Purification and some important characters of extracellular inulinase of
*Alternaria alternata* (Fr.) Keissler

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Microbial inulinas are an important class of industrial enzymes that have gained much attention recently. Inulinase (2,1-ß-fructan fructanohydrolases, EC 3.2.1.7) hydrolyze inulin to fructose in a single step. It finds several applications, for example, in the production of high fructose syrup, alcohol, acetone and butanol; as a tool in diagnosing kidney problems, for the saccharification of fructans of plant origin (Jerusalem artichoke, Chicory) and for the preparation of fructose syrup from inulin.

Different fungal species have been reported as extracellular inulinase producers i.e. Aspergillus fumigatus — a thermotolerant fungus, A. flavus, A. niger, A. oryzae, A. versicolor, Cladosporium cladosporioides, Fusarium oxysporum, Penicillium janczewski, P. purpuragenum and P. trzebinskii.

The present study was undertaken to purify and study some important characters of extracellular inulinase of *Alternaria alternata* (Fr.) Keissler which has not been reported earlier as an inulinase producer.

**Materials and Methods**

**Organism and medium**

*Alternaria alternata* (Fr.) Keissler used in this work is an Egyptian soil isolate previously identified by the authority of the Commonwealth Mycological Institute. It was maintained on Czapek’s-agar medium and the spore suspension was prepared in 10 ml of 0.85 saline from 7-days-old cultures. One ml containing 34 × 10^5 spores was used to inoculate a triplicate set of 250-Erlenmeyer flasks, each containing 50 ml of the following fermentation medium (g/100 ml): sucrose, 1.6; NaNO₃, 1.8%; KH₂PO₄, 0.1; KCl, 0.05; MgSO₄, 7H₂O, 0.05 and initially adjusted to pH 5.5. Submerged incubation (150 rev min⁻¹) last for 108 hr at 30°C.

**Enzyme assay**

Extracellular inulinase was assayed as follows: to 2 ml of 0.2% inulin suspension (inulin from Dahlia tubers, Sigma, Germany) and 2 ml of 0.2 M acetate buffer (pH 4.6), 0.5 ml of appropriately diluted crude enzyme preparation (culture filtrate) was added and incubated at 50°C for 20 min. The tubes were then boiled for 10 min to inactivate the enzyme, cooled, centrifuged and assayed for reducing sugars as fructose by the dinitrosaliclyic acid method using fructose as standard. One unit of inulinase (U) was defined as the amount of enzyme that produced 1μmol min⁻¹ of fructose under the assay conditions. Sucrose replaced inulin in case of assaying invertase activity. One unit of invertase was considered as the amount of enzyme which produces 1 μmol min⁻¹ of reducing sugar as an equimolecular mixture of glucose and fructose that was utilized as standard.

**Protein assay**

Protein content was assayed by the method of Bradford.
Enzyme purification and characterization

Aliquots of cell-free dialysate (CFD) were separately treated with ammonium sulfate using range of saturation from 0.5 to 0.9; methanol or iso-propanol in a ratio of 1:1, 2:1, 3:1, 4:1 or 5:1 (alcohol : CFD). All samples were left overnight at 4°C and the precipitates were collected by centrifugation at 12 x 10^3 g for 15 min., dissolved in 10 ml acetate buffer (0.01 M, pH, 4.6) and dialyzed overnight against the same buffer.

Protein precipitate obtained from methanol 1:1, containing most of the enzyme activity, was then fractionated on Sephadex G150 (Pharmacia product). Sephadex was suspended to swell in distilled water for 24 hr. During this time, it was stirred gently and the fine particles, if any, were removed by decantation. The water phase was then removed and the gel was suspended in 0.2 M acetate buffer (pH, 4.6). The gel suspension was carefully poured into a column (2.5 x 82 cm) of Fraction Collector (Fra-100, Pharmacia-Finh Chemicals) to avoid formation of air bubbles, then allowed to settle while slow flow rate of the buffer was maintained through the column. Two ml of the enzyme preparation obtained from methanol (1:1) was cautiously applied to the column. Active fractions (5 ml each) were pooled, lyophilized and subjected for further purification on DEAE-cellulose column (Diethylaminoethyl-cellulose, fast flow, fibrous form —Sigma product) prepared according to the procedure of Peterson and Sober21, in which: DEDE-cellulose was thoroughly washed with distilled water followed by washing with 1 N HCl and water till pH of the suspension was about 6.0. It was then washed several times with 0.5 N NaOH until no more colour was removed from the gel. After the last alkaline wash, the resin was rinsed with distilled water until it was alkali-free. The gel was allowed to settle and the fine particles were removed. The column was packed with the gel and injected with the enzyme preparation as in sephadex.

