Recent Research in Science and Technology 2010, 2(3): 64-70
ISSN: 2076-5061
www.recent-science.com

MICROBIOLOGY

CHARACTERIZATION OF RAW STARCH DIGESTING AND ADSORBING EXTRA CELLULAR ISOAMYLASE FROM *RHIZOPUS ORYZAE*

Barnita Ghosh and Rina Rani Ray*

Microbiology Laboratory, Post Graduate Department of Zoology, Molecular Biology and Genetics, Presidency College, 86/1, College Street, Kolkata 700 073, India

Abstract

The partially purified extra cellular isoamylase produced by *Rhizopus oryzae* PR7 MTCC 9642, was characterized for various parameters. It showed highest affinity towards oyster glycogen followed by starch and amylopectin. The temperature and pH optima were found to be at 55° C and 5 respectively. The enzyme was found to be stable at 55°C for 10 minutes and at a broad pH range of 4-8. Increase in stability in presence of thiol additives and deactivation in presence of thiol inhibitors indicated the existence of thiol groups at the active site of the enzyme. The enzyme could digest raw native starches collected from various wastes of which rice extract and bread dust showed the highest extent of saccharification. Glucose and maltose were the major end products of starch bioconversion by the isoamylase. The isoamylase was found to be adsorbed onto various raw starch molecules, the rate of adsorption and desorption was best onto corn starch molecules.

Keywords: adsorption; isoamylase; *Rhizopus oryzae;* saccharification

Introduction

Amylases are one of the most important industrial enzymes of which isoamylase (E.C.3.2.1.68, glycogen-6-glucanohydrolase) hydrolyses 1, 6- α-D-glycosidic linkages of glycogen, amylopectin and α and β limit dextrins, producing linear malto oligosaccharides (Fang *et al,* 1994). This enzyme is used primarily in the production of food ingredients from starch, like glucose syrup, maltose, maltitol, trehalose, cyclodextrin and resistant starch, for elucidation of fine structures of polysaccharides and related α –glucans (Gunja–smith *et al*, 1970; Akai *et al*, 1971, Fujita *et al,* 2003). It can also be used as effective additives in dishwashing and laundry detergents (Ara *et al*, 1993).

Isoamylase is known to be produced by fewer types of micro organisms in comparison to that of other amylases. Known microorganisms capable of producing isoamylases include mainly *Pseudomonas* (Harada *et al* 1972; Fang *et al*, 1994), *Bacillus* (Ara *et al*, 1993; Castro *et al*, 1992), Cytophaga (Gunja–Smith *et al*, 1970; Gunja–Smith, 1974) and yeast (Gunja *et al,* 1961; Sakano *et al,*1969; Spencer Martins, 1982).

According to Bisgard-Frantzen and Svendsen, 2008, these conventional isoamylases had several disadvantages in industrial use as isoamylase of yeast origin had poor heat stability, isoamylase of *Cytophaga* origin showed insufficient heat stability and acid resistance and isoamylase of *Pseudomonas* origin, although did not have the problem of heat stability and acid resistance, but involved disadvantages in productivity, i.e., used to require a long cultivation time and low activity attained and thus, conventional isoamylases were unsatisfactory for use on an industrial scale. Therefore, from industrial point of view, extensive research is needed to find out an isoamylase producing micro organism with high productivity, higher temperature and pH stability and ability to digest raw starches of native or non expensive origin.

The present study deals with the characterization of a raw starch adsorbing and digesting extra cellular isoamylase synthesised by *Rhizopus oryzae* MTCC 9642,

Materials and Methods

Organism

Rhizopus oryzae PR7 MTCC 9642, a saccharifying isoamylase producing strain (Ghosh and Ray, 2010), isolated from the decaying vegetation enriched soil of India was used for further characterization of the enzyme synthesized. All experiments were done in triplicate and their values were averaged.

Chemicals

All chemicals used were of analytical grade. The native starches were collected from domestic effluents and kitchen wastes, pulverized to fine dust and used for further studies.

* Corresponding Author, *Email*: rina_ray64@yahoo.co.in

Cultivation of the strain

The strain was cultivated in 100 ml Erlenmeyer flasks each containing 20 ml Basal Medium (BM) composed of (gl^{-1}): peptone 0.9; $(NH_4)_2HPO_4$ 0.4; KCl 0.1; $MgSO_4.H_2O$ 0.1 and starch 0.25 (pH 8) at 28°C for 72 hrs in static condition.

