PURIFICATION AND PROPERTIES OF POTATO ALPHA-AMYLASE

MAY LAY FAN

Abstract: Alpha-amylase was purified from fresh potato tuber homogenate by extraction at pH 5.0 with acetate buffer followed by glycogen precipitation and chromatography on Sephadex G75. The purified enzyme was homogeneous as judged by polyacrylamide gel electrophoresis. From gel filtration, the molecular weight was determined as 46,000. The optimum pH was 5.5-6.0 and optimum temperature was 42°C. Activation energy between 26°-42°C was 8,200 calories per mole and the Km was 5.2×10^{-3} g/ml on modified soluble starch.

INTRODUCTION

The starch-sugar interconversion in potato tubers during the storage and sprouting period is complex and detailed information in this area is lacking. An extensive study of the physiological and biochemical changes in potato tubers during the storage period was undertaken, a portion of which is presented here. The present paper describes the procedures for isolation and purification of potato alpha-amylase and some properties of purified enzyme.

MATERIALS AND METHODS

(a) Potato tubers: The mature potato tubers of variety Kennebec were maintained at 4°C in a well aerated storage condition.

(b) Substrate: Substrate was modified soluble starch prepared by reduction of the end groups of commercial soluble starch with sodium borohydride (Strumeyer, 1967).

(c) Enzyme assay: The standard reaction mixture for the alpha-amylase assay comprised 0.5 ml acetate buffer (0.2 M, pH 5.5), 1 ml 2% modified soluble starch and 0.5 ml enzyme extract. The mixture was incubated for 16 hours at 38°C. The rate of the reaction was linear with time. The reaction was stopped by addition of 1 ml Nelson reagent (Nelson, 1944). The mixture was boiled for 20 minutes, followed by addition of 1 ml arsenomolybdate reagent, mixed thoroughly, then diluted to 25 ml. The absorbance was determined at 525 mu 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.

(d) Protein determination: The method used was that described by Lowry (1951). A standard curve was prepared using fraction V bovine albumin.

(e) Vertical disc analytical electrophoresis: The electrophoresis was run at pH 9.3 in tris-glycine buffer with a current of approximately 4 ma per tube. The procedure was according to Davis (Davis, 1964) except that the sample and sucrose were not mixed with the upper gel. Protein bands were located by staining with amido black (1%) in 7% acetic acid.

(f) Paper chromatography of reaction products: The solvent was water: ethanol: nitromethane 23:44:35 (V/V). Separation was for three hours by ascending chromatography. Color was developed with the following three reagents to this order: (1) $AgNO_3$ in acetone (2) methanolic NaOH and (3) a mixture of sodium thio-sulfate, sodium sulfite and sodium bisulfite in water. Treatment with each solution was followed by washing in running water. (Trevelyan *et al.* 1950, Robyt and French, 1963).

EXPERIMENTS AND RESULTS

(a) Purification procedure:

1. Homogenate: Amylases in potato tubers were extracted with acetate buffer. The homogenates were prepared by grinding 500 g peeled potato tubers in Waring blender with 100 ml acetate buffer (0.05 M, pH 5.0) and 0.01 M calcium acetate. Sodium sulfite (0.5 g) was added to prevent polyphenol formation in the potato extract (Muneta, 1966). The mixture was blended vigourously for 30 seconds and the extract was filtered through 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 supernant.

2. Glycogen precipitation (Loyter and Schramm, 1964): Enzyme extract (100 ml) and 5 ml phosphate buffer, 0.2 M, pH 8.0 were mixed in a 250 ml fiask in an ice bath. Six ml of a 2% glycogen solution was added slowly with shaking, after which the mixture was centrifuged at 2500 g for 10 minutes. The solid material which separated was dissolved in 5 ml 0.05 M, pH 5.5 acetate buffer. The above procedures were repeated 4 times and 20 ml of enzyme mixture were collected. All procedures were carried out at 4°C.