The enzyme was eluted with a linear gradient of NaCl concentration (0.0 to 0.5 M) prepared in 0.2 M acetate buffer (pH, 4.6). pH value should be kept at this level or lower because inulinase bound firmly with DEAE-cellulose at pH 5.4 and could not be eluted even on raising NaCl concentration up to 1 M. Active fractions were pooled, dialyzed, lyophilized and kept cool for subsequent work.

The molecular mass (Mr) of the enzyme preparation was estimated by gel filtration22, in which a superose 12 column was calibrated with standard proteins: carbonic anhydrase, 29 kDa; ovalbumin, 43 kDa; phosphorylase, 94 kDa; alcoholic dehydrogenase, 150 kDa and catalase 232 kDa. Elution takes place with 0.2M acetate buffer (pH, 4.6) and a standard calibration graph was constructed representing the relation between elution volume against Mr of the standard proteins. The Mr of the enzyme was estimated by reference to the calibration graph.

Results and Discussion

Steps of the procedure used for purification of extracellular inulinase from A. alternata have been summarized in Table 1. The 1st step of purification was the dialysis of cell-free filtrate (CFD) against 0.2 M acetate buffer (pH 4.6). The results show a decrease of inulinase activity by 90.5%. This can be attributed to the loss of certain activating ions during the dialysis. The results show the superiority of methanol (1:1) in obtaining protein fraction having the highest total enzyme activity (~ 135 x 10^3 U/ml) with a yield of 81.6% of the original activity. Its specific activity was calculated to be 11.6 x 10^3 U/mg protein. This finding confirmed that this enzyme has a particular structure which makes it resist the known denaturing effect of organic solvents. Suitability of

<table>
<thead>
<tr>
<th>Step</th>
<th>Total activity (U x 10^3)</th>
<th>Total protein (mg)</th>
<th>Specific activity (U/mg x 10^3)</th>
<th>Yield (%)</th>
<th>Purification fold(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell-free filtrate (CFD)</td>
<td>165.00</td>
<td>127.00</td>
<td>1.3</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>Cell-free dialysate CFD</td>
<td>149.50</td>
<td>127.00</td>
<td>1.2</td>
<td>90.5</td>
<td>0.90</td>
</tr>
<tr>
<td>Protein precipitate of methanol 1:1</td>
<td>134.71</td>
<td>11.60</td>
<td>11.6</td>
<td>81.6</td>
<td>9</td>
</tr>
<tr>
<td>Gel filtration</td>
<td>115.50</td>
<td>3.51</td>
<td>32.9</td>
<td>70.0</td>
<td>25</td>
</tr>
<tr>
<td>Ion exchange chromatography</td>
<td>114.00</td>
<td>1.41</td>
<td>80.7</td>
<td>69.1</td>
<td>62</td>
</tr>
</tbody>
</table>

Total volume used was 500 ml of CFF
organicsolvents in this regard was previously recorded by Vullo et al.24 and Viswanathan et al.25.

The purification was followed by gel-filtration through Sephadex G150. The results obtained indicated the presence of two peaks. Only the major one (fractions 6 to 10, containing $33 \times 10^6$ U/mg protein with 25-fold purification) was subjected to ion exchange chromatography. This resulted in single sharp peak possessing about $81 \times 10^6$ U/mg protein with 62-fold purification (Table 1). This enzyme preparation was diluted to a final concentration of 100 U/ml to facilitate presentation of results, kept at 4°C and used in the subsequent experiments.