Enzyme extraction and enzyme assay

The culture broth was centrifuged at 10,000 rpm for 5 min and the supernatant was used as the crude enzyme. The supernatant was subjected to fractionated ammonium sulfate precipitation for partial purification of the enzyme. Ammonium sulfate (60-80% w/v) was gradually added to the supernatant followed by centrifugation (10,000 rpm for 10mins at 4°C). The pellet was resuspended in 50 mM phosphate buffer (pH 5) and the suspension was dialyzed against the same buffer for desalting (Aygan *et al,* 2008).

To measure the activity of isoamylase, the assay mixture (1ml) containing an equal volume of properly diluted enzyme and 1%(w/v) glycogen in 50mM phosphate buffer (pH-5.0) was incubated at 55°C for 5 min. The reducing sugar released was measured by DNS method (Bernfeld,1955). One unit of isoamylase activity was expressed as the amount of enzyme releasing 1 µmol of reducing sugar per ml per minute using glucose as standard.

Characterization of the enzyme

To detect the optimum pH, the partially purified isoamylase (0.5ml) was incubated with equal amount of 1% (w/v) glycogen with buffers presenting various pH values (4.0 to 9.0), at 55°C and the stability of the enzyme at different pH values was studied by keeping the partially purified isoamylase (0.5ml) with 50mM of acetate buffer (for pH 4-6), phosphate buffer (for pH 5-8), Tris glycine buffer (for pH 8-9) kept at 33°C for 120 minutes followed by estimation of the residual activity. To study the effect of temperature, the isoamylase (0.5ml) was incubated with substrate, at various temperatures (25°C to 75°C) at a constant pH. The thermo-inactivation kinetics of the enzyme could be studied by exposing the enzyme at various temperatures (25°C to 75°C) for 10 minutes followed by an estimation of the residual activity of the enzyme. The preference of the partially purified isoamylase for different substrate was tested by incubating the enzyme with various substrates (starch, amylopectin, glycogen, and dextrin) of various concentrations at fixed temperature and pH. The effect of metal ions and thiol compounds was measured by incubating the enzyme at 33°C for 30 minutes with the additives at a concentration of 10mM followed by the assay of enzyme activity in usual procedure.

Analysis of iodine staining value (ISV) and reducing value (RV, % glucose)

The iodine staining value and reducing value of the enzyme hydrolysate were determined by incubating the reaction mixture containing 1% (w/v) starch or amylopectin solution and properly diluted enzyme at 55° C followed by withdrawal of samples (0.5 ml) at regular intervals. The aliquots were mixed with 0.5 ml of iodine solution (0.01N I_2 and 0.1N KI) and diluted with 2 ml of deionized water followed by the measurement of absorbance at 540nm (Fang et al, 1994). The corresponding reducing power of the aliquotes were measured following DNSA method of Bernfeld (1955).

End product analysis

The end products of hydrolysis of various substrates by isoamylase were analysed by TLC on a pre coated TLC plate (Merck) using a solvent system of butanol: acetic acid: water (3:3:1v/v), developing it with 0.1% methanolic orcinol in 10% H_2SO_4 followed by heating the plate at 110°C (Murashima *et al,* 2003) .

Adsorption and resorption of the enzyme

To calculate the adsorption capacity of the enzyme, 0.1 gm of raw starch was suspended in 1 ml of suitably diluted crude isoamylase solution (pH-5) for 120 minutes at 4°C preceded by a thorough mixing. After centrifugation at 6000 rpm for 5 minutes, the residual activity (A) was measured and the adsorption percentage was determined by the formula: [{(original isoamylase activity, O)-(residual isoamylase activity, A)}/(original isoamylase activity, O)] x 100 (Fang et al, 1994). The enzyme bound starch was washed twice with phosphate buffer (pH 5) and suspended in elutant solution (1ml) containing cold gelatinized starch (1% w/v) at 4°C for 120 minutes with occasional shaking. After centrifugation at 6000 rpm for 5 minutes, the isoamylase activity was measured (D) and the desorption percentage was calculated by a simple mathematical expression as: D / (O- A) (Ray *et al,* 1994).

