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Research Article

Aqueous Two Phase Purification of *Vigna radiata* Amylase and its Characterization

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ABSTRACT

Amylase is digested the starch from the non-reducing end and thus producing limit dextrins and maltose too which has industrial applications such as in food, pharmaceutical, paper, leather, detergent and textile industries. Amylase was extracted and purified from *Vigna radiata* (moong seeds) by a streamline method without the use of proteolytic and lipolytic enzymes. The study features of present method were partial purification of crude enzyme extract done by ammonium sulphate precipitation at low temperature followed by aqueous two phase extraction by potassium phosphate-bi-phospahte buffer system with polyethylene glycol to remove impurities. Enzyme activity of the extracted & purified *Vigna radiata* amylase was done by dinitrosalicylic acid and absorbance was taken at 570 nm.

Keywords: *Vigna radiata*; amylase; polyethylene glycol; aqueous two phase extraction

INTRODUCTION

Amylases are widely distributed in microbial, plant and animal kingdoms and involved in the hydrolysis of starch molecules[9]. Initially the term amylase was used originally to those enzymes which are capable of hydrolyzing α-1,4- glucosidic bonds of amylose, amylopectin, glycogen andtheir degradation products[1,3,7]. Amylases are classified into three viz., α–amylase, ß–amylase and γ–amylase. α-Amylase (alpha→1-4-glucan 4-glucanhydrolase, EC 3.2.1.1) is one of the key enzymes hydrolysing reserve starch in the endosperm of germinating cereals and synthesized *de novo* in the aleurone[16]. *Vigna radiata* is an exalbuminous legume, in which the cotyledonary cells are filled with starchgrains as the main energy storage component. It is an annual food legume belonging to the subgenus *Ceratotropis* in the genus *Vigna*. Thermostable beta-amylase and pullulanase, secreted by the thermophilic anaerobic bacterium Clostridium thermosulfurogenes strain SV2 were purified by salting out with ammonium sulphate, DEAE-cellulose column

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chromatography, and gel filtration using Sephadex G-200[12]. The partial purification of five types of alpha amylases was done during germination[15]. Alpha amylase was produced, purified and characterized from *Bacillus subtilis*[2]. The β-Amylase is calcium metallo-enzymes, many times completely unable to function in the absence of calcium. Amylase is instrumental in starch digestion in animals resulting in the formation of sugars, which are subsequently used in various

metabolic activities[5]. In our present work was involved the extraction of amylase from *Vigna radidata* (moong seeds) and its further partial purification was done by ammonium sulphate

