Purification and characterization of beta-amylase from Curculigo pilosa

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Abstract Curculigo pilosa is traditionally used in the manufacture of sorghum beer in West Africa. β-Amylase was purified 100-fold with 38% yield from a crude extract, giving final specific activities of 4850 U/mg and 5650 U/mg using soluble starch and p-nitrophenyl maltopentaoside, respectively, as substrates. The molecular mass of the monomeric enzyme was 64 kDa and its pI 4.2. Both activity and thermostability are higher than reported for other plant β-amylases. The catalytic efficiency was lower for amylose than for starches and amylopectin. In contrast to other plant amylases, the β-amylase from C. pilosa is able to degrade raw starches from wheat, corn, potato and rice. In this respect, it resembles β-amylases from microbial origin. This property, and its high activity and stability, explain its traditional use in the manufacture of infant food and sorghum beer in Burkina Faso and could make it applicable for other biotechnological purposes.

Introduction

In Nigeria, sorghum has become the predominant cereal for industrial scale malting and brewing of beer, following legislation banning the importation of barley and wheat. The major disadvantage encountered using sorghum as brewing cereal is its low content of β-amylase (Okon and Uwaifo 1985) causing the incomplete saccharification of the starch (Swanston et al. 1993). This is a general problem of sorghum, for comparison of more than 60 sorghum malts in laboratory-scale brewing experiments has shown clearly that, in most cultivars, β-amylase was low or absent (Dufour et al. 1992).

Curculigo pilosa is added in Burkina Faso during the local manufacturing of infant food and sorghum beer. In the latter process, the aqueous extract of its tuber tissues is added to the sorghum flour at the mashing step or before fermentation. Given the relatively small amount of Curculigo extract added, it was envisaged that this extract may supply enzymes, e.g. β-amylase, for more efficient starch degradation during the brewing process. β-Amylase (α-1,4-glucan maltohydrolase) [E.C: 3.2.1.2] is an exo-enzyme hydrolysing the α-1,4-glucosidic linkages in polysaccharides so as to remove successive maltose units from the non-reducing ends of the chains. It cannot bypass the α-1,6 linkages, so its conversion of starch to β-maltose is incomplete. β-Amylase is widely distributed in plants but also occurs in fungi and bacteria. Plant β-amylases have no or hardly any activity on starch granules (Lizotte et al. 1990), whereas bacterial amylases are able to adsorb onto and to degrade raw starch (Saha et al. 1987; Sohn et al. 1996; Nagasaka et al. 1998).

The purpose of this study was to isolate and characterize the major amylolytic enzyme from C. pilosa tuber.

Materials and methods

Materials and chemicals

Tubers of C. pilosa (Schum. et Thonn.) Engl. (Hypoxidaceae or Liliaceae) were harvested at Ouagadougou (Burkina Faso) and, after thorough washing, kept in the dark at 4°C for 1 month before enzyme extraction.

Amylose and amylopectin were from Avebe (Veendam, The Netherlands), soluble starch from Merck (Darmstadt, Germany) and raw starches of wheat, corn, potato and rice from BDH Chemicals (The Netherlands). Chemicals for electrophoresis, column chromatography, ampholites, molecular-mass marker proteins (14.4–94 kDa) and pI marker proteins (pI 3.5–9.3) were
supplied by Pharmacia-LKB (Uppsala, Sweden). p-Nitrophenyl-
maltohexaose, azurine-crosslinked amylose and p-nitrophenyl-\(\alpha\)-glucopyranosidase were purchased from Megazyme (Sydney, 
Australia). Uitroel ACA54 was obtained from Sepracor (France) 
and disodium 2,2'-bicinchoninato was from Pierce (USA). All other 
chemicals were analytical grade.

**Enzyme purification**

All enzyme purification steps were carried out at 4 °C. All buffers 
contained 0.02% (mass/vol) sodium azide to prevent microbial 
growth.

A crude extract was prepared from the tubers (50 g) of *C. pilosa* 
by peeling, cutting and grinding with 150 ml of 0.05 M sodium 
malate, pH 6, containing 3 mM CaCl\(_2\), 20 mM cysteine, 0.1% 
(mass/vol) NaCl and 2% (mass/vol) poly(vinylpyrrolidone). From 
this crude extract, the \(\alpha\)-amylase was purified using ammonium 
sulphate precipitation, gel filtration and anion-exchange chro-
matography. SDS-PAGE, IEF electrophoresis and an electropho-
retic titration curve (pH 3.5–9.3) were performed with the 
PhastSystem unit (Pharmacia-LKB). The gels were either Coom-
massie brilliant blue R-250 or silver stained.

