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Research Article

Isolation, Purification and Characterisation of Alpha amylase From Phaseolus Aconitifolius

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Abstract:
Alpha amylase hydrolyses the starch. Alpha amylase (1,4-a-D-glucan glucanohydrolase, EC 3.2.1.1) was extracted from germinated Kidney beans, germinated for 3 days, with maximum activity 65.3 U/ml. After 3 days fungal growth was observed. The enzyme pH optima was 7.0. The optimum temperature for activity was found to be 55 ^{0}C. Mg2+ ions enhanced the activity of enzyme up to 101%. 1.5% substrate concentration of Starch solution was found to be optimum. Alpha amylase extracted from germinated Kidney beans followed the Michaelis- Menten equation.

Keywords: Alpha amylase, Kidney beans.

INTRODUCTION:

Enzymes were first divided into two classes "unorganised" and "organized" ferments. The action of "organized ferments" was thought to be due to 'vital' activity. This view was encouraged by the rediscovery of bacteria by Pasture and Koch[1]. In 1897 Eduard Buchner discovered that yeast extracts could ferment sugar to alcohol, proving that fermentation was promoted by molecules that continue to function removed from cells. Frederick Kuhne called these molecules "ENZYMES". Enzymes promote and control the conversion of complex carbohydrates, fats and proteins of our body into simple substance, which the intestines can absorb, and also the various reactions by which these simple substances are used in the body for building up new tissues or producing energy. Enzymes are not broken down or changed in the process. They are as potent at the end of the reaction as at the beginning and very small amount can affect the conversion of large quantity of material. They are really true catalyst. For example hydrolysis of protein takes at least a day by the action of strong acid at 100^{0}C. The same change takes place in a few hours in alimentary canal at 37 ^{0}C[4].

MATERIALS AND METHODS:

MATERIALS:

Kidney Bean was purchased from local market of Latur, Soluble starch was purchased from ACME Traders, India. Iodine, Hydrochloric acid,Sodium hydroxide, Sodium acetate,Potassium dihydrogen phosphate purchased from Research – Lab Fine Chem Industries, India,3,5-dinitrosalicylic acid (DNS) were obtained from Sigma Chemical Co. India.

Other chemicals like potassium chloride, glacial acetic acid, sodium chloride, Ca^{++}, Mg^{++} and EDTA was used are Analytical grade.

The seeds of kidney bean was soaked in distilled water for 2 to 4 days. The enzyme extract is then prepared by blending seeds in a mixer & the slurry was dissolved in 4 fold acetate buffer of pH 5 containing 0.1M NaCl and 1%PVP. It is then stirred for 3hrs using magnetic stirrer. The suspension is then centrifuged at 10,000rpm for 20 minutes at 4°C and the supernatant was collected for further assay[26.]

To quantify the enzyme extraction, enzymatic assay were made to determine Alpha amylase activities[11, 12].

Optimization of germination period

For optimizing the germination period of kidney bean for maximum activity of Alpha amylase, four batches of 50 gm of presoaked kidney bean was kept for germination in closed chamber at room temperature. After every day enzyme was extracted from one batch of germinated kidney bean and enzymatic assay was performed as mentioned earlier[11].

CONFIRMATORY TESTS FOR ALPHA AMYLASE[23]

1. Enzyme extract 1ml added into 5 ml 1% starch solution, heat for 10 minutes at 40^0C then add to it 1ml Iodine solution Color change occurs through shades confirmed that presence of Alpha amylase .

2. Prepare viscous solution of raw starch, add to it enzyme extract after some times viscosity decreases indicates that presence of Alpha amylase confirmed.

Preparation of the crude enzyme from germinated *Kidney bean.*

ASSAY OF ENZYME:-

In enzyme assay ,the rate of reaction can be known by measuring the amount of substrates (s) utilized or the amount of product (p) formed in unit time. Enzyme activity are usually expressed in terms of specific activity which is defined as the amount of substrates utilized or product formed per mg protein per minute at definite temperature.

Amylase activity was determined according to the method of Bernfeld (1955)[27]. The enzyme assay mixture contained 0.5 ml of enzyme extract, 0.5 ml of 0.05 M sodium phosphate buffer, pH 7.0 and 1.0 ml of 1% starch. The reaction mixture was incubated for 15 min at 55° followed by arresting the reaction by the addition of 1.0 ml of dinitrosalicilic acid reagent (DNS reagent). The optical density was read at 540 nm against reagent blank after addition of 3.0 ml of distilled water.

ENZYME UNITS

One unit of enzyme activity (DUN) is defined as the quantity of enzyme that causes 1% reduction of blue colour intensity of starch-iodine solution at 50°C in 1 min[13].

Protein Estimation

The protein content of the extract was determined following Lowry's method [28.]