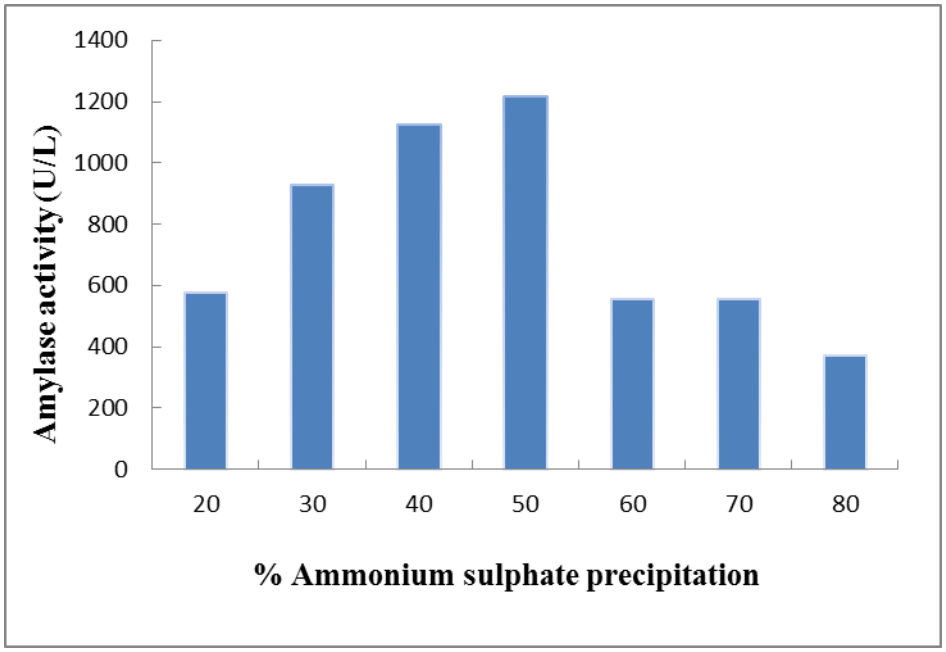

Fig 1: Comparative % of partial purification of *Vigna radiata* amylase by ammonium sulphate precipitation

precipitation followed by its purification by aqueous two phase extraction method as reported earlier for extracted alkaline phosphatase from plant sources[10].

MATERIALS AND METHODS

Extraction *Vigna radiata* Amylase: 8-10 grams of 3-days old seedlings from germinating seeds of *Vigna radiata* were homogenized using mortar and pestle in 4- 6 ml of 0.05M sodium phosphate buffer (pH 7.0). Enzyme extract was filtered through two layers of cloth and centrifuged for 15min at 4°C. The supernatant was collected which contained crude enzyme and stored at 4°C.

Amylase assay:Amylase was assayed according to the procedure followed by Bernfeld, 1955 and the activity of extracellular amylase was estimated by determining the amount of reducing sugars released from starch according to Miller (1959). 0.5 ml of the enzyme extracts were added to 1 ml of the starch solution and the mixture incubated at 37ºC for 20 min. After that 2 ml of dinitrosalicylic acid was added to terminate the reaction and the reaction mixture was boiled at 100ºC for 5 minutes. The amount of reducing sugars in the final mixture was determined spectrophotometrically at 570 nm. One unit of enzymatic activity is defined as the amount of enzyme that produces 1 µmol of maltose per minute [14, 4].

Ammonium sulphate precipitation in crude extract of *Vigna radiata***:** Crude enzyme extract of *Vigna radiata* was placed in ice bath and 20-80% ammonium sulphate (gm/ 100 ml of crude enzyme extract) was added in

small proportions (two pinches) till then the saturation point reached. Continuous stirring was done with each every small addition of ammonium sulphate salt. Medium was continuously stirred for 30 minutes after complete addition of ammonium sulphate or complete saturation. Then, the medium was kept at least for one hour for complete precipitation. After one hour, medium containing enzyme precipitate was centrifuged at 10,000 rpm for 30 minutes at 4ºC. Supernatant was discarded. Pellet was collected carefully with the help of pipette and dissolved in 2-5ml of 0.05M 5 ml of sodium acetate buffer having.

Aqueous two phase extraction of partially purified enzyme**:** 20ml of 30% of K2HPO4 (7 parts) and KH2PO4 (3 parts) solution was taken in test tube and add 2ml 5% Polyethylene glycol solution. Drop wise addition of

polyethylene glycol was done and vortexed after every addition. Now, 1ml of partially purified enzyme extract was added and vorexed again for 15-20 minutes[10].

Characterization: The crude and aqueous two phase purified enzyme was characterized for its various kinetic properties i.e. effect of time of incubation, pH, temperature, substrate concentration and $CaCl_2$ concentration.

Optimum incubation time: The effect of incubation time on the activity of the enzyme was estimated by performing the enzyme assay at different time (5min-25min) with an interval of 5 min and carrying out the enzyme activity by dinitrosalicylic acid method.

Optimum pH: The effect of pH on activity of the enzyme was determined by performing the enzyme assay at different pH using acetate buffer, phosphate buffer and carbonate buffer (pH rang of 2.5-10.5), the optimum pH of the enzyme was determined by incubating the enzyme with varying of buffer described above and then carried out the enzyme activity by dinitrosalicylic acid method.

Optimum temperature: Optimal temperature needed for enzyme activity was performed by incubating the reaction mixture at different temperature (20°C-80°C) by dinitrosalicylic acid method.