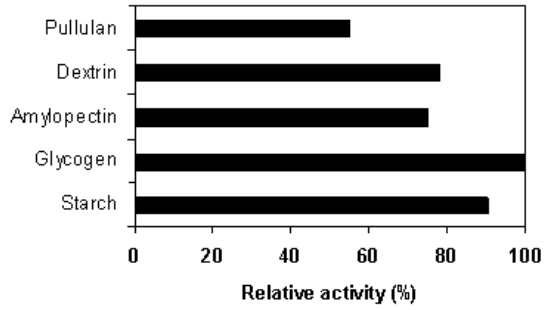
Results and Discussion

Affinity of the enzyme towards various substrates

The enzyme showed highest activity towards oyster glycogen, followed by starch, dextrin and amylopectin (Fig. 1), a result similar to the isoamylases of *Pseudomonads (*Amemura *et al*, 1980, Kato *et al*, 1977, Kitagawa *et al*, 1975,

Yokobayshi *et al*, 1970) and rice endosperm (Fujita *et al*, 2003). But it showed less affinity towards pullulan, a characteristic feature of isoamylase.

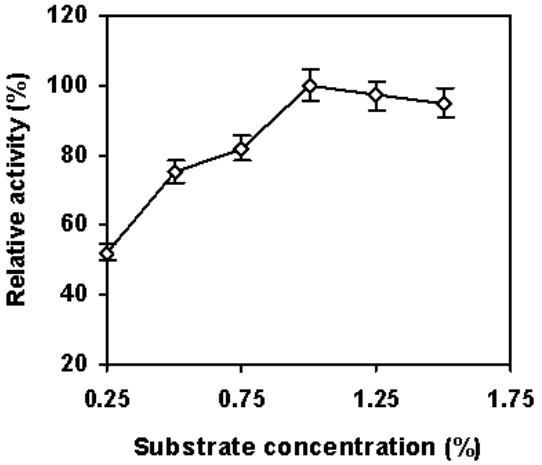
Fig 1. Effect of various substrates on the activity of isoamylase from *R. oryzae*.



Effect of substrate concentration

The enzyme showed highest hydrolytic activity in presence of 1% (w/v) oyster glycogen. Further increase in glycogen concentration could not bring about any remarkable increase in enzyme activity (Fig. 2), which might be due to enzyme limitation (Ghosh and Ray, 2010).

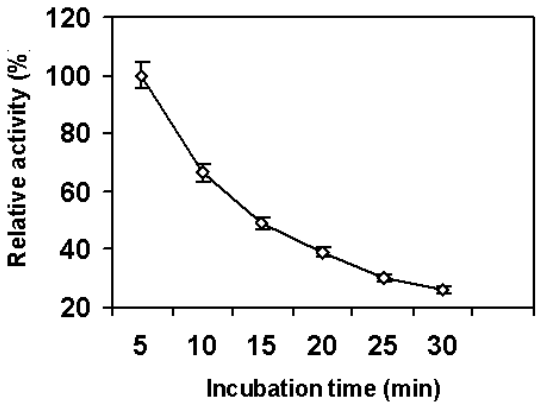
Fig 2. Effect of substrate concentration on the activity of isoamylase from *R. oryzae*



Effect of incubation time

Maximum activity of the enzyme was achieved within 5 minutes of incubation, above which no further increase in activity was detected. (Fig. 3).

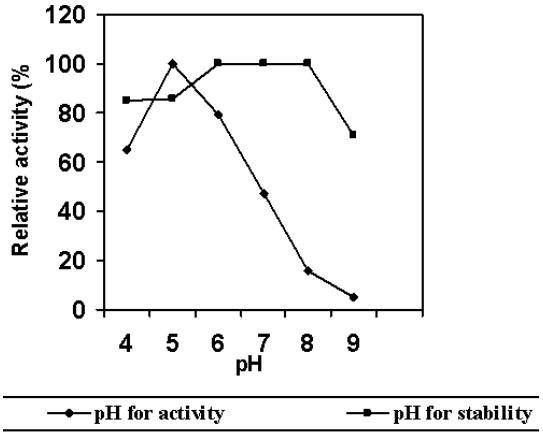
Fig 3 Effect of incubation time on the activity of isoamylase from *R. oryzae*.