3. Gel filtration: A column 2×34 cm was packed with Sephadex G75 which was pre-equilibrated with acetate buffer (0.05 M, pH 5.5) according to the manufacture's instructions. About 10 ml (about 13 mg of protein) of enzyme mixture from step 2 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. Fractions of approximately 12 ml were collected at room temperature. Fractions 6 and 7 had most of the enzyme activity and showed a 30 fold level of purification (Table 1). This value represented a minimum purification level, since the specific activity of the crude enzyme was based on total activity of both alpha- and beta- amylase. Enzyme extracts from various purification steps were checked by analytical disc electrophoresis on polyacrylamide gel. Fraction 6 from Sephadex G75 preparation showed a single band.

Table 1. Purification of alpha-amylase

Fraction	Total amylase activity (unit)	Total Protein (mg)	Specific Activity	Yield %	Purification (fold)
Crude enzyme	11,820	936	12.6	100	1
Glycogen ppt.	3,060	27	113	28	9.0
Sephadex G75	2,300	6.16	374	19.4	29.6

(b) Properties:

The purified enzyme was incubated with beta-amylase limit dextrin (derived from starch incubated with commercial beta-amylase at 38°C for 48 hours). The

blue color formed by addition of I_2-KI solution decreased gradually as observed visually. After 30 hours, only slight brown color was left. This observation was taken as evidence for the presence of alpha-amylase.

The ratio of the alpha-amylase elution volume to elution volume of blue dextran (Ve/Vo) as described by Andrews (1965) was used for determination of molecular weight. The molecular weight of alpha-amylase was estimated to be 46,000 (Fig. 1).

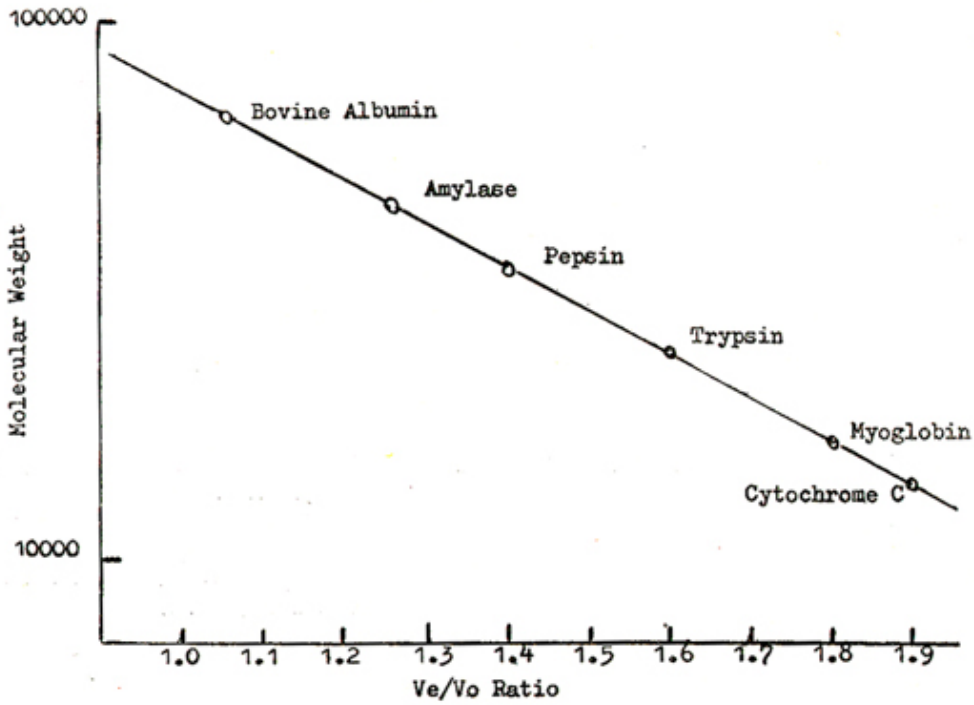

Fig. 1. Calibration curve for Sephadex G75 column and location of alpha-amylase elution ratio. The elution volume of blue dextran was used as the void volume. Column size was 2×34 cm and eluant was 0.05 M acetate buffer, pH 5.5.

