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Effect of Organic Solvents and Effectors on Radish Alpha Amylase

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Abstract: In recent years, organic solvents have attracted large avenue of interest to explore the potential of enzyme to produce highly specific active chemical molecule of industrial advantage. The present study discerns the role of organic solvents as a reaction medium for enzymatic catalysis. The stability of alpha amylase in organic medium promises its major application in starch processing food industries and pharmaceutics. The enzyme appeared to be stable and retained considerable activity in the presence of various aprotic organic solvents such as acetone, acetonitrile and DMSO. The anomalous behavior of acetone showed concentration dependent increase in activity with 1.9 times enhancement at 50 % (v/v) concentration. Various polar organic solvents such as methanol, ethanol, propanol and ethylene glycol showed activation of enzyme except for n-butanol. Non-polar solvents also activated the enzymatic activity. The n-hexane showed about 1.2 folds activation at 30 % (v/v) concentration. Further, Cr^{3+}, Al^{3+}, was found to be activator while $Cd^{2+}$and Ba^{2+}also could not show any significant inhibitory effect. The sulfhydryl-modifying reagents such as NEM showed activation whereas partial inhibition occurred in the presence of IAA and pHMB. In addition, PMSF showed moderate activity of enzyme while urea appeared to be strong inhibitor of enzyme. However, the characteristic properties of stable alpha amylase in non-aqueous medium have potential utility in non-aqueous enzymology for various biotechnological processes.

Key words: Alpha amylase, Organic solvents, Sulfhydryl group, Denaturing agent.

Abbreviations

DNS: 3, 5- Dinitrosalicylic acid.
DMSO: Dimethyl sulfoxide.
IAA: Iodoacetic acid.
p-HMB: p-hyroxymercuribenzoic acid.
NEM: N-ethylmaleimide.
PMSF: Phenylmethylsulfonyl fluoride.

Introduction

The alpha amylases are starch hydrolyzing enzyme which holds major applications in various starch processing industries, for liquefaction, pharmaceutics as digestive aid and for various analytical purposes such as detergent additive, including biotechnological treatment of waste, desizing agent in textiles, paper and pulp processing etc.,[1]. The alpha amylases are industrial important enzyme accounting 30 % of world's enzyme production[2]. However, enzymes being biological catalyst are highly unstable to withstand the harsh reaction conditions such as temperature, pH, pressure and ionic strength of the solvent[3]. Hence, from the industrial point of view, it is of crucial importance to develop highly stable enzyme with desired properties, offering diverse range of biotechnological applications.

In last few decades, the enzymes possessing enhanced catalytic activity and stability in non-aqueous reaction conditions has attracted tremendous interest[4]. Further, the non-aqueous media as a reaction medium provides various advantages; altering the enantio- and regio selectivity

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as well as thermodynamic equilibrium of the enzyme. Hence, favors the solubility of hydrophobic substrate in the reaction medium, minimizing the use of aqueous phase and prevents the bacterial contamination [5-7]. Nevertheless, the stabilization of enzyme structure and its activity in organic solvent requires certain tools such as either isolating intrinsically stable enzyme from extremophiles or manipulating the enzyme at genetic level, protein engineering, enzyme immobilization, physical and chemical alteration of enzyme, as well as reverse micelles entrapment and addition of additives etc [8]. Perhaps, the naturally occurring enzyme that remains stable in the presence of organic solvent without requirement of any unique stabilization technique would be highly efficient for various biotechnological applications [9]. Therefore, the search for organic solvent tolerant enzyme has been the major domain of research. Recently, the organic solvent tolerant alpha amylase have been reported from halophiles such as *Nesterenkonia* sp. and *Thalassobacillus* sp. LY18 [4,10]. However, very few studies relevant to organic solvent stable alpha amylase are reported from plants.

Apart from this, the earlier reports have already deciphered the role of sulfhydryl group of cysteine residue for the catalysis in amylase independent of their origin [11]. The thiol group of cysteine residue sometimes acts as a ligand for metal ions [12]. The amylases are susceptible towards sulfhydryl group reagents and exhibit various chemical modification. The chemically modified form of the cysteine residue possesses its own specific chemical and biochemical properties. This includes its structural stability, catalytic activity, metal binding, redox-activity, nucleophilicity, acidity etc.[13].