**Assay of enzyme activities**

\(\beta\)-Amylase activity was determined at 55 °C in 0.05 M sodium 
acetate, pH 5, by measuring the release of reducing sugar from 
glucose substrates using the disodium 2,2'-bicinchoninate assay 
(Garcia et al. 1993) with a modification to adapt it to microtitre 
plate format. The \(\beta\)-amylase activity was also determined with 
p-nitrophenylmaltohexaose as a specific substrate for \(\beta\)-amylase 
in the presence of \(\alpha\)-glucosidase (McCleary and Codd 1989). 
Azurine-crosslinked amylose was used to determine \(\alpha\)-amylase ac-

**Determination of raw starch digestion**

Forty milligrams of each starch (unboiled) and \(\beta\)-amylase (0.8 
units) in 1.5 ml 0.1 M sodium acetate, pH 5, was incubated at 
40 °C with slow shaking for 2 h and 70 h. The reaction mixture was 
centrifuged and sugars released in the supernatant were analysed by 
the disodium 2,2'-bicinchoninate assay and by high-performance 
anion-exchange chromatography using a Dionex Bio-LC GMP-11 
HPLC System (Sunnyvale, Calif.) equipped with a Dionex Car-
boPac PA-100 column (4 × 20.05 M) and a Dionex detector in the 
pulsed amperometric detection mode. Samples (19 \(\mu\)l) were eluted 
with a linear gradient (20 °C, 1 ml/min) 0-0.25 M CH\(_3\)COONa in 
0.1 M NaOH over 35 min, followed by an increase to 1 M over 
15 min. Then, the CH\(_3\)COONa concentration was kept constant at 
1 M for 10 min.

**Enzymic properties**

The optimum temperature was determined in 0.1 M sodium ace-
tate, pH 5. The pH optimum was determined at 55 °C using 
Mellivaine buffers (pH 2.5–9). Soluble starch (0.5%) was used as 
substrate. The residual activity of the enzyme was measured after 
pre-incubation at 30 °C for 1.5 h in buffers of different pH values. 
The thermostability of the enzyme was determined by measuring 
the residual activity after incubation of the enzyme in 0.1 M 
sodium acetate, pH 5, at different temperatures for 1.5 h.

Changes of optical rotation of sugars formed by the enzyme 
reaction were determined as a function of time as previously de-
scribed by Kolho et al. (1989), using a Ceti Polaris polarimeter 
(Belgium).

**Results**

**Purification of \(\beta\)-amylase**

In the crude extract of *C. pilosa*, only \(\beta\)-amylase but no 
\(\alpha\)-amylase or \(\alpha\)-glucosidase activity could be detected at 
the limit of the sensitivity of the assays used.

\(\beta\)-Amylase was purified 100-fold with a yield of 38% 
from the crude extract (Table 1). The final specific 
activities were 4850 U/mg and 5650 U/mg using soluble 
and p-nitrophenylmaltohexaose, respectively, 
as substrates. Purified \(\beta\)-amylase was monomeric and 
and had an apparent molecular mass of 64 kDa and a pI 
of 4.2.

The purified enzyme was unable to release dye from 
azurine-crosslinked amylose or to hydrolyse p-nitro-
phenyl-\(\alpha\)-glucopyranoside, confirming that it does not 
belong to the \(\alpha\)-amylase or \(\alpha\)-glucosidase groups. In 
addition, its ability to hydrolyse p-nitrophenylmalto-
hexaose is characteristic for a \(\beta\)-amylase (McCleary 
and Codd 1989). Furthermore, hydrolysis products from 
starches, amylose and amylopectin gave only maltose as 
detectable product and no high molecular-mass oligo-
mers were found. These hydrolysis patterns are typical 
for exo-acting enzymes. On the basis of changes in 
rotation, the hydrolysates formed by *C. pilosa* 
\(\beta\)-amylase were identified as \(\beta\)-anomers.

**Effect of pH and temperature on enzyme activity 
and stability**

The enzyme activity showed a rather sharp pH optimum 
around pH 5, while it lost no activity in the pH range 
4.5–7.5 after incubation for 1.5 h at 30 °C.

The \(\beta\)-amylase of *C. pilosa* retained about 80% of its 
activity after 1.5 h incubation at 65 °C and displayed the 
highest activity at 55 °C. Addition of substrate had a 
stabilizing effect. The enzyme was fully active for more 
than 2 h in the presence of substrate at 55 °C.

