Streptomyces matensis laminaripentaose hydrolase is an 'inverting' β-1,3-glucanase

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Abstract The laminaripentaose-producing β-1,3-glucanase of *Streptomyces matensis* is a member of the glycoside hydrolase family GH-64. We have constructed and purified a recombinant hexahistidine-tagged form of the enzyme for characterisation. The enzyme, which exists as a monomer in solution, hydrolyses β-1,3-glucan by a mechanism leading to overall inversion of the anomeric configuration. This is the first determination of the mechanism prevailing in glycoside hydrolase family GH-64 and this is the first characterisation of an 'inverting' β-1,3-glucanase.

Key words: β-1,3-Glucanase; Laminaripentaose hydrolase; Reaction mechanism; *Streptomyces matensis*

1. Introduction

O-glycoside hydrolases (EC 3.2.1.-) are a widespread group of enzymes which hydrolyse the glycosidic bond between two or more carbohydrates or between a carbohydrate and a non-carbohydrate moiety. To complement the traditional classification of these enzymes based on their substrate specificity, a classification of glycoside hydrolases in families based on amino acid sequence similarities has been proposed a few years ago [1–3]. These families are available on a permanently updated web site (http://afmb.cnrs-mrs.fr/~pedro/CAZY/db.html [4]). Amongst the 85 known families of glycoside hydrolases, β-1,3-glucanases (endo-β-1,3-glucanase, EC 3.2.1.39; exo-β-1,3-glucanase, EC 3.2.1.58) are found in several distinct families (GH-5, GH-16, GH-17, GH-55, GH-64 and GH-81). These enzymes cleave the β-1,3-bonds found in a variety of β-1,3-glucans such as laminarin or pachyman [5]. The bacterium *Streptomyces matensis* produces a β-1,3-glucanase which displays a unique particular product specificity, since it produces exclusively the pentasaccharide laminaripentaose from β-1,3-glucan [6]. Cloning and sequencing of the gene allowed to assign this enzyme to glycoside hydrolase family GH-64 [6].

Enzymatic hydrolysis of glycosidic bonds occurs with two possible stereochemical outcomes: inversion or retention of the anomeric configuration at the site of cleavage [7,8]. 'Inverting' enzymes utilise a single-displacement reaction where an activated water molecule performs a nucleophilic attack at the sugar C-1 while concomitant aglycone departure is achieved by protonation of the glycosidic oxygen. By contrast, 'retaining' enzymes utilise a double-displacement mechanism involving a covalent glycosyl–enzyme intermediate. In the first step, one carboxyl group functions as a general acid, protonating the glycosidic oxygen concomitantly with bond cleavage. A second carboxyl group acts as a nucleophile, forming the covalent intermediate. In the second step, the deprotonated acid catalyst can act as a base and activate an incoming water molecule which performs the second nucleophilic attack at the sugar anomeric carbon and thereby releases the free sugar with an anomeric stereochemistry identical to that of the substrate [7–10]. It has been shown that the molecular mechanism is conserved within the sequence-based families of glycoside hydrolases [11,12]. In consequence, an analysis of the stereochemical outcome of the reaction catalysed by one member of a given family is sufficient to ascertain the mechanism in this family. For β-1,3-glucanases, the mechanism has been established in families GH-16 [13] and GH-17 [14], and both families were found to operate with overall retention of the anomeric configuration. The (retaining) mechanism prevailing in family 5 has also been determined [11,15]. The mechanism is not known for the other families containing β-1,3-glucanases. We have therefore initiated a mechanistic study of the *S. matensis* laminaripentaose-producing β-1,3-glucanase (LPHase) in order to elucidate the molecular mechanism which prevails in glycoside hydrolase family GH-64.

2. Materials and methods

2.1. Subcloning

The native coding sequence of *S. matensis* DIC-108 LPHase was amplified, and its 5′ and 3′ ends modified, by PCR. In practice, 100 ng of plasmid pIHL (a pET28a+ vector bearing the *S. matensis* DIC-108 LPHase gene (1)) was used as template with 5′ primer CTGCAGGAATTCGGATCCGCGGTCCCGGCCACCATCCC and 3′ primer GTCGACGTTAACAAGCTTTCAGTCGAACGGGTCGAGCG in the following programme: (96°C 5 min)×1, (96°C 45 s, 68°C 2 min)×30, (68°C 10 min)×1, in a 50 μl reaction volume and 2.5 U of pFx DNA polymerase (Life Technology). The 5′ primer removed the original ATG and signal peptide, and incorporated an in-frame

