Poerio E., Silano V. and Tomasi M. (1976) Physical and catalytical properties of α-amylases from *Tenebrio molitor* L. larvae. *Biochem. J.* **153,** 621–625.

Chatterjee S. N. and Datta R. K. (1992) Hierarchical

clustering of 54 races and strains of the mulberry silkworm, *Bombyx mori* L: significance of biochemical parameters. *Theor. appl. Genet.* **85,** 394–402.

Daone W. W. (1969) Amylase variants in *Drosophila melanogaster*: linkage studies and characterization of enzyme extracts. *J. exp. Zool.* **171,** 321–342.

Daone W. W., Abraham I., Kolar M. M., Martenson R. E. and Deibler G. E. (1975) Purified *Drosophila* alpha-amylase isozyme. In *Isozyme IV* (Edited by Martet C. L.), pp. 585–607. Academic Press, New York.

Fisher E. H. and Stein E. A. (1960) α-Amylases. In *The Enzymes* (Edited by Boyer P. D., Lardy H. and Myrback K.), 2nd Edn, Vol. 4, pp. 313–343. Academic Press, New York.

Gabriel O. (1971) Analytical disc gel electrophoresis. In *Methods in Enzymology* (Edited by Joakoby W. B.), Vol. 22, pp. 565–578. Academic Press, New York.

Hirata Y. (1974) Relation between the amylase activity of the larval digestive juice and several quantitative characters in ae strains of silkworm, *Bombyx mori. J. Sericult. Sci. Jpn* **43,** 384–39.

Horie K. (1971) Physiological conditions in the midgut in relation to starch digestion and the salivary amylase of the bug *Lygus disponsi. J. Insect Physiol.* **17,** 1153–1167.

Horie K. (1972) Comparative study of a property of salivary amylase among various heteropterous insects. *Comp. Biochem. Physiol.* **42B,** 501–508.

Ishaaya I., Moore I. and Joseph D. (1971) Protease and amylase activity in the larvae of Egyptian cottonworm, *Spodoptera litoralis. J. Insect Physiol.* **17,** 945–953.

Janecek S. (1992) New conserved amino acid region of alpha amylases in the third loop of their (beta/alpha) 8-barrel domains. *Biochem. J.* **288,** 1069–1070.

Jolly M. S., Sen S. K. and Ahsan M. M. (1974) *Tasar Culture* Ambika, Bombay.

Jolly M. S., Sen S. K. Sonwalkar T. N. and Prasad G. K. (1979) *Nonmulberry Silks.* FAO Agricultural Series Bulletin No. 129.

Kanakatsu R. (1972) Purification and some properties of amylase in the digestive juice of silkworm larvae, *Bombyx mori. J. Sericult. Sci. Jpn.* **42,** 285–292.

Kanakatsu R. (1978) Studies on further properties for an alkaline amylase in the digestive juice of silkworm, *Bombyx mori. J. Fac. Text. Sci. Technol.* **76,** 1–21.

Karn R. C. and Rosenblum B. B. (1975) Genetic and posttranslational mechanism determining human amylase isozyme heterogeneity. In *Isozyme IV, Genetics and Evolution* (Edited by Markert C. L.), Vol. 4, pp. 745–776. Academic Press, New York.

Keller P. J., Kauffman D. L., Allan B. J. and Williams B. L. (1971) Further studies on the structural differences between the isozymes of human parotid α-amylase. *Biochemistry* **10,** 4867–4874.

Kusano T. and Tanabe S. (1986) Enzymatic properties of the midgut amylase activity and its changes during development in the cabbage armyworm, *Mammestra brassicae.* L. *Kontyu (Tokyo)* **54,** 12–24.

Lerner L. M. and Malacinski G. M. (1975) Genetics and structural studies of chicken α-amylase isozyme and their modified forms, and structural studies of HOG amylase. In *Isozymes, IV Genetics and Evolution* (Edited by Markert C. L.), pp. 727–743. Academic Press. New York.

Loyter A. and Schramm M. (1962) The glycogen-amylase complex as a means of obtaining highly purified α-amylases. *Biochim. biophys. Acta* **65,** 200–206.

Lowry O. H., Rosebrough N. J., Farr A. L. and Randall R. J. (1951) Protein measurement with the Folin phenol reagent. *J. biol. Chem.* **193,** 265–275.

Moon T. Y. and Seol G. Y. (1983) Varietal difference in amylase activity of larvae digestive fluid in the silkworm, *Bombyx mori. Seric. J. Korea* **24,** 73–80.

Podoler H. and Applebaum S. W. (1971) The alpha-amylase of beetle, *Callosobruchus chinensis. Biochem. J.* **121,** 321–325.

Robyt J. F and Whelan W. J. (1968) The alpha-amylase. In *Starch and its Derivatives* (Edited by Radley J. A.), pp. 430–476. Chapman & Hall, London.

Santos C. D., Riberio A. S. and Terra W. R. (1986) Differential centrifugation, calcium precipitation, ultrasonic disruption of midgut cells of *Erinnys ello* caterpillars. Purification of cell microvilli and inference concerning secretory mechanisms. *Can. J. Zool.* **64,** 490–500.

Sambrook J., Fritsch E. and Maniatis T.(1989) Molecular cloning—a laboratory manual. Cold Spring Harbor, Cold Spring Harbor Laboratory.

Spiro R. G. (1966) Analysis of sugars found in glycoproteins. In *Methods in Enzymology* (Edited by Neufeld E. F. and Ginsburg V.), Vol. 8, pp. 3–26. Academic Press, New York.

Takagi T., Toda H. and Isemura T. (1971) Bacterial and mold amylases. In *The Enzymes* (Edited bv Bover P. D.), 3rd Edn, Vol. 5, pp. 235–271. Academic Press, New York.

Terra W. R., Ferriera C. and De Bianchi A. G. (1977) Action pattern, kinetical properties electrophoretic studies on an alpha-amylase present in midgut homogenates from *Rhynchosciara americana* (Diptera) larvae. *Comp. Biochem. Physiol.* **56B,** 201– 209.