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A $HgCl_2$ INSENSITIVE AND THERMALLY STABLE INULINASE FROM *ASPERGILLUS ORYZAE*

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Abstract—An extracellular inulinase (EC 3.2.1.7) from *Aspergillus oryzae* growing on inulin-containing medium was purified by CM-cellulose and Sephadex G-200 column chromatographies to electrophoretic homogeneity. The purified inulinase cleaved β-linked fructose from inulin and sucrose with S/I ratio of 2.4. It had a remarkable stability in heat, losing only 41 and 32% of its inulin hydrolytic activity after 2 h at 90° and 100°, respectively. The pattern of thermal denaturation of both the inulin and sucrose hydrolytic activities was nearly the same. SO_4^{2-} and Fe^{3+} were strong inhibitors of inulin and sucrose hydrolytic activities, whereas Ba^{2+} was an activator. Hg^{2+} inhibited sucrose hydrolytic activity but increased inulin hydrolytic activity of inulinase. It is proposed that inulinase has different sites for binding of sucrose and inulin and these are either very close to or partially overlapping. The sulphydryl group is in the non-overlapping zone of the sucrose binding site and its modification by $HgCl_2$ results in an enhanced inulin hydrolytic activity. In the presence of inulin and sucrose together, both substrates compete with each other resulting in reduced activity compared with that observed with individual substrates. Inulinase had a temperature optima of 55° and a mass of 38 kd.

INTRODUCTION

Fructose syrup is produced mainly from starch by the action of amyloglucosidase and amylases and subsequent conversion of glucose to fructose by glucose isomerase. However, the yield of fructose in the final sugar syrup is about 45% [1]. The separation of fructose from the sugar syrup adds to the cost of its production. Inulin, a β 2-1 linked polymer of about 30 fructose units terminated by α-D-glucose, is a reserve carbohydrate of many plants like tubers of *Helianthus tuberosus* [2] and *Taraxacum officinale* [3], roots of *Cichorium intybus* [4] and stem of *Agave* species [5]. Acid hydrolysis of inulin is not preferred because of the formation of difructose anhydride which does not have any sweetening capacity [6]. Inulinase can hydrolyse inulin to almost pure fructose. In this context, microbial inulinases are receiving attention in various laboratories [7–10]. However, for any inulinase to be successful in industry it must be stable at high temperatures because of the possibility of microbial contamination at room temperature [11]. We screened the literature and found that inulinases from *Aspergillus* species are more stable to heat than those from other microbial sources. Therefore, we screened a number of *Aspergillus* species for their capacity to produce thermally stable inulinases and found that inulinase from *Aspergillus oryzae* (MTCC 152) is highly stable to heat and also that it possesses some unique properties like low M_r and insensitivity towards Hg^{2+}.

RESULTS AND DISCUSSION

Aspergillus oryzae produced maximum extracelllular inulinase (1–1.2 units ml^{-1} of medium) after 9 days of growth on a medium containing inulin. Approximately 70–80% of the enzyme was extracellular.

The inulinase preparation obtained on precipitating the extracellular enzyme from the medium after 9 days of growth, either with ammonium sulphate or two volumes of chilled ethanol, was not active. Later it was found that sulphate ions were strong inhibitors of inulinase. Therefore, crude inulinase was loaded on a DEAE cellulose column. However, inulinase bound firmly with DEAE cellulose at pH 5.4 and was not eluted even on raising the NaCl concentration to 1 M in 20 mM sodium acetate buffer pH 5.4. In this respect, inulinase of *A. oryzae* behaved like the extracellular inulinase of *Fusarium oxysporum* which also binds firmly with DEAE cellulose [12]. Inulinase was then directly loaded on CM cellulose and was eluted as a

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Table 1. Effect of heating on activity of inulinase

Pre-incubation time (min)	% inulin hydrolytic activity after pre-incubation		% sucrose hydrolytic activity after pre-incubation	
	90°	100°	90°	100°
0	100	100	100	100
30	70	66	95	73
60	60	58	91	65
90	56	48	83	60
120	41	32	66	46

Values have been obtained from three replicates.