Optimum substrate concentration: Optimum substrate concentration was estimated by incubating the reaction mixture for 15 minutes at different concentrations of starch solution (0.25% - 1.75%) by dinitrosalicylic acid method.

Effect of $CaCl_{2:}$ The effect of CaCl2 on activity of the enzyme was estimated by performing the enzyme assay at different CaCl2 concentrations (2%-8%) by dinitrosalicylic acid method.

RESULTS

Partial purification and aqueous two phase purification: The crude amylase *Vigna radiata* amylase was purified at 50% of ammonium sulphate concentration. Then, partially purified *Vigna radiata* amylase was subjected to aqueous two phase extraction for further purification. It was purified and devoid of impurities at 30% of

potassium phosphate-biphosphate buffer system with polyethylene glycol solution. Hence, this purified enzyme was utmost pure and proceeded further for its characterization.

Optimum incubation time: The reaction mixture of aqueous two phase purified and crude amylase was incubated for varied time intervals from 5 to 25 minutes and optimum incubation time was 20 minutes (Table 1). Our present study was showed that incubation time of aqueous two phase purified enzyme was same as that of crude enzyme (20min).

Optimum pH: The pH of the reaction mixture of aqueous two phase purified and crude enzyme was varied from 2.5 to 10.5 and optimum pH 6.5 was obtained indicating that optimum pH was higher than that of crude enzyme (Table 1).

Optimum temperature: Optimum temperature of aqueous two phase purified and crude enzyme was determined by various temperatures from 20°C to 80°C. The enzyme was found to show maximum activity at 40°C and thermal stability at 50°C (Table 1) which was similar to crude enzyme (40°C).

Optimum substrate Concentration: The starch concentration was varied from 0.25 to 1.75 and there was no change in substrate concentration on aqueous two phase purified and crude enzyme activity (Table 1)

Effect of $CaCl_{2:}$ The reaction mixture of aqueous two phase purified and crude enzyme was incubated for varied $CaCl_2$ concentration. The 8-10% of $CaCl_2$ concentration was found optimum for aqueous two phase purified enzyme which was higher than that of crude enzyme (4%) (Table 1).

DISCUSSION

Optimum incubation time: Optimum incubation time of aqueous two phase purified enzyme is same as that of crude enzyme (20min) as well as to comparable to earlier reports[15, 11].

Optimum pH: The obtained optimum pH of aqueous two phase purified enzyme was 6.5 which considerably higher than that to crude enzyme as well as comparable to previous reports (Saleh, A. *et al*, 2009 and [2]Rani, K., 2012) as well as comparable to pH 6.0 [2].

Optimum temperature: The aqueous two phase purified and crude enzyme had maximum activity at 40°C and thermal stability at around 50°e and it was pretty comparable to previous reports as well [15, 2, 11].

Optimum Substrate Concentration:There was no change in optimum substrate concentration after the purification for aqueous two phase purified and crude enzyme (1%).

Effect of $CaCl_2$: There was not too much change in $CaCl_2$ concentration which was of 8-10%. This optimum $CaCl_2$ concentration was pretty similar to that of the crude enzyme (6%) as well as earlier reports too which confirmed that calcium ions acted as activating factor for increased activity of enzyme [15, 2]. Beyond 6%, the activity of aqueous two phase purified enzymes was decreased due to deleterious effect of calcium ions[13].

CONCLUSION

Thermostable amylase is one of the most important and widely used enzymes whose spectrum of application has widened in food, paper and detergent industries[5, 8]. The activity of the purified amylase was increased by 3 folds as compared to crude enzyme. The conventional methods of purification like chromatography, dialysis are used to purify the industrial important enzymes but these methods are highly expensive and sophisticated as well. But, aqueous two phase systems was consisted of salt phase-polymer system which were enough efficient to remove the organic and inorganic impurities[10] and can be used in starch processing, starch liquefaction, fermentation, starch saccharification, cleaning, laundrying, textile desizing, baking, and biofilm removal. This method of purification was affordable too and did not require costly equipments too. Hence, we can take into consideration the large scale production and purification of commercially important enzymes.

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