Rather than this, metal ions and chemical additives such as urea (denaturing agent) and PMSF (serine protease inhibitor) alter the stability and catalytic activity of alpha amylase. Herein, the present study reports the organic solvent stable alpha amylase from radish seeds. Additionally, the study also includes the effect of various metal ions and effectors such as sulfhydryl-modifying reagents and chemical additives on alpha amylase activity.

Material and methods

Chemicals and plant material

Radish seeds were procured from the local market. Sodium alginate, soluble starch, maltose and 3,5-Dinitrosalicylic acid, PMSF (phenylmethylsulfonyl fluoride), IAA (iodoacetic acid), p-HMB (p-hyroxymercuribenzoic acid), NEM (N-ethylmaleimide) were purchased from Sigma Chemicals Co. (St. Louis, MO, USA). All other chemicals such as metal salts ($Cr_2(SO_4)_3$, $AlCl_3$, Cd $(CH_3COO)_2$, $BaCl_2$), urea, β-mercaptoethanol, and various organic solvents were of analytical grade either from Loba chemie or E. Merck, India.

Enzyme

Alpha amylase was isolated from radish (*Raphanus sativus*) seeds procured from the local market, and purified to apparent homogeneity as described by Prakash and Jaiswal [14].

Enzyme assay

The α-amylase activity assay was done using 3,5-Dinitrosalicylic acid as described by Bernfeld using starch as a substrate [15]. The reaction mixture containing 2 % starch and suitably diluted enzyme solution was incubated at 27°C for 3 min. The reaction was stopped by adding 3,5-Dinitrosalicylic acid solution followed by heating the reactants in a boiling water bath for 5 min and then cooling down to room temperature. Further, the reaction mixture was diluted by double distilled water and the amount of reducing sugar (maltose) released was determined spectrophotometrically at 540 nm. One unit of enzyme was defined as the amount of enzyme required to produce one μmol of maltose per minute from the soluble starch at 27°C and pH 6.5 under the specified conditions.

Effect of organic solvent

The various concentrations (10-50 % v/v) of organic solvents such as aprotic (acetone, acetonitrile and dimethyl sulfoxide); polar (methanol, ethanol, propanol, butanol and ethylene glycol) and non-polar (benzene, hexane & cyclohexane) were added to the enzyme sample and the activity was quantified after incubating for 30 min at room tem-

perature under standard assay condition using starch as the substrate. The enzyme activity in the absence of organic solvents was taken as 100% and residual activity was determined accordingly in each case.

Effect of metal ions

The effect of various metal ions such as (Al^{3+}, Cr^{3+}, Ba^{2+} and Cd^{2+}) of various concentrations (1-10 mM) were added to the suitably diluted enzyme and incubated for 30 min at room temperature. The enzyme activity was determined under standard assay condition using starch as substrate. The enzyme activity in the absence of metal ions was considered as 100% and residual activity for each case were calculated accordingly.

Effect of effectors

The effect of various effectors such as sulfhydryl modifying reagents (NEM, pHMB and IAA), denaturing agent urea and PMSF a protease inhibitor in the concentrations range of 1-10 mM were determined. The various effector were added to suitably diluted enzyme and incubated for 30 minutes at room temperature. The enzymatic activity was estimated under standard assay condition using starch as substrate. The enzyme activity in the absence of effectors was considered 100 % and residual activity for each effector was calculated accordingly.

Statistical analysis

All the experiments were performed in triplicates, the mean and standard deviation were estimated accordingly.

Results

Effect of organic solvents

The effect of various organic solvents on α-amylase activity were explored as shown in Fig. 1a-c. The enzyme appeared to be active in the presence of various aprotic organic solvents. The concentration dependent increase in enzymatic activity was observed in the presence of acetone. The higher concentration of acetone (50 % v/v) showed about 1.9 times enhancement in activity. However, radish amylase appeared to be stable in the presence of acetonitrile and dimethyl sulfoxide (DMSO) which showed moderate activity. Acetonitrile (30 % v/v) showed higher activity and thereafter decline in activity was observed. Further, DMSO showed partial inhibition retaining 83% of enzyme activity at higher concentration of 50 % v/v (Fig. 1a).