Electrophoretic pattern of the enzyme preparation that was carried out using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) revealed that the enzyme was not purified to electrophoretic homogeneity since a minor band in addition to a broad one were detected. Yet, purification to electrophoretic homogeneity is not required for industrial purposes26.

Effect of temperature on enzyme activity and stability

The purified enzyme preparation was subjected to different temperatures and the results were graphically represented in Fig. 1. It is apparent that the maximum enzyme activity was observed in the narrow range of 50°C to 60°C with its maximum at 55°C. This is in complete agreement with the results obtained for other inulinas from A. oryzae12; P. janczewskii20 and P. trzebiinski21. The noticed behavior of the enzyme activity in response to temperature, obviously obeyed the kinetic theory.

Enzyme preparation was incubated at the indicated temperature for 1 hr and the residual activity was measured. The enzyme appears to be completely stable up to 30°C, and also show high stability up to 50°C, after which there is a gradual loss in enzyme activity. In this respect, inulinas from A. fimicatus and P. purpureogenum2 and from A. oryzae12 MTCC-152 show higher thermal stability.

$T_{1/2}$ of the purified enzyme was found to be two weeks when kept in 0.2 M acetate buffer, pH 4.6, at either −15°C or at 4°C.

Effect of pH on the enzyme activity and stability

pH optimum for activity was found to be 4.5 (Fig. 2), identical to that recorded for inulina from A. niger-24526. It lies in acidic range as those recorded for A. niger, 5.027; P. janczewskii, 5.08 and A. versicolor, 5.513. As pH value diverged from the optimum level, the efficient functioning of the enzyme affected, most probably, due to change in active site conformation which is determined, in part, by ionic and hydrogen bonding which can be affected by pH.

The pH stability of the enzyme was studied in the pH range of 3.0 to 9.0 after 1 hr exposure to the indicated pH. The enzyme was stable in the acidic range and sharp decrease was observed at pH values higher than 5. It is also clear that at 4.5 (the optimum value for activity) the enzyme retained only 90% of its original activity. This may be for the absence of its substrate during the time of exposure that may affect its behavior.
Ongen-Baysal et al.\textsuperscript{28} and Gupta et al.\textsuperscript{12} reported the industrial importance of microbial inulinas that possess high activity and stability at relatively high temperatures and low pH because these conditions increase solubility of the substrate and help in avoidance of microbial contamination. As a conclusion, with its thermal and acidic stability and high temperature and acidic pH optima, inulinate from \textit{A. alternata} appears suitable for use in industrial applications.

**Effect of metal ions and inhibitors on inulinate activity**

With regard to the examined metal ions and inhibitors on inulinate activity, Table 2 demonstrates the fluctuating effects of these compounds. \(\text{K}^+\) and \(\text{Na}^+\) had insignificant effect while \(\text{Hg}^{2+}\), \(\text{Fe}^{3+}\), or \(\text{Cu}^{2+}\) exhibited strong inhibition especially at the higher concentrations. On the other hand, \(\text{Ba}^{2+}\) and \(\text{Ca}^{2+}\) exhibited a highly significant stimulatory effect. Similar results were reported for the effect of \(\text{Cu}^{2+}\), \(\text{Fe}^{3+}\), \(\text{Hg}^{2+}\), \(\text{Ba}^{2+}\) and \(\text{Ca}^{2+}\) on the activity of inulinas from \textit{A. niger}, \textit{A. oryzae} and \textit{A. versicolor}\textsuperscript{12,13,15}.

The inhibition caused by \(\text{Hg}^{2+}\) substantiates the view that an SH group participates in the enzyme structure. The inhibition effect of arsenate and arsenite in addition to the great inhibition due to the presence of the more specific sulfhydryl inhibitor i.e. iodoacetic acid were additional evidences for this possible participation. EDTA at 5 and 10 mM retarded the enzyme activity mostly due to binding with \(\text{Ba}^{2+}\) and/or \(\text{Ca}^{2+}\). Nakamura et al.\textsuperscript{16} also found that EDTA cause slight reduction in activity of inulinate from \textit{A. niger}.

