Biochemical characterization of *Aspergillus awamori* exoinulinase: substrate binding characteristics and regioselectivity of hydrolysis

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Abstract

\(^1\)H-NMR analysis was applied to investigate the hydrolytic activity of *Aspergillus awamori* inulinase. The obtained NMR signals and deduced metabolite pattern revealed that the enzyme cleaves off only fructose from inulin and does not possess transglycosylating activity. Kinetics for the enzyme hydrolysis of inulooligosaccharides with different degree of polymerization (d.p.) were recorded. The enzyme hydrolyzed both \(\beta\)2,1- as well as \(\beta\)2,6-fructosyl linkages in fructooligosaccharides. From the \(K_{cat}/K_m\) ratios obtained with inulooligosaccharides with d.p. from 2 to 7, we deduce that the catalytic site of the inulinase contains at least five fructosyl-binding sites and can be classified as exo-acting enzyme. Product analysis of inulopentaose and inulohexaose hydrolysis by the *Aspergillus* inulinase provided no evidence for a possible multiple-attack mode of action, suggesting that the enzyme acts exclusively as an exoinulinase.

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1. Introduction

Inulin, i.e. 2→1-linked \(\beta\)-D-fructofuranosyl residues with a terminal glucose unit, and levan consisting predominantly of \(\beta\)2→6-fructosyl-fructose linkages (Fig. 1), belong to a group of naturally occurring fructans comprising nondigestible fructooligosaccharides, which are commonly referred to as FOS in the nutrition industry. It was estimated that about one-third of the total vegetation on earth consists of plants that contain these carbohydrates. Pure \(\alpha\)-fructose or fructose syrup, which can be easily produced from inulin, take an important place in human diets today [1,2]. Levan-type FOS have a potential as emulsifying, thickening or stabilizing agents in various food products [3,4]. As such, fructan-hydrolyzing enzymes, especially from microbial sources, represent an effective tool in fructan digestion and nowadays find new industrial applications [5,6]. Microbial inulinases can be divided into two classes according to their action on inulin. Exoinulinases (2,1-\(\beta\)-D-fructan fructohydrolase, E.C. 3.2.1.80) sequentially cleave off terminal fructose unit from the inulin molecule. Endoinulinase (2,1-\(\beta\)-D-fructan-fructanohydrolase, E.C.3.2.1.7) degrades the inulin-type fructans with an endocleavage, producing a series of fructooligosaccharides [7,8].

In our previous paper [9], we reported that a strain of *Aspergillus awamori* var. 2250 produces an extracellular enzyme during solid-state fermentation converting inulin and levan to obtain mainly fructose. Based on its DNA sequence (EMBL accession number AJ315793), the enzyme was classified to belong to 32 glycoside hydrolase family Nucleotide Sequence Database (for the classification of glycoside hydrolase families see http://afmb.cnrs-mrs.fr/CAZY/db.html) [9]. Despite the numerous reports on isolation and cloning of exoinulinases and exolevanases from
fungal and bacterial sources, mainly their enzymatic properties in the hydrolysis of polymeric substrates, inulin and levan, were described so far [10,11]. Meanwhile, analysis of the mode of exoinulinase action towards fructooligosaccharides with different degree of polymerization (d.p.) is an effective tool for subsite mapping of the active site of this enzyme. Kinetic studies on the enzymatic hydrolysis of oligomeric substrates with different lengths allowed determination of affinities and number of subsites in the active center of β-glucanases and glucoamylases from various sources [12,13]. In the present study, we describe for the first time details on the mode of action of the exoinulinase from A. awamori on fructooligosaccharides using 1H-NMR analysis, thereby estimating the number of fructosyl-binding sites and judging on a possible multiple attack mode of action.

