PARTIAL PURIFICATION AND PROPERTIES OF POTATO BETA-AMYLASE

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Abstract: Beta amylase has been partially purified from fresh potato homogenate, by acetate buffer extraction, dialysis, ammonium sulfate fractionation, chromatography on Sephadex G75 and on CM-cellulose. After dialysis it appeared that some inhibitor was removed, since total activity increased 2.2 folds. This preparation was found to be contaminated with maltase. Approximate molecular weight calculated by Sephadex G150 was 122,000. The optimum pH was 5.1-5.5 and the optimum temperature 55°C. Activation energy calculated from 26°-42° was 6,700 cal., and the Km 4.35×10^{-3} g/ml for modified soluble starch.

INTRODUCTION

In the previous communication, I described the purification of alpha-amylase from potato tuber by methods of glycogen precipitation. Here, I studied the amylase which remained after glycogen precipitation. This enzyme was classified as an exoamylase based on blue value determinations.

In this paper, purification procedures and some properties of this beta-amylase are described.

METERIAL AND METHODS

The mature potato tubers variety Kennebec were stored at 4°C prior to use. Substrate was modified soluble starch. It was prepared by reducing the end groups of commercial soluble starch with sodium borohydride (Strumeyer 1967).

The standard reaction mixture for beta-amylase contained 0.5 ml acetate buffer (0.2 M, pH 5.5) 1 ml 2% modified soluble starch and 0.5 ml enzyme extract. Incubations were carried out at 38°C for two hours. The reaction was linear with time. The reaction was stopped by addition of 1 ml Nelson reagent (Nelson, 1944), boiling for 20 minutes, then addition of 1 ml arsenomolybdate reagent. The sample was mixed thoroughly and diluted to 25 ml. The optical density of the stable blue color was determined at 525 mμ with a Zeiss spectrophotometer. A standard curve was prepared using maltose. One unit of amylase activity is defined as the amount of enzyme which will produce 1 μg of maltose from a 1% starch solution in one hour at pH 5.5 in sodium acetate buffer.

The protein was determined by Lowry's method (Lowry, 1951). A standard curve was prepared using fraction V bovine albumin protein.

EXPERIMENTAL RESULTS

(a) Purification procedure:

(1) Homogenate: Amylases in potato tubers were extracts with acetate buffer.

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They were prepared by grinding 500 g peeled potato tubers in a waring blender with 100 ml acetate buffer (0.05 M, pH 5.0) and 0.5 g sodium sulfite added to prevent polyphenol formation in the potato extract (Muneta 1966). The mixture was blended vigorouly for 30 seconds and the extract was filtered throug a buchner funnel. Approximately 400 ml of potato juice was obtained. This was centrifuged ten minutes at 17,300 g at 4°C to yield a clear supernatant. The procedure was repeated three times to yield 1000 ml of potato extract.

(2) Dialysis: The potato extract (1000 ml) was dialysed against running cold tap water for 24 hours. The small amount of precipitate formed during dialysis was removed by centrifugation.

(3) Ammonium sulfate precipitation: Concentrated sodium acetate buffer pH 4.5 was added until a final concentration of 0.05 M was obtainde. Dixon's monogram was used to determine the amount of ammonium sulfate to be added. 50–65% ammonium sulfate fractionation was collect. The effect of pH on ammonium sulfate precipitation of beta-amylase was shown in following Table 1.

pH	4.0	4.5	5.0	5.5	6.0
Specific Activity (unit/mg protein)	31.3	62.0	39.0	28.2	31.8

Ammonium sulfate concentration was 50–65%.

(4) Gel filtration column chromatography: A column 5×50 cm was packed with Sephadex G75 which was pre-equilibrated with acetate buffer (0.05 M, pH 5.0) according to the manufacture's instructions. About 50 ml of enzyme mixture from ammonium sulfate precipitation was carefully added on the top of the column. The sample was eluted with the same buffer used for equilibration at a flow rate of about 48 ml per hour. Fraction 13 to 18 were collected.

(5) CM-cellulose chromatography: Column bed dimension were 2.5×16 cm., CM-cellulose was washed in a Buchner funnel with 0.5 M NaOH, distilled water 0.5 N HCl and distilled water sequentially until the yellow color of cellulose disappeared. The CM-cellulose was equilibrated with starting buffer (0.05 M, pH 4.71 acetate buffer) about 8–10 times, then packed into the column. Before the sample was loaded on the column, starting buffer was run over night at 45 ml per hour. A 10 ml sample from the preceeding step was carefully added on the top of the column. The unabsorbed material was eluted with starting buffer about 6 hours at a flow rate of approximate 30 minute interval.