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Abbreviations: LPHase, laminaripentaose-producing β-1,3-glucanase; SDS–PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis; IPTG, isopropyl 1-thio-β-D-galactoside

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*Bam*HI restriction site immediately upstream of the codon encoding the first residue of the mature protein (A36, (1)). The 3′ primer introduced a *Hin*dIII restriction site immediately after the natural TGA stop codon (1). A 5 μl aliquot was run on agarose gel to verify the efficiency and specificity of the reaction, and the amplification product contained in the remaining 45 μl was purified with a QIAquick PCR purification kit (Qiagen). The DNA was then digested with *Bam*HI and *Hin*dIII and, after separation from the restriction digestion by-products by preparative agarose gel electrophoresis, it was ligated to *Bam*HI/*Hin*dIII-digested pQE-30 (Qiagen). *Escherichia coli* DH5α cells were transformed with the ligation product. Positive clones were screened by ampicillin resistance and colony PCR using the primers described above.

2.2. Protein expression

The plasmidic DNA from one positive clone was recovered by miniprep (Qiagen), sequenced to assert the lack of misincorporation by the polymerase and then used to transform *E. coli* M15pREP4 cells (Qiagen). Transformed cells underwent overnight double antibiotic selection on LB plates containing 100 μg/ml ampicillin and 30 μg/ml kanamycin. Subsequently, 10 randomly picked colonies were grown individually at 37°C in 1 ml of LB containing the same antibiotics (LBAmpKana), and expression of LPHase was induced by addition of 1 mM isopropyl 1-thio-β-D-galactoside (IPTG) for 2 h. The result of the expression was checked by running 50 μl aliquots of the culture on sodium dodecyl sulphate (SDS) 12% acrylamide gel, after boiling in denaturing and reducing sample buffer.

The solubility of the protein produced under these experimental conditions was tested by performing the same experiment, except that soluble and insoluble materials were separated prior to electrophoresis by sonicating the bacteria in 20 mM Tris pH 8.0, 200 mM NaCl followed by centrifugation for 5 min at 15 000 rpm in a bench minicentrifuge. For large scale production, an M15pREP4 clone was grown at 37°C in 1 l of LBAmpKana up to an absorbance of 1 at 600 nm. At this point, expression of the protein was induced for 2 h by addition of 1 mM IPTG. Bacteria were centrifuged and the pellet stored at −20°C until use.

2.3. Protein purification

The bacterial pellet was resuspended in 30 ml of 20 mM Tris pH 8.0, 200 mM NaCl, 1 mM pefabloc (Roche) and the cells were lysed with a French press. The lysate was centrifuged for 20 min at 17 000×*g* and the supernatant further clarified by filtration over Nalgene cellulose acetate 25 mm syringe filters (0.8 and then 0.2 μm pore size). His-tagged LPHase was isolated from the filtered supernatant by immobilised metal ion adsorption chromatography (IMAC) using a 5 ml Hitrap chelatin column (Amersham-Pharmacia Biotech) and a linear 0–500 mM imidazole gradient. The fractions containing a major protein of the expected size were pooled, dialysed on Amicon membrane (cut-off: 30 kDa) against 20 mM MES pH 6.0, 20 mM NaCl and loaded on a 1 ml Hitrap SP Sepharose column (Amersham-Pharmacia Biotech) from which the protein was eluted by a 20–500 mM NaCl linear gradient in the same buffer.

The mono/oligomericity of the purified protein was assessed by gel filtration on a Superdex 200 column (Amersham-Pharmacia Biotech). The elution was run at 0.5 ml per min in 20 mM Tris pH 8.0, 200 mM NaCl. The void volume was defined as the elution volume of dextran blue (2000 kDa). Ferritin (440 kDa), nitrite reductase (120 kDa), bovine serum albumin (68 kDa), ovalbumin (45 kDa) chymotrypsinogen (19 kDa) and ribonuclease (14 kDa) were used as standards of molecular mass (Amersham-Pharmacia Biotech). A calibration curve was obtained by reporting the log of molecular mass as a function of the retention coefficient (elution volume/void volume: Ve/Vo) for each protein. The straight line $y = -1.16x + 6.735$, with correlation coefficient of 0.95 with six experimental points, was used to calculate the molecular mass of recombinant LPHase on the basis of its retention coefficient ($Ve/Vo = 2$) on the same column and under the same conditions. N-terminal sequencing and MALDI-TOF mass spectroscopy were performed using the facilities provided by the Institute of Structural Biology and Microbiology.