Effect of pH

The pH profile study indicated that the optimum pH for enzyme activity was 5.0, similar to the optimum pH of some bacterial isoamylase (Castro *et al* 1992, Fang *et al,* 2005) but higher than that from *Pseudomonas amylodermamosa* (Olempska-Beer, 2007) and *Hendersonula toruloidea* (Odibo *et al*, 1992). About 80% activity of the enzyme was restored at a pH range of 4-8 (Fig. 4). This broad range of pH tolerance would increase the applicability of the enzyme at industrial level.

Fig 4. Effect of pH on the activity and stability of isoamylase from *R. oryzae*.

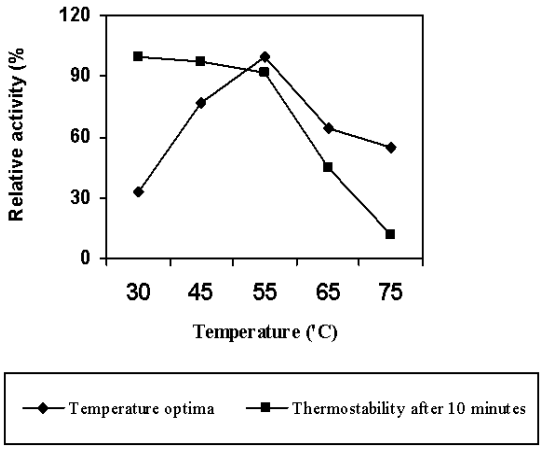


Effect of temperature

Maximum activity was found at 55°C (Fig. 5), slightly higher temperature optima reported from other microbial isoamylases (Ueda and Nanri, 1967; Fang *et al*, 1994, Ara *et al*, 1993, Spancer-Martins, 1982, Olemposka-Beer, 2007), but lower than that of *Sulfobolobus solfataricus* (Fang *et al*, 2005). The thermo inactivation kinetics of the enzyme indicated that the enzyme was almost 100% stable at 55°C even after10 minutes of exposure and about 45%

activity was retained at 65°C for 10 minutes and was found to be more thermo stable than the isoamylases reported from *Xanthomonas maltophilia (*Yamada *et al*, 1994) and from *Bacillus* sp (Ara *et al*, 1993) .

Fig.5. Effect of temperature on activity and stability of Isoamylase from *R. oryzae*



Effect of time on debranching of amylopectin

In the initial phase of the amylopectin degradation kinetics (Fig 6), increase in the iodine staining capacity (Blue value) indicated the mode of action of isoamylase, the amylase with a totally different mode of action from that of α and β amylases. The increased blue value could be explained by debranching of amylopecin by isoamylase (Yokobayashi, 1988). Maximum substrate utilization took place within 10-20 minutes of reaction, as indicated by the liberation of highest blue value and maximum amount of sugar released.

Effect of metal ions and additives

Although the activity of isoamylase from *R.oryzae* was not increased in presence of any metal ion (Table 1), about 50% of activity was reduced in presence of $Cu^{2+,}$ a thiol inhibiting ion, but it was remarkably enhanced in presence of thiols like dithiothreitol (DTT), reduced glutathione (GSH), and Cysteine HCl. This result indicated the presence of thiol group at the active site of the enzyme. This was confirmed by the deactivation of enzyme in presence of p chloro mercuribenzoate (pCMB) a potent thiol inhibitor. This observation was similar to that from *Pseudomonas* (Yokobayashi *et al,* 1970) *and Xanthomonas maltophilia* (Yamada *et al*, 1994, but was contrary to that reported from *Hendersonula toruloidea* (Odibo *et al,*1992).

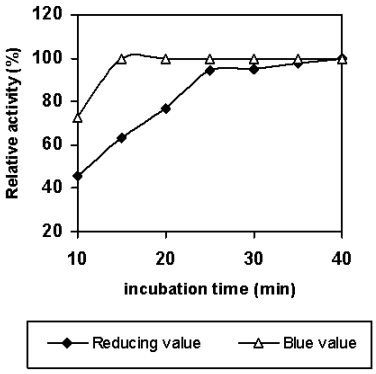


Fig.6. Effect of incubation time on blue value and sugar production kinetics of isoamylase from *R. oryzae*

Table 1. Effect of metal ions and additives on the activity of isoamylase of *R. oryzae*

Additives (10mM)	Relative activity (%)
None	100
Na^{+}	85
K^{+}	94
Ca^{2+}	94
Mg^{2+}	86
Mn^{2+}	78
Cu^{2+}	51
Ba^{2+}	82
Sn^{2+}	82
Cysteine HCl	125
GSH	134
pCMB	36
DTT	157

100% activity: 1800U/ml

Digestion of various raw starches

The enzyme was found to digest various waste starches (Table 2) collected from kitchen effluents. The enzyme showed highest potency in bioconversion of starch from rice extract followed by bread dust. This saccharifying ability of the enzyme would definitely increase its applicability in industries related to sugar production.