2. Materials and methods

2.1. Substrate production

Inulin was purchased from Merck (cat. number 1.04733.0010; Germany). Levan from Microbacterium levaniformans was a gift from Dr. A. Miasnikov, Danisco Global Innovation, Finland. Average inulin d.p. was calculated by 1H-NMR from the ratio \( n \) of the signals H3 (\( \delta = 4.3–4.17 \) ppm) in fructose and H1 (\( \delta = 5.43 \) ppm) in glucose residues following the relation \( \text{d.p.} = n + 1 \). Inulin used in these experiments had \( \text{d.p.} = 23.8 \pm 0.5 \) and average molecular mass \( 3850 \pm 80 \) Da. 1-Kestose (\( \beta-d\text{-Fru}\text{-(2\text{→1})-} \beta-d\text{-Fru}\text{-(2\text{→1})-} \alpha-d\text{-Glc} \)) was obtained with the aid of the fructosyltransferase from Aspergillus foetidus according to the procedure described in Ref. [14]. Inulooligosaccharides with d.p. from 2 to 4 were produced by digestion of inulin with Aspergillus ficuum endoinulinase, which was kindly provided by Prof. Chae K.S., Chonbuk National University, Korea. To produce inulooligosaccharides, 400 mg of inulin was dissolved in 3.5 ml of 20 mM sodium acetate buffer, pH 5.0, and incubated with approximately 0.05 U of the endoinulinase per 1 mg of inulin at 37 °C for 60 min. The hydrolysate was applied onto a Biogel P2 Super Fine column from Bio-Rad, USA (10 × 1200 mm, flow rate 9 ml/h) to separate high molecular weight fractions. Specific fractions with d.p. from 2 to 4 were rechromatographed on a Dextro-Pak™ cartridge column (8 × 10 mm) WATO85650 from Millipore-Waters, USA, using isocratic elution in water (flow rate 1 ml/min) with refractometric detection. To produce inulooligosaccharides with d.p. 5–7, inulin (200 mg) was dissolved with 5% (v/v) of TFA and incubated at 40 °C for 30 min. After incubation, the mixture was neutralized by mixed Dowex MR-3 (Sigma Chemical Co., St. Louis, MO) and concentrated by evaporation. Individual fractions of inulooligosaccharides with d.p. 5–7 were isolated chromatographically as described above. \( \beta\text{-2,6-fructooligosaccharides} \) were produced by digestion of levan by levanase [15], which was kindly donated by Dr. Miasnikov, Danisco Global Innovation, Finland. The reaction mixture consisted of levan (100 mg/ml) in 20 mM sodium acetate buffer, pH 5.0, and was incubated with approximately

![Fig. 1. Structures of β2,6-linked fructan (levan) (A) and β2,1-linked fructan (inulin) (B).](image-url)
0.1 unit of levanase per 1 mg of fructan at 37 °C for 60 min. After incubation, specific fractions of levanoooligosaccharides with d.p. 2–7 were isolated as described for inulooligosaccharides. Completion of the hydrolysis and purity of levanoooligosaccharides obtained were tested by TLC on Kieselgel 60 plates (Merck) with a mobile phase of butan-1-ol/acetic acid/water (3:1:1, by vol), HPLC on a Dextro-Pak column as described above and by ¹H- and ¹³C-NMR spectroscopy techniques using data on fructooligosaccharides given in Refs. [16,17]. The purity of the compounds obtained was greater than 95% as judged by HPLC and NMR analyses.

2.2. Enzyme and enzyme assays

Exoinulinase was isolated from the solid-state culture of A. awamori var. 2250 according to the procedures described previously [9]. The concentration of pure exoinulinase was estimated by UV absorption spectrophotometry at 280 nm using a specific absorption coefficient of 2.2 l g⁻¹ cm⁻¹. Purified exoinulinase contained less than 0.01% of contaminating exo- and endoglycosidase activities. Exoinulinase activity was determined by Somogyi–Nelson measurement of reducing sugars released from inulin (2.5 mg/ml) in 20 mM sodium acetate buffer, pH 4.5, 37 °C, as described in Ref. [9]. One unit of enzymatic activity was defined as the amount of inulinase required to produce 1 μmol of fructose per minute at pH 4.5, 37 °C, using inulin as the substrate at a concentration of 2.5 mg/ml. Alternatively, exoinulinase action on fructooligosaccharides was evaluated from analysis of hydrolytic products by HPLC on a Dextro-Pak column using refractometric detection. Products were quantified by integration of the peaks that corresponded to inulooligosaccharides with a d.p. of 2–7 or fructose, using the respective standards. Peak areas were proportional to the amounts of oligosaccharide loaded for standards up to a d.p. of 7. Activity of the enzyme in the hydrolysis of sucrose was estimated by UV absorption spectrophotometry at 280 nm using a specific absorption coefficient of 2.2 l g⁻¹ cm⁻¹.