<table>
<thead>
<tr>
<th>Fractionation step</th>
<th>Total activity (U)</th>
<th>Specific activity (U/mg)</th>
<th>Purification fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>14100</td>
<td>48.5</td>
<td>1</td>
</tr>
<tr>
<td>Ammonium sulphate</td>
<td>9050</td>
<td>76</td>
<td>1.6</td>
</tr>
<tr>
<td>precipitation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gel filtration (ACA54)</td>
<td>6480</td>
<td>900</td>
<td>18.6</td>
</tr>
<tr>
<td>FPLC MonoQ</td>
<td>5345</td>
<td>4850</td>
<td>100</td>
</tr>
</tbody>
</table>

Table 1 Purification summary of \(\beta\)-amylase from *C. pilosa*
Kinetic parameters

For β-amylase from *C. pilosa*, the apparent $K_m$ for soluble starch, potato starch and amylose was 1.5–1.8 mg/ml, but the $K_m$ for amylpectin was 2.3 mg/ml (Table 2). The enzyme had $k_{cat}$ values ranging over 5460–6500 s$^{-1}$ for potato starch, amylpectin and soluble starch, and a $k_{cat}$ of 2400 s$^{-1}$ for amylose. The catalytic efficiency ($k_{cat}/K_m$) was lowest for amylose and highest for soluble starch. The activation energies ($E_a$) for these substrates ranged from 18.3 kJ/mol (soluble starch) to 29.5 kJ/mol (amylose).

Raw starch digestion

No oligomers were detectable in the supernatant of raw starches from wheat, corn, potato and rice before enzyme incubation as analysed by high-performance anion-exchange chromatography. However, after enzymatic hydrolysis, one single peak, identified as maltose, was observed in the high-performance anion-exchange chromatography pattern of the four starches. After 70 h, 4.5% (corn) to 6.2% (wheat) of the raw starch had been degraded.

Discussion

The β-amylase purified from *C. pilosa* had a much higher specific activity than mostly found for plant β-amylases (Kohno et al. 1989; Monroe and Preiss 1990), including the very active β-amylases from activated sweet potatoes (4664 U/mg) (Chang et al. 1996) and the enzyme from *Clostridium thermosulfurogenes* (4215U/mg) (Shen et al. 1988). With respect to molecular mass, pi, kinetic parameters and inhibition by thiol reagents, it resembled other plant amyloses, but it was more thermostable than other β-amylases from plants (Kohno et al. 1989; Lizotte et al. 1990; Hagenimana et al. 1994; Chang et al. 1996).

In contrast to other plant β-amylases (Lizotte et al. 1990; Hagenimana et al. 1992), the β-amylase from *C. pilosa* displayed hydrolytic activity towards raw starches, comparable to raw-starch-degrading amylases from microbial sources (Saha et al. 1987; Sohn et al. 1996; Nagasaka et al. 1998). Whereas the microbial β-amylases hardly degrade raw potato starch, β-amylase from *C. pilosa* degraded it equally well as the other starches. Degradation of raw starch might explain the absence of α-amylase and α-glucosidase activities in the tuber *C. pilosa* (before germination) and suggests a role for β-amylase in starch splitting in vegetative tissues. Since raw-starch-degrading enzymes from microbial origin show a high sequence similarity in the C-terminal domain that is responsible for binding to raw starch (Svensson et al. 1989) and since this domain has not been found in plant-derived β-amylases, it would be interesting to determine the primary structure of the β-amylase from *C. pilosa*.

The presence of high amylolytic activity in extracts of *C. pilosa* explains its traditional use in the preparation of easily digestible infant food and in the traditional method for the preparation of sorghum beer. To increase the diatasic power of sorghum malt, cloning the β-amylase gene from barley or microbes into sorghum has been urged (Okafor 1995), but the use of partially purified *C. pilosa* β-amylase as an exogenous source of enzyme in sorghum beer processing may provide an alternative.

Acknowledgements. The Netherlands Organization for Coöperation in Higher Education (NUFFIC) is acknowledged for financial support to M.H.D. The authors are very grateful to Prof. Dr. F. M. Rombouts for stimulating discussions, and to Prof. Dr. A. G. J. Voragen for his support.

References


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Table 2 Kinetic parameters

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$K_m$ (mg/ml)</th>
<th>$V_{max}$ (U/mg)</th>
<th>$k_{cat} \times 10^{-3}$ (s$^{-1}$)</th>
<th>$k_{cat}/K_m$ (s$^{-1}$ ml mg$^{-1}$)</th>
<th>$E_a$ (kJ mol$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soluble starch</td>
<td>1.5 ± 0.1</td>
<td>6135 ± 215</td>
<td>6.54 ± 0.23</td>
<td>4.36 ± 0.15</td>
<td>18.3 ± 0.9</td>
</tr>
<tr>
<td>Potato starch</td>
<td>1.8 ± 0.1</td>
<td>5125 ± 205</td>
<td>5.46 ± 0.22</td>
<td>3.03 ± 0.12</td>
<td>26.5 ± 1.3</td>
</tr>
<tr>
<td>Amylose</td>
<td>1.7 ± 0.1</td>
<td>2254 ± 113</td>
<td>2.40 ± 0.12</td>
<td>1.41 ± 0.07</td>
<td>29.5 ± 1.5</td>
</tr>
<tr>
<td>Amylopectin</td>
<td>2.3 ± 0.1</td>
<td>5208 ± 260</td>
<td>5.55 ± 0.27</td>
<td>2.3 ± 0.12</td>
<td>21.4 ± 1.5</td>
</tr>
</tbody>
</table>