The enzyme activity appeared to be sensitive to acid conditions lower than pH 5.0. There seemed to be no marked effects of pH in the range of 5.0 to 8.5: the optimum, however, appeared at pH 5.5 (Fig. 2).

The optimum temperature was found to be 42°C. (Fig. 3). According to the Arrhenius equation, the activation energy calculated from the slope between 26°–42°C was determined to be 8,200 cal. From the Lineweaver-Burk plot, the Km for soluble starch was 5.3×10^{-3} g/ml (Fig. 4).

Various chemicals which might serve as inhibitors or protective agents, were incubated with the enzyme and acetate buffer 0.05 M, pH 5.5 at 38°C for 40 minutes. The substrate was then added and incubated under standard conditions. The effect of a number of different reagents on the amylase activity is shown in Table 2.

Reaction products from soluble starch produced by the purified enzyme preparation were analyzed by paper chromatography. The main product was maltose. A summary of the characteristics of potato alpha-amylase and comparison of its properties with those of the same enzyme from other sources is presented in Table 3.

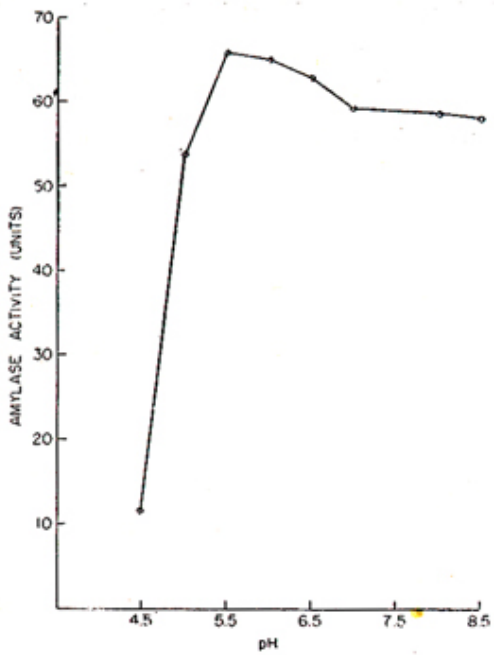

Fig. 2. Effect of pH on alpha-amylase activity. pH 3.0–pH 5.0: Acetate buffer. pH 6.0–pH 8.5: phosphate buffer.

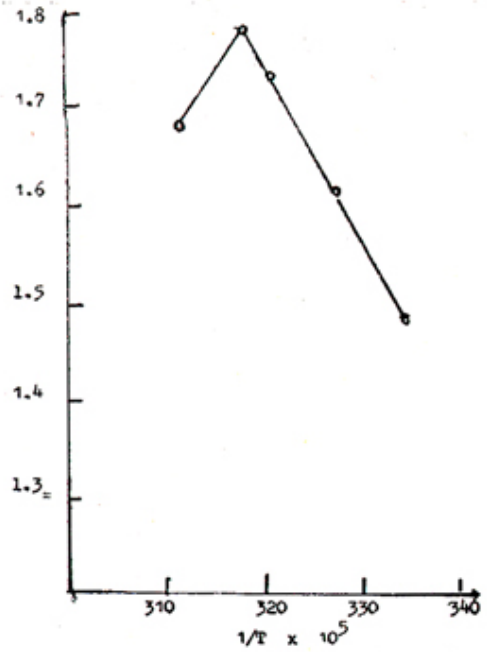

Fig. 3. Effect of temperature on alpha-amylase activity. The slope between 26°–42°C was used to calculate the energy of activation, according to Arrhenius equation slope =E/2.303 R. The value obtain was 8,200 cal.