2.3. Calculation of subsite affinities

Kinetic parameters, Michaelis constants (Kₘ) and catalytic rate constants (kₘ) of the exoinulinase during the hydrolysis of β₂,₁-fructooligosaccharides of d.p. 2–7 were used to determine subsite affinities of the enzyme. Kₘ and kₘ were determined by fitting the initial rate of a substrate hydrolysis to the Lineweaver–Burk equation. Rates were determined under the assay conditions described above with 10–15 different fructo-oligosaccharide concentrations ranging from 0.1 × Kₘ to 4 × Kₘ. The Michaelis constants were determined by nonlinear regression analysis [21]. The equation system developed by Hiromi [22] for exo-acting enzymes was used as a base for subsite analysis of the exoinulinase assuming Davies’ nomenclature [23] from −1 to +1, +2, etc.:

\[
\ln\left(\frac{k_{\text{cat}}}{K_{\text{m}}} - \ln\left(\frac{k_{\text{cat}}}{K_{\text{m}}}\right)_n\right) = -\left(A_n + \frac{1}{RT}\right)
\]

where Kₘ and kₘ are the kinetic parameters of the exoinulinase-catalyzed hydrolysis of β₂,₁-fructooligosaccharides of d.p. 2–7, and A is the respective subsite affinity. Assuming that there is only one nonproductive complex, A₋₁ can be obtained from the Hiromi dependence of 1/kₘ on \(\exp\left(-A_{n+1}/(RT)\right)\) [22]. The value for A₁ can be estimated from Eq. (2) based on preliminary calculated affinities A₂–A₅:

\[
\left(A_n\right)_m = \frac{55}{\exp\left(-\sum_{i=1}^{n} A_i/RT\right) + \exp\left(-\sum_{i=2}^{n+1} A_i/RT\right) + \ldots}
\]

3. ¹H-NMR investigation of the exoinulinase hydrolysis of inulin

¹H-NMR spectra were recorded with an AMX-500 Bruker spectrometer. Inulin, buffer materials and the enzyme were freeze-dried from D₂O twice prior to use. Reaction was carried out with inulin (5.4–20 mM) in 50 mM sodium phosphate buffer containing D₂O (pH 6.5). After the accumulation of the initial spectrum, approx. 10 units of the inulinase was added to complete hydrolysis of the substrate in 2–20 min. The spectra of the inulin hydrolysis were recorded at 1-min time interval during the reaction. Concentrations of substrate and products of the reaction were determined integrating the peaks characteristic for each compound: the signal at 5.43 ppm corresponds to H1 proton in glucose residue of inulin and its glucose-containing fragments, including sucrose; the signal at 5.23 ppm corresponds to H1 proton of a free α-glucose; the signal at 4.23 ppm corresponds to the H3 proton of the terminal fructofuranose of inulin; the signal at 4.32 ppm corresponds to the H3 proton of the fructose residue following the glucose; the signal at 4.29 ppm corresponds...
4. Results and discussion

4.1. Mode of action of the exoinulinase

To investigate the mode of exoinulinase action on inulin as the substrate, $^1$H-NMR analysis as an effective method was used. Results of the NMR study of hydrolysis of the inulin are shown in Fig. 2 where $^1$H-NMR spectra of the reaction mixture at the initial and intermediate stages (0 and 5 min, Fig. 2A,B, respectively), and after the hydrolysis is near completion (20 min, Fig. 2C), are given. The weak signal with the chemical shift around $\delta=5.23$ ppm corresponds to the anemic proton of $\alpha$-glucose. The figure insert shows the time course of the reaction. It demonstrates that the decrease in the concentration of inulin (squares) is closely followed by the increase in the concentration of fructose divided by the d.p. $-1$ (circles), indicating that fructose is likely the only major product of the hydrolysis. Moreover, the absence of signals in the area of 4.3–4.1 ppm that are typical for fructooligosaccharides [17] also confirms this suggestion.