A linear gradient of NaCl ranging from 0.0 to 1.0 M in acetate buffer (pH 4.71, 0.05 M) was used to elute the protein. The gradient was obtaind by connecting two identical 750 ml flasks with a bridge. The mixing flask leading to the column contained buffer only, while the limiting flask contained 1.0 M NaCl in buffer. Effluent and fractionation patterns are shown in Fig. 1. Both pH range and buffer concentration must controlled within narrow limits to preserve enzyme activity. Acetate buffer at 0.005 M, 0.09 M, 0.1 M and pH 4.3, 4.5, 5.0, 5.5, respectively, and phosphate buffer of pH 5 to 7 all failed to give active fractions. Only 0.05 M acetate buffer and a pH range of 4.71 to 4.87 eluted enzymatically active fractions. Glutathione, oxidized form (10^{-4} M) in the elution buffer appeared to have a protecting function.

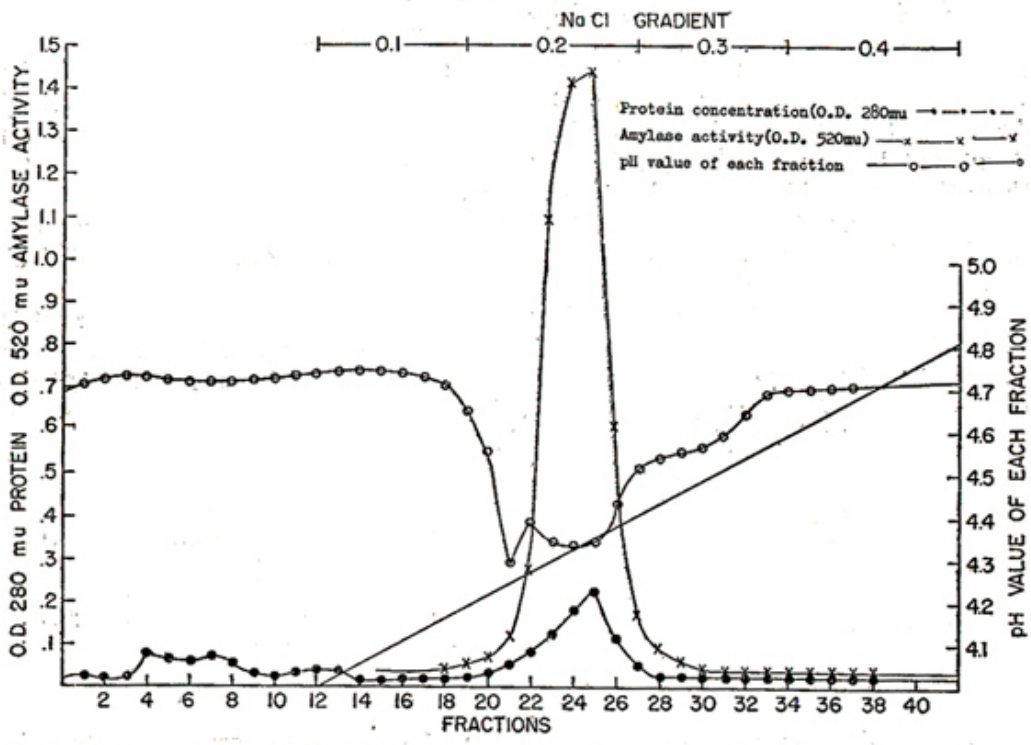

Fig. 1. Elution pattern of CM-cellulose column chromatography. The colnmn was 2.5×16 cm. Starting buffer was acetate (0.05 M, pH 5.0). A linear gradient of NaCl ranging from 0.0 to 1.0 M in acetate buffer was started at fraction 12.

(b) Properties of beta-amylase:

Every fraction purification was checked by disc electrophoresis in polyacrylamide gel. The gel was run at pH 9.3 in tris-glycine buffer with a current of approximately 4 ma/tube. The fractions with the highest degree of purity (CM-cellulose fraction 24–26) contained three protein bands. These fractions, however, gave only one peak in the analytical ultracentrifuge. The S_{20w} was calculated as 5.01. Fraction 24 to 26 were combined and used for all studies of enzyme properties.

The approximate molecular weight estimated from Ve/Vo ratio by gel filtration (G150 Sephadex) was 122,000 (Fig. 2). The optimum temperature was found to be 55°C (Fig. 3). The activation energy calculated from Arrhenius plot was 6,700 cal per mole as determined by the slope between 26°–49°C. A Lineweaver-Burk plot of starch concentration gave a Km value for the enzyme of 4.35×10^{-3} g of soluble starch. The optimum pH was 5.1–5.5. This enzyme appeared to be highly sensitive to acid conditions lower than pH 4.5 and basic conditions higher than pH 6.5 (Fig. 4).