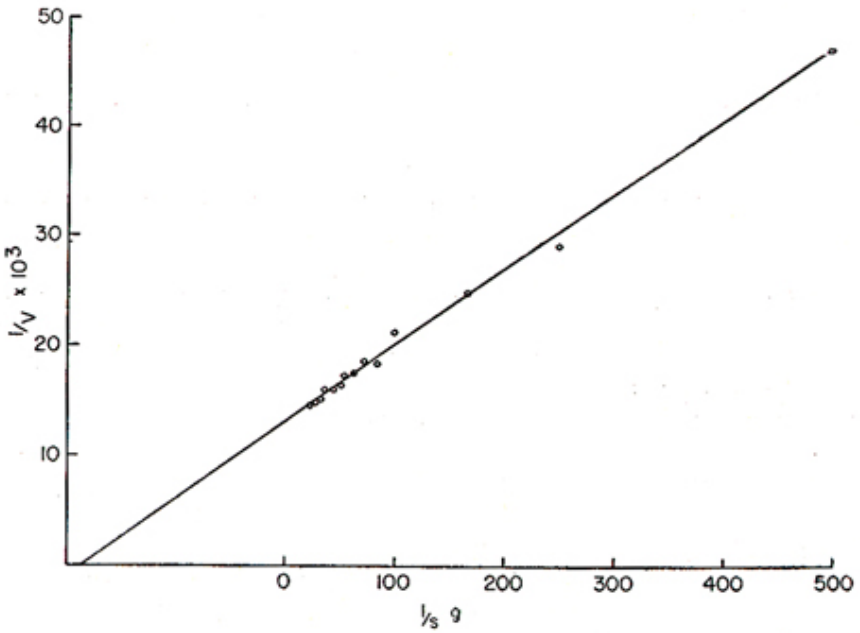

Fig. 4. Effect of substrate concentration on alpha-amylase activity. V was measured as μg of maltose produced per hour. S is expressed as g/ml of soluble starch.

Table 2. Effect of various reagents on alpha-amylase activity

Supplement	Concentration (mM)	Inhibition (%)
Control	—	0
Cleland reagent	2.0	0.75
EDTA	6.8	0.75
Glutathione (oxidized)	0.72	42.5
p-Chloromercuribenzoic acid	2.0	100

Table 3. Properties of alpha-amylases from various sources*

Source	Enzyme Characteristics				
	Optimum pH	Optimum Temp. (C°)	Activation energy (25°-40°)	Molecular weight	Km g/ml soluble starch
Potato	5.5—6.0	42°	8,200	46,000	5.45×10^{-3}
Malt	—	—	—	59,500	—
Human saliva	6.9	—	13,000	69,000	0.6×10^{-3}
B. subtilis	6.0	—	11,000	48,000	—
P. saccharophila	5.25—5.75	40	—	—	0.6×10^{-3}
Hog pancreas	—	—	8,500	45,000	0.6×10^{-3}
B. Macerans	—	—	—	—	3.3×10^{-3}

* Fischer, E. H., & E. A. Stein, 1961.

DISCUSSION

Potato tuber alpha-amylase can be readily purified in three relatively simple steps that can be completed in three days. The procedure involves extraction, glycogen precipitation and Sephadex gel filtration.

Glycogen precipitation appeared to be highly specific for the enzyme and may bear a similarity to the antigen-antibody reaction as proposed by Loyter and Schramm (1964). The subsequent gel filtration step was found to be more efficient for separating the enzyme from its contaminants than charcoal-celite chromatography which has been used by other researchers. The detection of a single band by acrylamide disc electrophoresis indicated that these procedures resulted in a preparation with a high level of purity.

Potato alpha-amylase appeared similar to amylases from other sources in a number of properties (Table 3). The Km value of 5.45×10^{-3} g/ml of soluble starch was in the high range of values reported for other amylases, 0.6×10^{-3} to 3.3×10^{-3} (Manning & Campbell, 1961: Depinto & Campbell, 1968).

Evidence was obtained that enzyme activity is dependent on the integrity of SH groups, since both oxidized glutathione and *p*-chloromercuribenzoic acid were strong inhibitors. Cleland reagent, however, had little or no effect on enzyme activity. EDTA did not produce any inhibition, suggesting that the calcium ions required for activity are probably tightly bound to the enzyme.

The appearnce of alpha-amylase with the onset of sprouting in potato tubers, suggests a functional role of starch degradation for seedling growth. The levels

of activity at early stages of sprouting, however, appear to be insufficient to meet physiological requirements and other amylolytic enzymes such as phosphorylase (Fan, 1970), may play a more dominant role in starch degradation.

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