The possible transglycosylating activity of the exoinulinase can be evaluated by the analysis of the product pattern of inulin hydrolysis. Transglycosylation is most likely to
occur at high substrate concentrations towards the end of the reaction, because under these conditions the concentration of potential acceptor molecules is particularly high. The indicative products of such reaction would be short fructooligosaccharides, the presence of which could be detected by the recording of differential NMR spectra, which are differences between spectrum of the reaction mixture at time \( t \) and those at time \( t_0 \), taking into consideration the degree of substrate conversion. At an inulin concentration of 20 mM and a substrate conversion of 80%, no signals at 4.3–4.1 ppm that would be characteristic for fructobioside and fructo-oligosaccharides with higher d.p. [17] were observed. Instead, the differential \(^1\)H-NMR spectra of the reaction mixture at the intermediate stage of the reaction (1 min, Fig. 3A), and after the hydrolysis is near completion (20 min, Fig. 3B) closely resemble the spectrum of fructose (Fig. 3C). Therefore, only fructose and glucose are produced as the final results of this reaction. Noteworthy, the use of \(^1\)H-NMR analysis of the reaction mixture allows detection of down to 40–60 \( \mu \)mol of an oligosaccharide formed during the reaction, which corresponds to less than 0.3% of initial inulin concentration.

The approach introduced here allowed us to employ \(^1\)H-NMR analysis to study the kinetics of inulin and inulooligosaccharide hydrolysis carried out by the exoinulinase. The method can be applied as a continuous assay without the need for termination of the reaction and isolation of intermediate and final products. The sensitivity of NMR analysis gives an indisputable advantage over TLC, HPTLC and HPLC methods [7,8] and, moreover, allows to exclude the presence of more than 1% of contaminating endoinulinase activity in the hydrolysis of inulin.

Additionally, transglycosylating activity of the exoinulinase was investigated in the hydrolysis of sucrose as a substrate in a concentration range from 40 up to 200 mM and at several pH values from 3.5 to 7.0. Despite of 91% similarity with \( A. \) foetidus fructosyltransferase according to their amino acid sequences [9], the \( A. \) awamori exoinulinase was found not to produce 1-kestose, the product of fructosyltransferase reaction expected to be most prevalent in the considered substrate concentration range [14], as evidenced by HPLC analysis on a Dextra-Pac column and TLC methods. Moreover, inulotriose, inulotetraose and levano- triose investigated as substrates in the transglycosylation reaction at the concentrations up to 50 mM and pH values from 3.5 to 7.0 did not yield the predicted corresponding products, namely inulotetraose, inulopentaose and levanto- triose. Difructosides were also not formed in the exoinulinase reactions when the enzyme was incubated with fructose in the concentration range of 50–200 mM. In all these analyses, the level of substrate hydrolysis was at least 20–25%. Therefore, based on these results, we have no evidence that the exoinulinase from \( A. \) awamori 2250 possesses transglycosylating activity.

Fig. 3. The differential \(^1\)H-NMR spectra of the reaction mixture of the inulin hydrolysis at the intermediate stage (A) and after the hydrolysis is near completion (B). The spectrum (C) is the \(^1\)H-NMR spectrum of fructose.
4.2. Multiplicity of the exoinulinase hydrolytic action

As it was reported earlier for starch-degrading enzymes, endo-acting α-amylases [24,25], exo-acting β-amylases and glucoamylase [26–28], hydrolysis of polymeric substrate can occur with multiplicity of attack. Here, the primary product of the enzymic action serves as the secondary substrate for the next act of the hydrolysis. After the initial catalytic action, the product can recover itself on the enzyme surface giving a new productive enzyme–substrate complex. This can be repeated several times before the final dissociation of the enzyme–substrate complex. This can be repeated several times before the final dissociation of the enzyme–substrate complex. Besides polymeric substrates, starch and glycogen [24,25], shorter ones, maltooligosaccharides [27,28], were used to investigate the phenomenon of multiple-attack mode of action. The concept of multiple-attack mode of action was studied with the exoinulinase from A. awamori using fructooligosaccharides as substrates.