The enzyme was activated in the presence of varying concentration (10-50 % v/v) of various polar organic solvents such as methanol, ethanol, propanol and ethylene glycol except for n-butanol

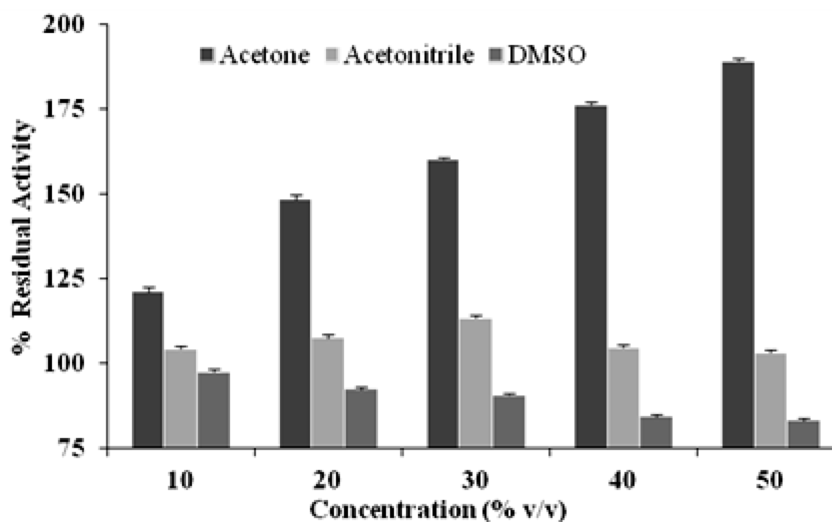

Figure 1a. Effect of various aprotic organic solvents on alpha amylase activity. Percent residual activity in each case represents enzyme activity relative to control (without any organic solvents) was taken as hundred percent. The values are presented as the mean ± SD of triplicate tests

(Fig. 1b). The enzymatic activity at higher concentration of 50 % (v/v) of methanol, ethanol, propanol and ethylene glycol was 128 %, 135 %, 126 % and 111 % respectively. Moreover, n-butanol showed partial loss in enzymatic activity retaining 82 % of activity. The effect of various non-polar solvents such as hexane, cyclohexane and benzene were also investigated which showed activating effect (Fig. 1c). The n-hexane showed 121 % activation at 30% (v/v) concentration and thereafter there was decline in activity. Cyclohexane showed gradual increase in enzymatic activity in concentration dependent manner with 1.2 fold increase at higher concentration of 50 % (v/v).