2.4. NMR experiments

The stereochemistry of the hydrolysis reaction was determined by ^{1}H-NMR performed at 27°C in a Bruker DRX 500 spectrometer equipped with a ^{1}H 5 mm probe and operating at 500 MHz. The β-1,3-glucan substrate was laminarin from *Laminaria digitata* (Sigma). In preparation for NMR, 5 mg substrate was exchanged by lyophilisation from 500 μl 99.8% D_2O (once) and 500 μl 99.96% D_2O (twice), then redissolved in 400 μl 99.96% D_2O and placed in a dry 5 mm NMR tube. Purified recombinant LPHase (8.7 μl, 6.83 mg/ml in 20 mM Tris pH 8.0, 200 mM NaCl) was diluted with 50 μl of sodium acetate buffer (50 mM, pH 6.0) also exchanged against D_2O using the same procedure as the substrate. The enzyme was finally redissolved in 50 μl 99.96% D_2O. To further remove the residual water signal during spectra acquisition, a moderate water presaturation (70 dB attenuation) was applied. After recording the spectrum of the substrate, the 50 μl D_2O-exchanged LPHase was added to the tube which was immediately placed back in the spectrometer. The first spectrum was recorded 3 min after the addition of the enzyme, then spectra were recorded every 3 min during 15 min. Spectra were then recorded 30 min, 60 min, 90 min, 120 min, 150 min and 210 min after the addition of the enzyme. A last spectrum was recorded 72 h after the beginning of the reaction. All spectra were recorded over a 7500 Hz sweep width with eight scans for a total recording time of 25 s each.

3. Results and discussion

3.1. Expression and purification of His-tagged LPHase

For a straightforward purification of recombinant LPHase expressed in *E. coli*, the full length sequence encoding *S. matensis* DIC-108 LPHase was subcloned into a prokaryotic expression vector encoding a hexahistidine stretch at the N-terminus of the enzyme. As described in Section 2, the plasmid construct was transferred to *E. coli* M15pREP4 cells for expression. Surprisingly, only one clone out of 10 proved to express the protein after induction by IPTG.

Most of the protein was expressed in a soluble form in the cytosol of the bacteria under these basic experimental conditions (incubation temperature 37°C and IPTG concentration 1 mM, data not shown). This indicated that export to the periplasmic compartment was dispensable to produce a soluble (and functional, see below) protein. We therefore kept these parameters unchanged for large scale production.

As shown in Fig. 1, the first affinity column (IMAC) was sufficient to obtain an almost pure protein. However, an additional cation exchange chromatography was performed to eliminate some high and low molecular weight contaminants that were detectable on heavily loaded gels. N-terminal se-

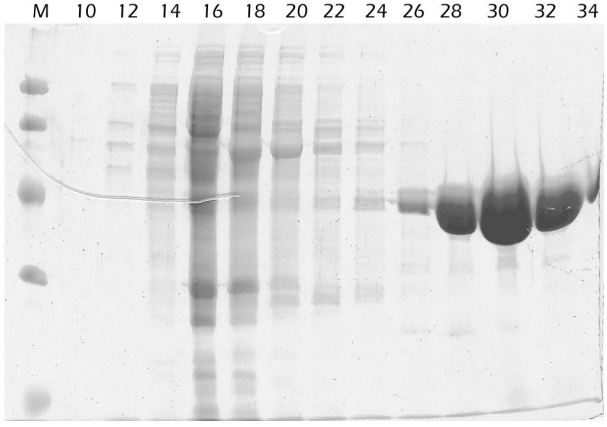

Fig. 1. Purification of His-tagged LPHase on Ni affinity column. The experiment was conducted as described in Section 2. The proteins eluted by the imidazole gradient were recovered as 1 ml fractions. A 30 μl aliquot of fractions 10–34 was run on 12% SDS–acrylamide gel, and the gel was stained with Coomassie blue. Low molecular weight markers were run in the 'M' lane (from top to bottom: 97.4, 66.2, 45, 31 and 21.5 kDa).

quencing revealed that the following sequence HHHHGSAVPATI accounted for 90% of the protein content after the cation exchange step and corresponded to the last four His residues of the His tag, followed by the two residues (GS) encoded by the two codons constitutive of the *Bam*HI cloning site (GGATCC), and the first six residues (AVPATI) of the mature (i.e. after removal of the signal sequence) LPHase. It is interesting to note that four (instead of six) His residues are sufficient for efficient binding to (and elution from) Ni affinity column over the non-specific binding/elution of *E. coli* His-rich proteins. Taking into account this N-terminal truncation, the molecular mass calculated from the coding sequence was 40 113.5 Da. On the other hand, mass spectroscopy revealed the existence of two isoforms of 40 834 and 41 364 Da, respectively (data not shown). Surprisingly, although the purified protein definitely behaved as a single species in molecular filtration, its retention coefficient accounted for a molecular mass of only 26 kDa (Fig. 2). However, the protein eluted from the molecular filtration migrated on SDS–polyacrylamide gel electrophoresis (PAGE) at a position (around 40 kDa, see inset in Fig. 2) in close agreement to that measured by mass spectroscopy or that predicted from the amino acid sequence.