The end products produced from soluble starch by the purified enzyme preparation were analyzed by paper chromatography (Robyt and French, 1963). The main product was maltose while glucose was a minor product. If the enzyme was incubated with maltose, a small amount of glucose was produced. This was taken as evidence that maltase was a contaminant in the enzyme preparation.

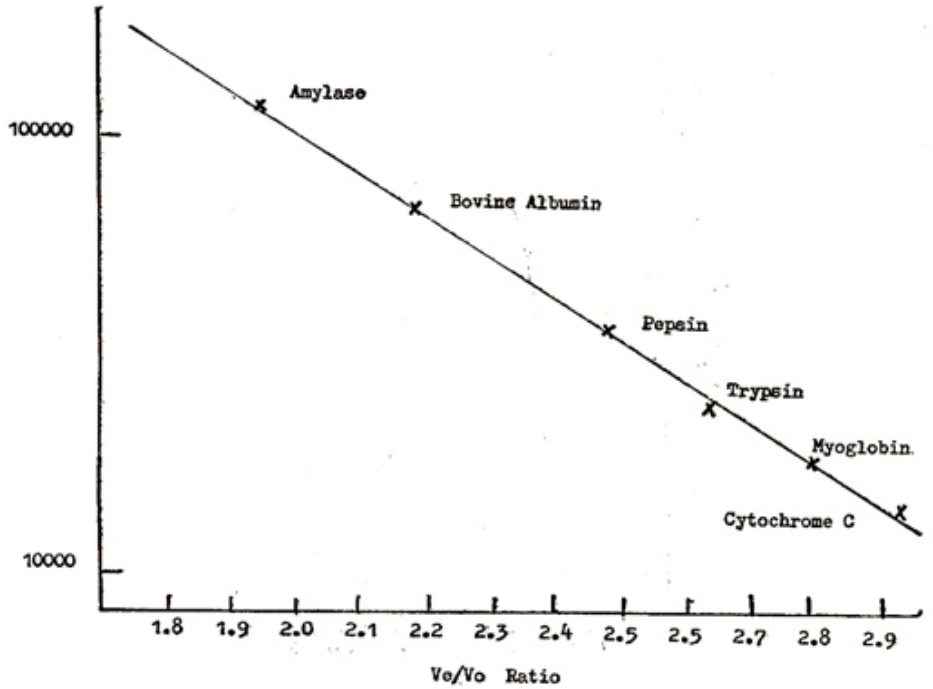

Fig. 2. Calibration curve for Sephadex G150 column and location of beta-amylase elution ratio. The amount applied was approximately 2 ml of 1% solution. The elution volume of blue dextran (molecular weight 2,000,000) was used as the void volume.

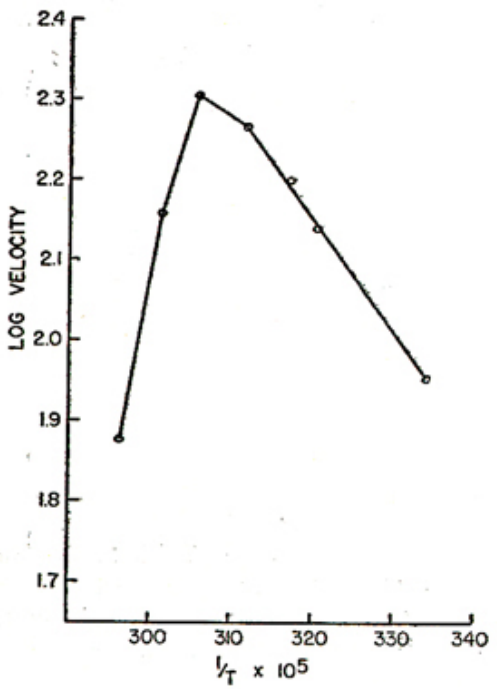

Fig. 3. Effect of temperature on beta-amylase activity. The slope between 26°-49°C was used to calculate the energy of activation according to the Arrhenius equation. Slope = $-E/2.303\,R$. The value obtained was 6,700 cal.