Taking the possible multiplicity of attack into account, the reaction of fructooligosaccharide hydrolysis effected by the exoinulinase may occur in two ways:

\[
(F\text{ru})_n + E \xrightleftharpoons{k_{-1}} (F\text{ru})_n E \xrightarrow{k_2} E + (F\text{ru})_{n+1} + \text{Fru}
\]

where \((F\text{ru})_n\) is a fructooligosaccharide with d.p. = \(n\), E is the enzyme, \(k_{-1}\) and \(k_{+1}\) are dissociation constants, \((F\text{ru})_{n-1}\) is a fructooligosaccharide with d.p. = \(n - 1\), and Fru is fructose. Such minimal kinetic scheme can be discussed in case of the absence of transglycosylation as was proven above for the exoinulinase described here. If multiplicity of attack occurs, \(k_3\) becomes greater than zero. Both pathways can exist in parallel and be of similar relevance if constants \(k_2\) and \(k_3\) are in the same range. The extent of multiplicity of attack by the exoinulinase can be calculated using Eq. (3) developed earlier [29]:

\[
\Theta = \frac{zN}{-\ln \left( \frac{[SN_t]}{[SN_0]} \right)}
\]

where \(\Theta\) is the extent of multiplicity of attack, \(z\) is the extent of hydrolysis of the substrate consisting of \(n\) fructose units at the time \(t\), \([SN_0]\) and \([SN_t]\) are concentrations of the substrate at the initial point \(t_0\) and time \(t\). Hydrolysis of inulooligosaccharides with d.p. 4–7 and levanopentaose was analyzed by HPLC of the reaction products after different time intervals (data are given in Fig. 4). The value for the multiplicity extent \(\Theta\), which was calculated according to Eq. (3), was about 1 for all tested compounds (Table 1). Therefore, multiple-attack mechanism of the exoinulinase towards inulooligosaccharides with d.p. 4–6 was not observed.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Multiplicity extent, (\Theta)</th>
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<tbody>
<tr>
<td>Inulotetraose</td>
<td>1.05 ± 0.04</td>
</tr>
<tr>
<td>Inulopentaose</td>
<td>1.03 ± 0.04</td>
</tr>
<tr>
<td>Inulohexaose</td>
<td>1.08 ± 0.02</td>
</tr>
<tr>
<td>Inuloseptaose</td>
<td>1.07 ± 0.02</td>
</tr>
<tr>
<td>Levanopentaose</td>
<td>1.15 ± 0.12</td>
</tr>
</tbody>
</table>

Fig. 4. (A) Time-dependent HPLC analysis of the exoinulinase-catalyzed hydrolysis of inulopentaose (○); formation of inulotetraose (□); formation of fructose (▲). (B) Time-dependent HPLC analysis of the exoinulinase-catalyzed hydrolysis of inulohexaose (■); formation of inulopentaose (○); and formation of fructose (▲).
4.3. Hydrolytic activity and subsite structure of the active center