Partial Purification of Alpha Amylase:

Ammonium Sulphate Precipitation

The crude enzyme extract was subjected to ammonium sulphate precipitation[29].

Solid ammonium sulphate was added slowly with constant stirring over magnetic stirrer at 4°C to obtain 80% saturation. The solution was allowed to stand for 1 hr at 4°C, and the mixture was then kept inside refrigerator at 4°C for 1hr. The precipitated protein (0–80%) was recovered from solution by centrifugation at 8,000 rpm for 30 min. The protein pellet dissolved in 20mM Phosphate buffer(pH 6) and it was then dialyzed against same buffer at 4°C.

Acetone Precipitation[30]

The crude enzyme extract was taken in a glass beaker and to it chilled acetone was added slowly, with continuous stirring, up to 70% (v/v) concentration and kept at 20°C for 4 h to allow protein precipitation. The precipitates were then harvested by centrifugation at 4°C and 10,000 rpm for 30 min. The pellet thus obtained was resuspended in 34 mL of 20 mM Phosphate buffer (pH 6) to allow the solubilization of proteins. The unsolubilized proteins were then removed by centrifugation at 4°C, 10,000 rpm for 30 min. Supernatant was then subjected to dialyzed overnight against same buffer at 4°C. The protein content and enzyme activity were determined.

CHARACTERIZATION OF PURIFIED ALPHA AMYLASE

Determination of optimum Substrate Concentration

For the determination of optimum substrate concentration for maximum enzymatic activity, E was measured at different concentrations of starch (0.1% to 4.0 %). Then assay was carried out as mentioned earlier, the enzyme assay mixture contained 0.5 ml of enzyme extract, 0.5 ml of 0.05 M sodium phosphate buffer, pH 7.0 and 1.0 ml of 1% starch. The reaction mixture was incubated for 15 min at 55° followed by arresting the reaction by the addition of 1.0 ml of dinitrosalicilic acid reagent (DNS reagent). The optical density was read at 540 nm against reagent blank after addition of 3.0 ml of distilled water.[11]

Table 1: Determination of optimum Substrate Concentration

Sr.No.	Substrate concentration	Activity (U/ml)*	%Relative Activity
1	0.1	3.0411	4.65
2	0.2	18.3	27.97
3	0.5	29.8	45.54
4	0.7	49.3	75.34
5	1	61.8	94.50
6	1.5	62.1	94.95
7	1.8	65.3	99.78
8	2	64.9	99.16
9	2.5	65.1	99.48
10	3	65.4	99.93
11	3.5	64.8	99.01

*** Average value of three determinations**

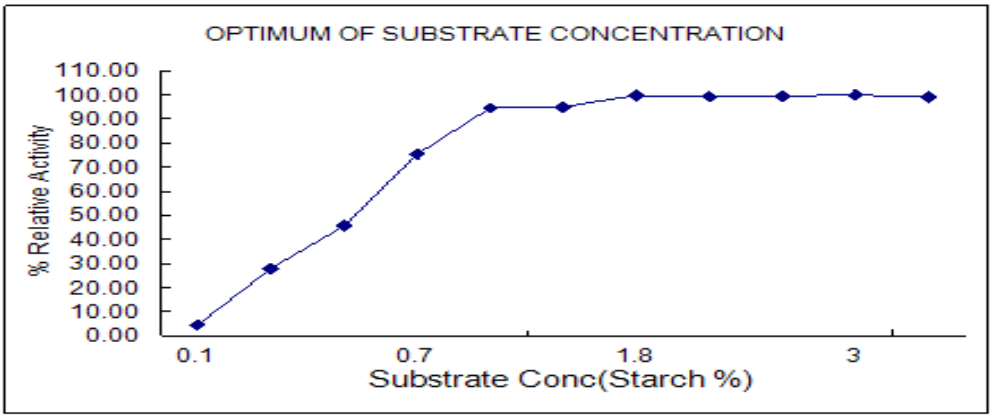

Figure 1 Determination of optimum Substrate Concentration

Determination of optimum pH

The effect of pH on activity of purified Alpha amylase, buffer solution from pH range of 3.5 to 10 were prepared by using Acetate buffer of pH 5.0; Phosphate buffer of pH 6.0; and alkaline borate buffer in the pH range of 8 to 10. For pH 4 and pH 7, buffer tablets were used; then, the catalytic activity of the enzymes in the above buffers was determined with starch as substrate then the assay was carried out for each buffer solution[11] with an incubation period of 10 min[11].