Inulinase was heated for different time periods in the absence of substrates before measuring its activity with inulin and sucrose.

single peak with 0.1 M sodium phosphate buffer (pH 6.0). Subsequent purification of inulinase on Sephadex G-200 column gave a single low M_r peak containing inulinase as well as invertase activities. The specific activity of purified fraction was 3.7 μmol fructose produced min^{-1} mg^{-1} protein. The purified inulinase gave a single protein band of R_f 0.82 by polyacrylamide gel electrophoresis. Guiraud *et al.* and Nahm and Byun, were also unable to separate inulinase from its sucrose hydrolytic activity though the enzyme was electrophoretically homogeneous [13, 14].

The naming of a β-fructosidase as an inulinase or invertase is based on its relative hydrolytic capacity for sucrose and inulin (S/I ratio). A true invertase has S/I ratio of more than 1000 whereas for inulinase this ratio is only 10–15. The S/I ratio of purified inulinase was 2.4 so it can be classified as a true inulinase. The inulin and sucrose hydrolytic activities in the purified preparation could either be due to two different enzymes or one enzyme showing broad specificity or one enzyme having two different active sites. There are some plant inulinases which do not show significant activity with sucrose [2, 15] but to the best of our knowledge no microbial inulinase preparation completely free from invertase activity, has so far been reported.

Both the inulin and sucrose cleavage activities of inulinase were extraordinarily stable to heat. Heating the purified preparation at 90° and 100° for 2 h caused a loss of 59% and 68% of inulin hydrolytic activity, respectively and 34% and 54% of sucrose hydrolytic activity, respectively (Table 1). If inulin hydrolytic and sucrose hydrolytic activities are due to two different enzymes, one would not expect such closeness in the thermal stabilities of the two activities. Ettalibi and Baratti suggested inulinase from *Aspergillus ficuum* to have a common catalytic site for sucrose and inulin hydrolysis but different binding sites for these substrates [10]. There is a strong possibility that separate binding sites for both the substrates may be a general feature of microbial inulinases. Gupta *et al.* were also unable to separate invertase and inulinase activities of *Kluyveromyces fragilis* from each other using a series of chromatographic techniques [9]. If indeed the activities with sucrose and inulin are due to same catalytic site, one would expect a similar response of these two activities to exogenous additives. The presence of Cl^-, F^-, NO_3^-, Mg^{2+}, K^+ and Ca^{2+} (5 mM) did not affect the sucrose and inulin hydrolytic activities. Sulphate ions inhibited both the activities (Table 2). Ba^{2+} was an activator of both sucrose and inulin hydrolytic activities whereas Fe^{3+} inhibited both the activities (Table 2). In general, all compounds had similar effects on both the activities. One exception found was of $HgCl_2$ which, though it inhibited sucrose hydrolytic activity, activated inulin hydrolysis. This was contrary to expectations because all known inulinases and invertases are inhibited by $HgCl_2$, suggesting the requirement of an -SH group for catalytic activities of β-fructosidases. In four different

Table 2. Effect of various compounds on inulin and sucrose hydrolytic activities of the purified preparation

Compound	Concentration (mM)	% activity with	
		Inulin	Sucrose
Na_2SO_4	1	92	85
Na_2SO_4	5	5	0
$FeCl_3$	1	73	68
$FeCl_3$	5	18	5
$HgCl_2$	1	121	2
$HgCl_2$	5	152	0
$BaCl_2$	1	126	124
$BaCl_2$	5	139	135
$(NH_4)_2SO_4$	1	31	32

Activity of the control experiment in which no additional compound was added has been considered as 100. The required compound was incubated with the enzyme for 30 min at 30° before adding the required substrate. NaCl, NaF, $NaNO_3$, $MgCl_2$, KCl and $CaCl_2$ had no effect on the activity at 1 mM and 5 mM concentrations.

Data are the mean of at least two replicates.

Table 3. Effect of $HgCl_2$ on inulin and sucrose hydrolytic activities of inulinase

Substrate	$HgCl_2$ (2 mM)	nmol of reducing sugar formed min^{-1} ml^{-1} of enzyme
Inulin	+	20.1
Sucrose	+	0.0
Inulin and sucrose	+	14.1
Inulin	−	14.6
Sucrose	−	15.1
Inulin and sucrose	−	9.8

Mean of the triplicate assays.

Experiment was replicated three times.

Concentrations of inulin and sucrose were 2 and 50 mM, respectively.