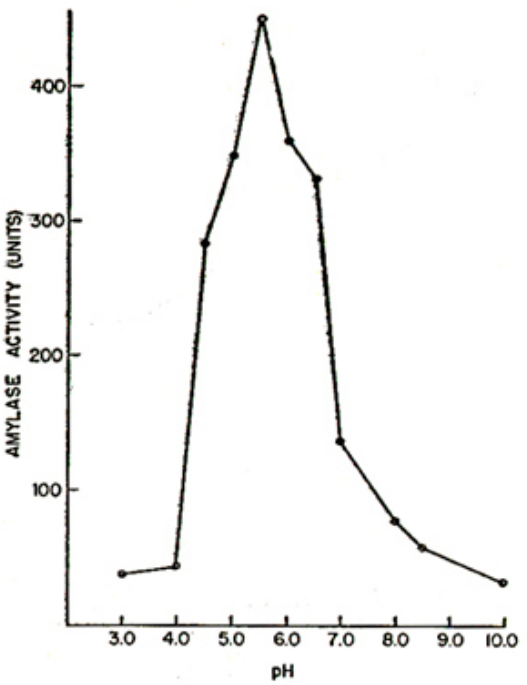

Fig. 4. Effect of pH on beta-amylase activity. pH 3.0-3.5: glycine-HCl buffer. pH 4.0-6.5: Acetate buffer. pH 7.0-8.5: Phosphate buffer. pH 9.0-10.0 glycine-NaOH buffer.

Table 2. The effect of various additions on beta-amylase activity

Supplements	Concentration	Inhibition %
Control	—	0
EDTA	6.8	0
EDTA and sodium lauryl sulfate	—	87.6
Sodium lauryl sulfate	1%	87.6
4-chloromercuribenzoic acid	2	97.5
Cleland reagent	2	0
Cleland reagent, sodium lauryl sulfate, EDTA	—	80.5
Thioglycerol	10.4	0
2-mercaptoethanol	0.58	0
Glutathione (reduced)	0.56	0
Glutathione (oxidized)	0.72	2.5

The effect of various additions on enzyme activity is shown in Table 2. Sodium lauryl sulfate and p-chloromercuribenzoic acid were strong inhibitors. EDTA and various SH reagents had little effect. A summary of the characteristic of potato amylase and a comparison of its properties with those of the same enzyme from other sources is presented in Table 3.

Table 3. Comparison of the properties of beta-amylase from potato and from other sources

Source	Enzyme Properties*			
	Functional SH groups	Optimum pH	Activation energy (25°-40°C) (Cal.)	Molecular wt.
Potato	+	5.1-5.5	6,700	122,000
Wheat	+	5.3	9,300	—
Malt	+	5.2	5,530	—
Sweet potato	+	4.0-5.0		152,000
Soybean	+	6.0		—

* Characteristics of beta-amylase from all sources other than potatoes were taken from French (1961).

DISCUSSION

Low levels of amylase activities were found in the crude extract of potato tubers. This may have been due in part to the presence of amylase inhibitors, since a 2.2 fold increase in the total activity was obtained after dialysis.

Beta-amylase was found to be extremely sensitive to conditions during CM-cellulose chromatography. A slight variation in pH and/or buffer concentration resulted in enzyme inactivation. This inactive enzyme had similar electrophoretic patterns to the active enzyme and on this basis, a minor moification is presumed, possibly a change in tertiary structure of the protein. These effects warrant further study.

The reaction products of the purified enzyme were consistently maltose and a readily detectabe amount of glucose. Glucose production could be attributed to contamination by phosphorylase, maltase or glucose transferase. Phosphorylase does not appear to be the contaimnant, since under conditions of pH and available inorganic phosphate which are highly unfavorable for phosphorylase activity, glucose is still produced. With maltose as the sole substrate, glucose is produced in detectable amounts, On this basis, it is assumed that the purified beta-amylase preparation contains a low level of maltase with physico-chemical propeties very similar to the beta-amylase.

The characteristics of bata-amylase from potato tubers were quite similar to those from other higher plants, (Table 4). PCMB strongly inhibited the enzyme, but cystine and oxidized glutathione had little effect. These results suggest the

Table 4. Summary of beta-amylase purification

Fraction	Total protein (mg)	Total activity (unit)	Specific activity (unit/mg)	Purification (fold)
Crude enzyme	11,600	38,000	3.28	1.0
Dialysis	6,600	85,000	12.88	3.9
Ammonium sulfate precipitation	920	53,000	57.61	17.6
Sephadex G75	280	29,000	103.57	31.6
CM-cellulose	33	18,200	551.52	168.1

specificity of the SH groups to SH reagents. The strongly inhibitory effect of sodium lauryl sulfate supports the contention that tertiary structure of the protein in critical to functioning of the enzyme.

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