Table 2 Determination of Enzyme optimum pH

Sr.No	pH	Activity (U/ml)*	% Relative Activity
1	3.5	21.73	32.99
2	4	39.51	59.99
3	5	48	72.89
4	6	58	88.07
5	7	65.3	99.16
6	8	63.2	95.97
7	9	48.07	72.99
8	10	26.99	40.98

* **Average value of three determinations.**

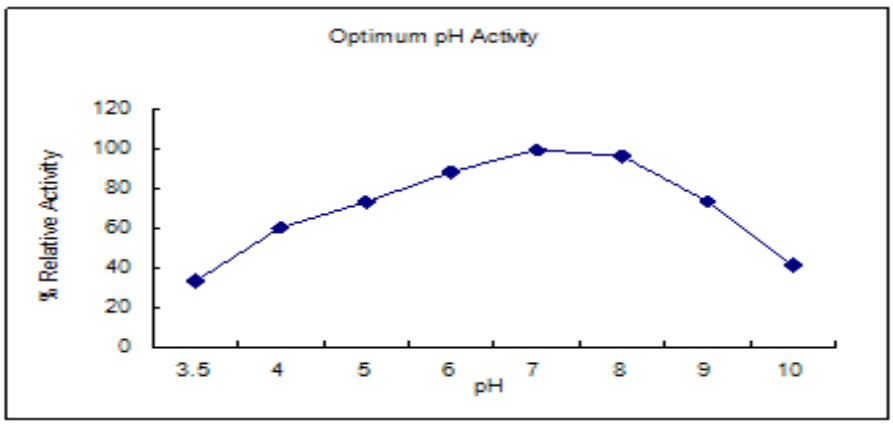

Figure 2 Determination of optimum pH

Determination of optimum temperature

For determination of optimum temperature for activity of the enzyme, The assay was carried out as per the procedure from the incubation temperature of 40°C to 90°C at pH 7.0 with an incubation period of 10 min[11].

Table 3 Determination of optimum Temperature

Sr.No	Temp. 0C	Activity (U/ml)*	% Relative Activity
1	40	31.2	48.83
2	45	42.8	66.99
3	50	57.2	89.52
4	55	65.2	102.04
5	60	62.1	97.19
6	65	55.3	86.55
7	70	48.2	75.44
8	75	36.2	56.66
9	80	26.3	41.16
10	85	22.1	34.59
11	90	12.3	19.25

* **Average value of three determinations.**

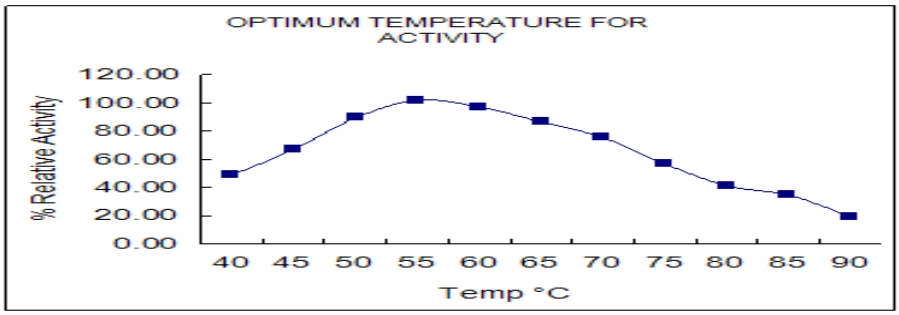

Figure 3 Determination of optimum Temperature

Effect of metal ions and other compounds

The effect of various metal ions like Mg^{2+}, Ca^{2+}, and EDTA on enzymatic activity carried out by adding various concentration from 1 to 10 mM. The volume taken was 1ml in reaction mixture described in the assay and the assay was carried out at °55C and optimum pH was taken 7.0

Table 4 Effect of metal ions and other compounds on Enzyme activity.

Sr.No	Concentration (mM)	% Relative activity		
		Mg++	Ca++	EDTA
1	1	78	86	64
2	5	101	93	48
3	10	78	113	17