+, in the presence of $HgCl_2$ and −, in the absence of $HgCl_2$.

replicates using $HgCl_2$ from different companies we obtained the same results. Therefore, the effect of $HgCl_2$ on production of reducing sugars from sucrose, inulin and inulin + sucrose was studied. The formation of reducing sugars was less when sucrose + inulin were used as a substrate than when sucrose and inulin were used separately (Table 3). In the presence of inulin and sucrose together, possibly both the substrates compete in binding to the enzyme; and this process inhibits their binding, resulting in a decreased formation of products compared with those formed by the substrates individually (Table 3). The different behaviour of $HgCl_2$ towards inulin and sucrose hydrolysis by inulinase could be explained by assuming its different binding sites for inulin and sucrose but having a common catalytic site. Sucrose binding site probably has an -SH group which after modification by $HgCl_2$ loses its affinity towards sucrose. The sucrose and inulin binding sites may either be very close to or partially overlapping. The sulphydryl group at the sucrose binding site may be in the non-overlapping zone. Modification of an -SH group by $HgCl_2$ may modify the environment at the catalytic or binding site of inulin leading to enhanced inulin hydrolytic activity.

The pH optima of inulinase with inulin and sucrose were 6.0 and 4.5, respectively; but the activity below and above the pH optima especially in the pH range of 4.0–6.5 was high, with minimium activity on either side of this range varying from 69–91% with inulin and 68–88% with surose (data not given). The temperature optima for this enzyme with both the substrates was 55°.

The inulinase showed a hyperbolic pattern of increase in velocity both with inulin and sucrose. In this respect the inulinase of *A. oryzae* differs from that of *Kluyveromyces fragilis* which showed a sigmoidal pattern of increase in velocity with increasing inulin concentrations and hyperbolic curve with increasing sucrose concentrations [9]. The *Km* of inulinase with inulin was 0.3 mM whereas with sucrose it was 4.8 mM. *Km* values of inulinase with inulin from different sources are 17 mM from *Candida salmenticensis* [16], 15 mM from *Debaryomyces canterelli* [17], 7 mM from *Closteridium acetobutylicum* [18] and less than 1 mM from *Fusarium oxysporum* [12]. Obviously inulinase from *A. oryzae* has an affinity with inulin.

The M_r of inulinase was about 38 ± 2 kd. The M_r of inulinase is dependent upon the source of this enzyme. Inulinase from *A. niger* and *F. oxysporum* is around 300 kd [19, 20] whereas its M_r from *Aspergillus* species varied from 43–81 kd [10, 21, 22]. The low M_r inulinase from *A. oryzae* makes it a better choice for introducing the gene for this enzyme into a fast growing microorganism in order to obtain a higher yield of thermally stable inulinase.

EXPERIMENTAL

The culture of *Aspergillus oryzae* (MTCC 152) was obtained from Institute of Microbial Technology, Chandigarh. The sterilized medium containing glucose, 2%; K_2HPO_4, 0.1%; $MgSO_47H_2O$, 0.05%; $NaNO_3$, 0.15%; KCl, 0.05%; $FeSO_4$, 0.01% and $NH_4H_2PO_4$, 0.2% was inoculated with freeze-dried culture of *Aspergillus oryzae* [7]. One ml of inoculum obtained after 2 days of growth at 25° was used to inoculate the medium in which glucose was replaced by an equivalent amount of inulin which was isolated from chicory roots [23].

The culture filtrate was centrifuged at 10,000 *g* for 15 min and the supernatant was passed through Sephadex G -25 column using a 0.05 M NaOAc buffer (pH 5.4) to remove sugars and low M_r components. Inulinase and invertase activities were determined [9]. M_r of inulin has been considered to be 5000. One inulinase unit is the amount of enzyme which produces 1 μmol fructose min^{-1}. One invertase unit is the amount of enzyme which hydrolyses 1 μmol sucrose min^{-1}. Protein content was estimated by procedure of Ref. [24].

The enzyme was purified by using CM-cellulose and Sephadex G-200 column chromatographies. The purity of the purified inulinase was checked by polyacrylamide gel electrophoresis using 7.5% acrylamide gel [25]. The protein band was visualized using Coomassie blue.

The M_r of inulinase was determined by plotting Ve/Vo values of standard proteins and purified inulinase obtained after the Sephadex G-150 column chromatography against log M_r of standard proteins. The standard protein M_r markers used were β-amylase (200 kd), alcohol dehydrogenase (150 kd), BSA (66 kd) and carbonic anhydrase (29 kd).

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