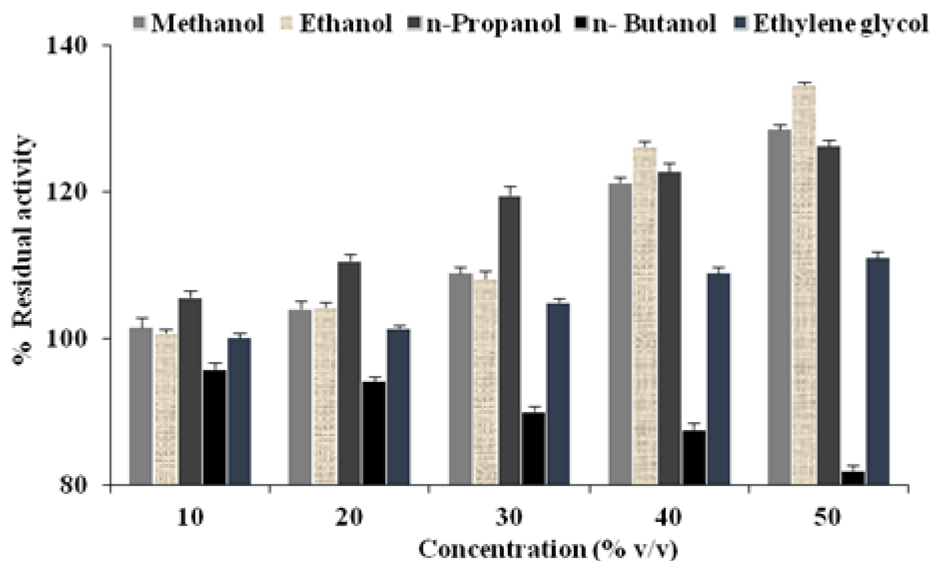

Figure 1b. Effect of various polar organic solvents on alpha amylase activity. Percent residual activity in each case represents enzyme activity relative to control (without any organic solvent) which was taken as hundred percent. The values are presented as the mean ± SD of triplicate tests

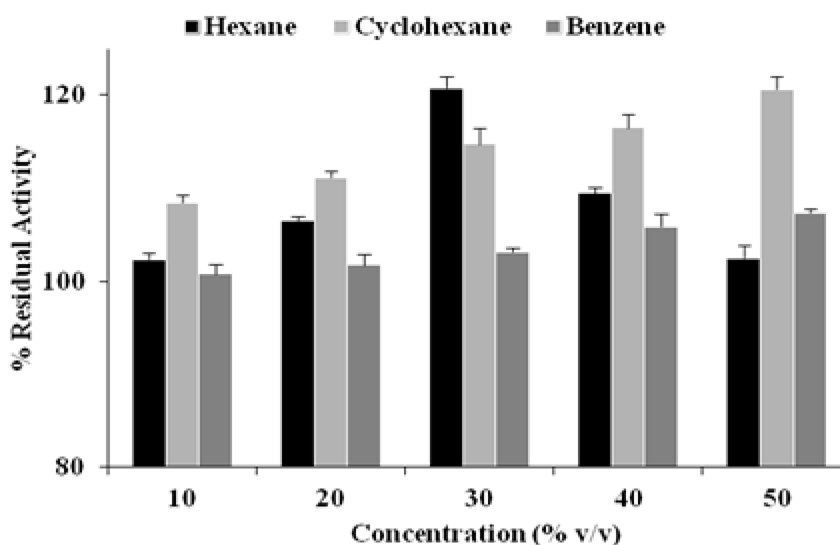

Figure 1c. Effect of various non-polar organic solvents on alpha amylase activity. Percent residual activity in each case represents enzyme activity relative to control (without any organic solvent) which was taken as hundred percent. The values are presented as the mean ± SD of triplicate tests

Effect of metal ions

The various metal ions such as (Al^{3+}, Cr^{3+}, Ba^{2+} and Cd^{2+}) was tested for activation / inhibition effect on amylase activity as shown in Fig. 2. Al^{3+} and Cr^{3+} ion showed slight activating effect on amylase activity with increased metal salt concentration (1-10 mM). The concentration of Cd^{2+} ion upto 5 mM showed enhanced enzymatic activity and thereafter decline in activity was observed. However, Ba^{2+} showed activation in enzymatic activity at lower concentration (2.5 mM) and further increasing concentration showed slight inhibitory effect.

Effect of effectors

The role of various effectors such as -SH modifying group (NEM, pHMB and IAA) and chemical additives (urea and PMSF) on amylase activity was investigated in the concentration range (1 - 10 mM) as shown in Table 1. The sulfhydryl modifying chemicals such as NEM (10 mM) showed approximately two folds increase in enzyme activity whereas pHMB and IAA (10 mM) showed 65 % and 53 % of activity respectively. PMSF and urea showed inhibitory effect, retaining about 65 % and 30 % enzymatic activity respectively.