**Other enzymatic properties**

Relation between inulin concentration and apparent activity of inulinate was also studied and Lineweaver-Burk plot of the reciprocals of initial velocities and inulin concentrations was constructed and the apparent \(K_m\) value of this enzyme was found to be 0.066 \(M\).

Free fructose was the only product released from inulin (as evidenced by adopting paper chromatography technique) indicating that this enzyme displays exo-inulinate activity. This finding is in complete ac-

![Table 2—Effect of metal ions and enzyme inhibitors on relative activity of the purified inulinate](image)

<table>
<thead>
<tr>
<th>Metal ions(^a)</th>
<th>Relative activity as affected by the concentration of</th>
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<tbody>
<tr>
<td></td>
<td>1 mM(\pm)</td>
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<tr>
<td>(\text{Ba}^{2+})</td>
<td>118 ± 5.44**</td>
</tr>
<tr>
<td>(\text{Ca}^{2+})</td>
<td>118 ± 7.44**</td>
</tr>
<tr>
<td>(\text{Co}^{2+})</td>
<td>83 ± 4.98**</td>
</tr>
<tr>
<td>(\text{Cu}^{2+})</td>
<td>77 ± 5.39**</td>
</tr>
<tr>
<td>(\text{Fe}^{3+})</td>
<td>81 ± 2.84**</td>
</tr>
<tr>
<td>(\text{Hg}^{2+})</td>
<td>74 ± 5.18**</td>
</tr>
<tr>
<td>(\text{K}^+)</td>
<td>99 ± 3.96**</td>
</tr>
<tr>
<td>(\text{Mg}^{2+})</td>
<td>110 ± 7.70**</td>
</tr>
<tr>
<td>(\text{Mn}^2+)</td>
<td>109 ± 4.25**</td>
</tr>
<tr>
<td>(\text{Na}^+)</td>
<td>100 ± 6.00**</td>
</tr>
<tr>
<td>(\text{Zn}^2+)</td>
<td>78 ± 6.24**</td>
</tr>
</tbody>
</table>

\('a\)The investigated metal ion as chloride was added at the indicated concentration was incubated with the enzyme for 30 min at 30°C before adding substrate. Activity of the enzyme in complete absence of such compounds served as control (100% activity) to which other data were statistically compared according to the mathematical principles described by Glantz\textsuperscript{32}. The results were considered non significant when \(P\) is greater than 0.05, significant when \(P\) is less than 0.05 and highly significant when \(P\) is less than 0.01 and expressed as " = Non significant, * = Significant and ** = Highly significant, respectively.
cordance with that of Olta et al. who stated that microbial inulinas are usually exo-acting.

Microbial inulinas were also reported to have the capability of hydrolyzing raffinose and sucrose. In this regard, inulina of A. alternata recorded similar capability against sucrose (198 U/ml) and raffinose (73 U/ml). Such substances were separately added under conditions completely similar to those described for inulase assay, but inulina was replaced with the substances under investigation.

Molecular mass of the enzyme was estimated by gel filtration and was found to be 115 ± 5. Gupta stated that the molecular mass of inulinas is dependent upon the source of the enzyme, for example 38 kDa was recorded for the inulina from A. oryzae, 34-81 kDa for inulinas from different Aspergillus species and 300 kDa for inulinas from A. niger and F. oxysporum.

As many microbial preparations of inulina possess remarkable invertase activity (S) accompanying the inulina activity (I), their catalytic activity is described in terms of I/S or S/I ratios. I/S of this enzyme preparation was found to be 0.3 (equals S/I of 1.9). I/S ratios for other inulinas were in the range of 0.31-0.39: Cladosporium cladosporioides; A. niger; 0.625, A. ficuum and 0.7, A. versicolor. Despite the above considerations, Uhm et al. determined a ratio of infinity for endo-inulina from A. ficuum. Naming of β-fructosidase as an inulina or invertase is based on such hydrolytic capacity for sucrose and inulina (S/I ratio) where true invertase has S/I ratio of more than 1000 and there is no microbial inulina preparation completely free from invertase activity has so far been reported. Moreover, during the purification of inulina, the sucrose and inulin-hydrolyzing activities are never separated. From that, this enzyme preparation could safely be named inulina.

References