* **Average value of three determinations.**

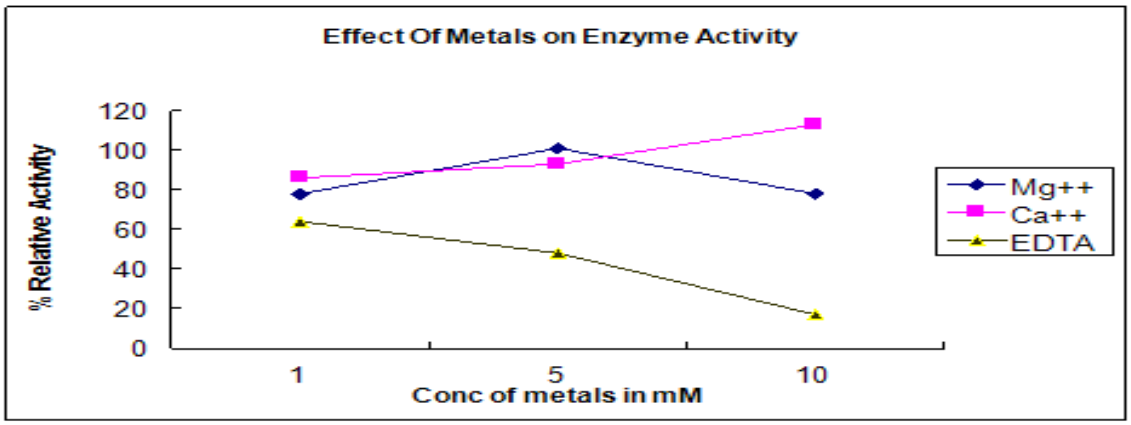

Figure 4 Effect of metal ions and other compounds on Enzyme activity.

RESULTS AND DISCUSSION

Confirmation of Alpha amylase

From the confirmatory tests presence of Alpha amylase is confirmed.

Optimization of Germination period for maximum activity

50 gm *kidney bean* presoaked for 3-4 hr and germinated at room temp (29°C) in closed chamber showed fungal growth after 4 days. Hence germination was not continued beyond 4 days. The enzymatic activity was assayed every day after partially purified enzyme isolated from germinated *kidney bean* in acetate buffer pH 5.0. The enzymatic activity was increased markedly with increasing period of germination. The crude extract of 3 days germinated *kidney bean* shows highest enzymatic activity of 65.3 U/ml as compare to 1st day & 2nd day germinated *kidney bean*.

Purification of α-amylases

The acidic crude enzyme extract was subjected to ammonium sulphate precipitation and by chilled acetone. Considerable loss of amylase activity was observed with acetone. On the other hand, ammonium sulphate precipitation resulted in good yield with an increase in fold purification. Hence, ammonium sulphate precipitation was selected for fractionation of amylases from the crude enzyme extract.

Characterization of partially purified alpha amylase enzymes

The optimum pH of purified amylase was 7.0 (Figure 2). The optimum pH for most of the purified α-amylases of animals ranged from 7-9. Among plant α-amylases, the result of the optimum pH for activity of Alpha amylase, it can be seen that maximum enzymatic activity in the pH range from 7.0 to 8.0 In this range more than 95% of the original activity was retained at given conditions.

Optimum Temperature

Table 3 and Figure 3 Illustrate the optimum temperature for Alpha amylase, as the temperature increased from 40^{0}C to 55°C enzyme activity increased progressively. Above 55°C enzyme activity decreased gradually upto 90°C. and at 120°C enzyme was totally denaturated. Thus the optimum temperature for activity was found to be 55°C

Effect of metal ions and other compounds

Table 4 and Figure 4 explain the effect of different metal ios on enzyme activity of

Alpha amylase. Of various divalent metal ions examined, 5mM Mg^{++} stimulated the enzyme activity upto 101%. 1mM and 10mM Mg^{++} stimulated the enzyme activity by 78% and 78% respectively. 10 mM Ca^{++} stimulated the enzyme activity upto 93%. 1mM and 5mM Ca^{++} stimulated the enzyme activity by 86% and 113% respectively. As per fig metal ion EDTA caused slight inhibition even at 1mM EDTA inhibited the enzyme activity drastically.

Optimization of substrate concentration

For the determination of optimum substrate concentration for Alpha amylase substrate, solution of soluble starch in the concentration range from 0.1% to 3.5% was used. Fig 1 and Table 1 Justifying that as the substrate concentration was increased above 0.1%, rate of reaction increased progressively upto 1.5%. When concentration increased above 1.5%, the rate of reaction remained constant suggesting that Alpha amylase obtained from germinated *kidney bean* followed the Michaelis Menten equation. Hence, optimum substrate concentration was found to be 1.5%, as in Graph justifying that, at this concentration enzyme remained saturated with substrate for 10 min and rate of reaction was maximum at this concentration.

CONCLUSION

Alpha amylase is the enzyme which hydrolyzes starch and hence have considerable commercial interest. In the present study Alpha amylase is extracted form germinated "Kidney beans".The maximum activity of Alpha amylase was found at the 3rd day of germination. The Alpha amylase extracted form Kidney beans having optimum activity at pH 7.0. & optimum temperature for activity was found to be 55^0C. In the effect of metal ions it was observed that enzymatic activity of Alpha amylase enhanced only by Mg^{2+} up to 101%, 1.5% substrate concentration (Starch solution) was found to be optimum, further increase in substrate concentration, the rate of reaction remains the same, indicates the Alpha amylase extracted from germinated Kidney beans following Michaelis-Menten equation .It can be concluded that Kidney bean is a good source of Alpha amylase and this enzyme could find promising application of hydrolysis of starch.

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