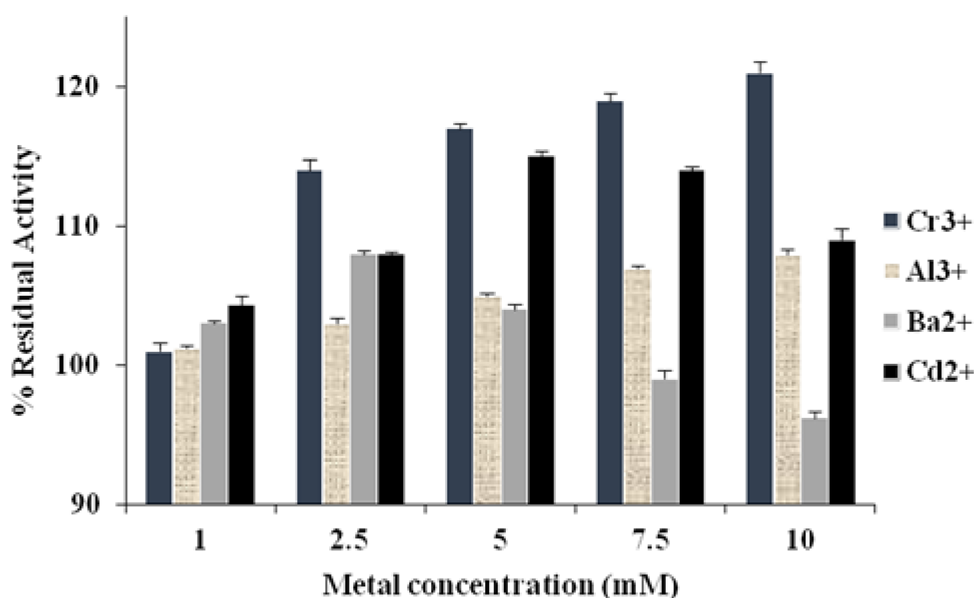

Figure 2. Effect of various metal ions on alpha amylase activity. Percent residual activity in each case represents enzyme activity relative to control (without any metal ions) which was taken as hundred percent. The values are presented as the mean ± SD of triplicate tests

Table 1: Effect of various effectors on alpha amylase activity. Percent residual activity in each case represents the enzymatic activity relative to control (without any effectors) which was taken as hundred percent. The values are represented as the mean ± SD of the triplicate tests

	% Residual activity at various concentrations			
Effectors	**1 (mM)**	**2 (mM)**	**5 (mM)**	**10 (mM)**
NEM	124.6±1.20	144.7±0.92	176.46±1.40	196.4±1.45
pHMB	93.2±0.95	81.0±0.725	70.6±1.4	65.6±1.2
IAA	88.5±0.82	81.6±0.778	56.0±1.36	53.3±1.17
PMSF	89.3±0.804	87.2±0.748	75.6±0.993	65.6±0.993
Urea	78.1±0.837	72.0±1.69	55.3±0.804	30.5±1.55

Discussion

The organic solvents have emerged as a novel reaction medium and have attracted immense interest for transformation of various enzymatic reactions. The enzyme appeared to be stable in the presence of various aprotic solvents (Fig. 1a). The acetonitrile and dimethyl sulfoxide (DMSO) could not show any significant effect. The suppressed enzymatic activity in the presence of acetonitrile and dimethyl sulfoxide (DMSO) at higher concentration is due to alteration in enzyme active site. Zhu, *et al.*, reported the alteration in active site of lipase by acetonitrile, leading to change in enzyme structure, which results in loss in enzymatic activity [16]. The result produced that DMSO acts differently when exposed to protein surface. It may be due to presence of sufficient hydration shell around the protein surface helps in maintaining the protein structure as well as the enzyme activity. Worth mentioning, bulky methyl group of DMSO strip water molecules from the surface of the protein and competes for hydrogen bonding. This leads to structural transformation in enzyme molecule causing denaturation and complete loss in enzymatic activity has been mentioned [17-18]. Apart from this, the anomalous behavior of acetone showed concentration dependent increase in amylase activity. The aprotic solvents lacks hydrophilic group. Hence, the carbonyl group of acetone molecules facilitates hydrogen bonding when exposed to protein molecules and leads to increased activity.