Table.2. Ability of the enzyme to digest raw starchy wastes.

Starch used (5mg/ml)	Sugar produced (mg/ml)			
	15min	30min	45min	60min
Rice powder	2.3	2.4	2.6	2.6
Rice extract	2.9	3.2	3.8	3.9
Bread dust	2.8	3.1	3.1	3.4
Wheat dust	1.6	1.8	1.9	1.9

Table 3. Adsorption of the isoamylase of *R. oryzae* on to various waste starches.

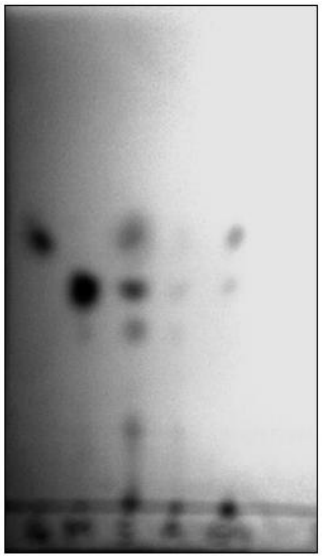
Starch type (1% w/v)	Adsorption (%)	Desorption (%)
Soluble potato starch (Merck)	50.4	24
Arrowroot	50	24
Corn	50	50.7
Rice dust	37.5	40.3
Pulse powder	12.5	28.2

Analysis of end product

The end product of isoamylase action on various substrates like starch, glycogen, and amylopectin was tested, which was found to be mainly glucose, maltose and maltooligosaccharide (Fig 7). Maltose, maltotriose and maltotetraose were detected as the main hydrolysis product of *Hendersonula* isoamylase (Odibo *et al,* 1992), *Bacillus* sp (Ara *et al*, 1993) and from *Lipomyces* (Spancer Martin, 1982). On the other hand, degradation product of glycogen by isoamylase of *Bacillus stearothermophilus* was detected as a single spot of glucose on thin layer chromatogram (Prayitno *et al,* 1996).

Adsorption of isoamylase onto various starches

Isoamylase synthesized by present working strain like other isoamylases (Kato et al, 1977; Fang *et al,* 1994) was able to get adsorbed on various native starches. Although highest rate of adsorption was found in soluble potato starch (Merck) followed by corn and arrowroot starches (Table 3), highest desorption was found in corn and rice dust. This differential rate of adsorption was dependent on the arrangement of starch molecules, which was in turn dependent on the botanical source of the starch. However, the moderately higher rate of adsorption and recovery of the enzyme from corn starch could be utilized in further purification of the enzyme.



G M St Am Gly

G: glucose, M: maltose, Gly: glycogen, St: soluble potato starch, Am: amylopectin

Fig 7. Thin layer chromatographic analysis of the end products of isoamylase action on various substrates

Conclusion

Although a few yeast and mould strains were reported to have isoamylase producing ability (Spancer Martins, 1982, Odibo *et al,* 1992), so far the literature survey is concerned, there was no

report of isoamylase production by a strain of *Rhizopus* or related fungi. Therefore the present report is the first report of fungal isoamylase that had properties which were somewhat different from those of the already reported yeast strains. Obviously, the properties of the present strain were found to be more advantageous from industrial point of view. Isoamylase produced from *Rhizopus oryzae* showed higher temperature optima than other yeast and mould isoamylases reported, and except thermophilic bacterial strains, showed higher thermostability (Yokobayashi *et al*, 1970 , Ara *et al* , 1993 and Yamada *et al*, 1994). It also possessed a broader range of pH stability. The saccharification potential of the enzyme may make it more applicable in sugar and food processing industries. The ability of the enzyme to digest mainly raw starch residues collected from wastes will surely add economy in sugar production. Further the property of adsoption onto raw starch molecules and desorption thereof will ameliorate the physical immobilization of the enzyme and make the purification process more simplified.

Acknowledgement

The authors wish to thank University Grants Commission, India for the financial support.

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