Previously, we determined kinetic parameters of the *Aspergillus* exoinulinase in the hydrolysis of inulin, levan, stachyose and raffinose [9]. The exoinulinase hydrolyzed sucrose with $K_m = 40 \text{ mM}$ and $k_{cat} = 1150 \pm 10 \text{ s}^{-1}$. The specificity of the inulinase action was also investigated in the hydrolysis of β2,6-fructooligosaccharides (levanooligosaccharides) with d.p. 2–6. Kinetics of levanooligosaccharide hydrolysis of the exoinulinase followed Michaelis–Menten kinetics. The values for $k_{cat}$ and $K_m$ obtained with these substrates are given in the Table 2. Rates were determined under the assay conditions described above with 12–16 different levanooligosaccharide concentrations ranging from $0.1 \times K_m$ to $4 \times K_m$. The initial rates of hydrolysis of β2,1-fructooligosaccharides (inulooligosaccharides) with d.p. 2–7 were measured as a function of substrate concentration. Plots of the hydrolytic rates versus oligosaccharide concentrations were linear at the initial stages of the reaction and, therefore, obeyed Michaelis–Menten kinetics ranging from at least $0.1 \times K_m$ to $4 \times K_m$ (Table 3). Kinetic analysis of the inulinase indicates that hydrolytic reactions of inulooligosaccharides with lower d.p. occur with a lower rate. The catalytic efficiency factor, $k_{cat}/K_m$, also decreases depending on the d.p. of the substrates in the same way. The preference of the exoinulinase for β2,1-fructooligosaccharides of increasing chain length suggested that it has an extended substrate-binding region. This was confirmed by calculation of binding energies based on kinetic results in accordance with Hiromi et al. [12,22] and as presented in Fig. 5. The results indicate that the exoinulinase has an extended active center consisting of at least five fructosyl-binding sites. The highest values for binding energies were at subsites $-1$ and $+1$. Similar high affinities for subsites $-1$ and $+1$ were reported for retaining barley β-D-glucosidase/(1,4)-β-D-glucan exohydrolase, which belongs to glycoside hydrolase family 3 [30] and inverting exo-β-1,3-glucanase from *Trichoderma viride* [13]; retaining α-glucosidase of family 13 and β-glucosidase of family 1 [31]. Unfortunately, these data do not allow us to speculate about the relationships between retention/inversion mechanism of the *A. awamori* inulinase action and its binding energies in the particular sites of the active center. However, kinetic and subsite structure data confirm our earlier suggestion that the enzyme can be classified as exo-acting inulinase and not β-fructofuranosidase [32].

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$K_m$ [mM]</th>
<th>$k_{cat}$ [s$^{-1}$]</th>
</tr>
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<tbody>
<tr>
<td>Levanotriose</td>
<td>0.21 ± 0.01</td>
<td>1840 ± 92</td>
</tr>
<tr>
<td>Levanotetraose</td>
<td>2.74 ± 0.14</td>
<td>1990 ± 89</td>
</tr>
<tr>
<td>Levanopentaose</td>
<td>10.2 ± 0.31</td>
<td>575 ± 18</td>
</tr>
<tr>
<td>Levanohexaose</td>
<td>1.01 ± 0.02</td>
<td>570 ± 6</td>
</tr>
</tbody>
</table>

As seen from Tables 2 and 3, the exoinulinase hydrolyzes levanooligosaccharides and inulooligosaccharides with comparable rate constants suggesting that the enzyme is built to act similarly well on both β-2,6- as well as β-2,1-fructosyl bonds. However, the rate of the hydrolysis for levan is much lower than that for inulin [9]. This may be explained by differences of space structures of levan and inulin [17]. Another possible explanation might be interactions between the enzyme surface and polysaccharides (e.g. inulin and levan) as it is described for the hydrolysis of laminarin by *Candida albicans* β-1,3-exoglucanase [33]. It should be noted that the $K_m$ for the hydrolysis of inulin by the exoinulinase has the value of 0.05 mM [9], which is lower than the relative constants for the hydrolytic reactions of inulooligosaccharides with d.p. 5–7 (Table 2). On the other hand, the application of Hiromi approaches showed the presence of at least five binding sites. Similar dependence was reported earlier for glucoamylase from fungal sources [12,22] in the hydrolysis of maltooligosaccharides and...
starch. For both enzymes, the presence of additional non-catalytic binding sites for polymeric substrates, glycogen and starch was well documented [34]. Possibly, the exoinulinase considered here also has additional binding site for inulin located near the active center. Structure investigations of the enzyme, which are in progress now, will allow to answer this question.

Acknowledgements

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