The enzyme was activated in the presence of various polar organic solvents such as methanol, ethanol, propanol and ethylene glycol (Fig. 1b) except for n-butanol. The increased hydroxyl group leads to hydrophilicity of the polar organic solvent. This facilitates the interaction between the solvent and enzyme molecules, which is attributes to increased enzymatic activity. However, the higher alcohols such as n-butanol have higher hydrophobicity and solvation capacity. Hence, higher alcohols are strong denaturant causing inactivation even at 10-30 % (v/v) of concentration [8]. Jaiswal and Prakash also reported organic solvent stable α-amylase from soybean except for n-butanol that showed inhibitory effect [19].

The enzyme showed higher activity with the increased concentration of non-polar solvents except for n-hexane (Fig. 1c). In general, the enzyme exhibited higher activity in hydrophobic (non-polar) solvents in contrast to hydrophilic solvents [7,20]. However, in the presence of hexane amylase activity followed a bell-shaped mechanism. The hydration shell and the hydrophobic group of organic solvent play a remarkable role in enzymatic activity [7]. The enzyme unfolds when water content is too high and exhibited inefficient catalytic activity. At 30 % (v/v) of hexane, there was optimum water content so enzyme showed maximum activity and thereafter it declined. Moreover, with the increased hydrophobicity of the solvent, there was inadequate water in the reaction medium. This leads to rigidity of enzyme causing decline in enzymatic activity.

The effect of various metal ions such as (Al^{3+}, Cr^{3+}, Ba^{2+} and Cd^{2+}) on radish alpha amylase activity was determined as shown in Fig. 2. Al^{3+}, Cr^{3+} showed activating effect while Ba^{2+} (2.5 mM) and Cd^{2+} (5 mM) showed slight activation and further decline in activity with increasing concentration was observed. Prakash and Jaiswal also observed slight activationg effect in the presence of Al^{3+} and Cd^{2+} [14]. The slight activation of amylase activity by Ba^{2+} might be due to replacement of Ca^{2+}. However, the effect of Ca^{2+} compared to Ba^{2+} is entirely different [21].

The role of various effectors on radish amylase activity was determined as shown in Table 1. The NEM activated the enzyme in the concentration dependent manner while pHMB and IAA showed reduced enzymatic activity with increasing concentration upto 10 mM. The result reported here is quite different from other plant amylases, which inhibited at lower concentration of sulfhydryl modifying reagents [22]. The sulfhydryl group reagents inhibits the amylase activity signifies the role of cysteine molecules in catalysis is noteworthy. Beside, the sulfhydryl modifying reagents should have completely inhibited the enzymatic activity, if cysteine residues were directly involved in catalysis. This signifies that sulfhyryl group reagent binds to the non-catalytic site other than the cysteine residue, which leads to change in the orientation of catalytic amino acids as proposed for soybean amylase [23].

The enzyme showed inhibitory effect in the presence of urea and PMSF (1 -10 mM). The PMSF a protease inhibitor showed reduced amylase activity with increasing concentration and retained about 65 % of activity at concentration of 10 mM of PMSF. The partial inactivation of enzyme by PMSF showed indirect involvement of serine residue in catalysis. In contrast, Zhang *et al*., also observed inhibition of amylase activity from *Anoxybacillus* sp. in the presence of PMSF [24]. The enzyme retained only 21 % (5 mM) of residual activity suggesting crucial role of serine residue. However, urea a chemical denaturant of protein appeared to be a strong inhibitor of amylase. The enzyme retained about 30 % of activity at 10 mM concentration of urea. Moreover, the amylase from *Amphibacillus* sp. retained 21.5% of activity (4 M) and complete inhibition (8M) in the presence of urea [25]. Buhan *et al.*, reported inactivation of α-amylase ANT-6 having 14 % of activity but at higher concentration of urea (8 M) [26].

Conclusion

The present study reports organic solvent stable alpha amylase facilitating considerable utility of enzyme in non- aqueous environment. This can also have significance for organic synthesis as well as to study the chiral resolution of enzyme. The enzyme was found to stable towards various metal ions. The sulfhydryl modifying reagents showed no marked inhibition of enzymatic activity and remained stable showed significantly indirect role of sulfhydryl group in enzyme catalysis. Consequently, the enzyme being stable in organic solvents, sulfhydryl modifying reagents, metal ions appears to holds its immense applications in biotechnological industries.

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