3.2. Stereochemical course of the reaction catalysed by LPHase

LPHase hydrolyses different types of β-1,3-glucans such as colloidal pachyman and laminarin [6]. After checking by TLC that His-tagged LPHase hydrolysed colloidal pachyman similarly to the wild-type recombinant enzyme (data not shown), laminarin was chosen as the substrate for NMR analysis of the stereochemistry of hydrolysis because of its better solubility. Fig. 3A shows the partial ^{1}H-NMR spectrum of the starting β-1,3-glucan in the 4.4–5.3 ppm region. Only traces of signals referable to a free hemiacetal H-1 are observed at 4.67 (β-anomer) and 5.24 (α-anomer) ppm in accord with the polymeric structure of the substrate. Spectra recorded 6 min, 12 min, 30 min, 150 min, 72 h after the addition of the enzyme and illustrating the stereochemical course of the reaction are shown in Fig. 3B–F. The spectrum corresponding to 6 min of hydrolysis (Fig. 3B) shows a new signal centred at 5.24 ppm ($J_{1,2}$ = 3.5 Hz) assignable to H-1 of a reducing oligosaccharide in α-configuration. As the reaction continued, the doublet at 5.24 ppm kept growing (Fig. 3C,D) and a new signal appeared and increased at 4.67 ppm ($J_{1,2}$ = 8.0 Hz) 150 min after the addition of the enzyme (Fig. 3E). This peak corresponds to reducing H-1 in β-configuration originating from spontaneous mutarotation of the α-anomer generated by the enzyme. At 150 min, the α/β ratio of 1.64 (calculated after integration of the signals at 4.67 and 5.24 ppm, respectively), far from the ∼0.66 value expected at mutarotation equilibrium [16], further demonstrates that the α-anomer was indeed initially formed. The peaks assignable to the β- and α-anomers continued to grow after 150 min reaction, with an α/β ratio of 0.92 after 72 h (Fig. 3F). This indicates that although the α-anomer is the primary product of the reaction, mutarotation progressively became dominant. This demonstrates that the enzymatic hydrolysis by LPHase occurs with inversion of the anomeric configuration and that LPHase operates with a single-displacement catalytic mechanism.

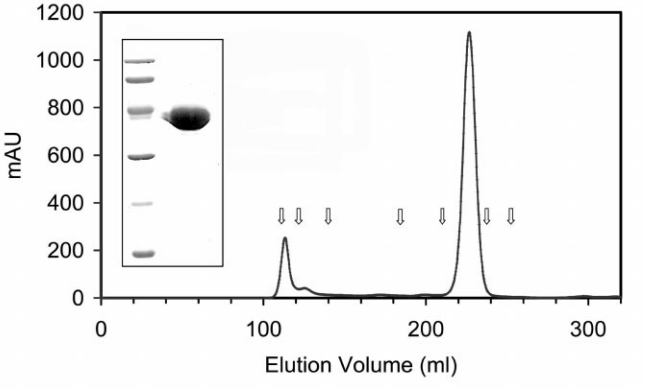

Fig. 2. Gel filtration analysis of the monomer/oligomer state of recombinant His-tagged LPHase. A 12 mg/ml solution of purified LPHase was resolved by gel filtration as described in Section 2. The figure represents the absorbance at 280 nm (arbitrary units) as a function of the elution volume (ml). The two main peaks at elution volumes 113 and 226 ml account for dextran blue and recombinant LPHase, respectively. Vertical arrows indicate the positions of the molecular mass standards. From left to right: dextran blue (Ve = 113 ml), ferritin (Ve = 123 ml), nitrite reductase (Ve = 140 ml), albumin (Ve = 184 ml), ovalbumin (Ve = 210 ml), chymotrypsinogen (Ve = 237 ml), ribonuclease (Ve = 252 ml). Inset: SDS–PAGE analysis of the purified His-tagged LPHase (right lane) along with the following molecular weight markers (left lane, from top to bottom: 97.4, 66.2, 45, 31, 21.5 and 14.4 kDa).

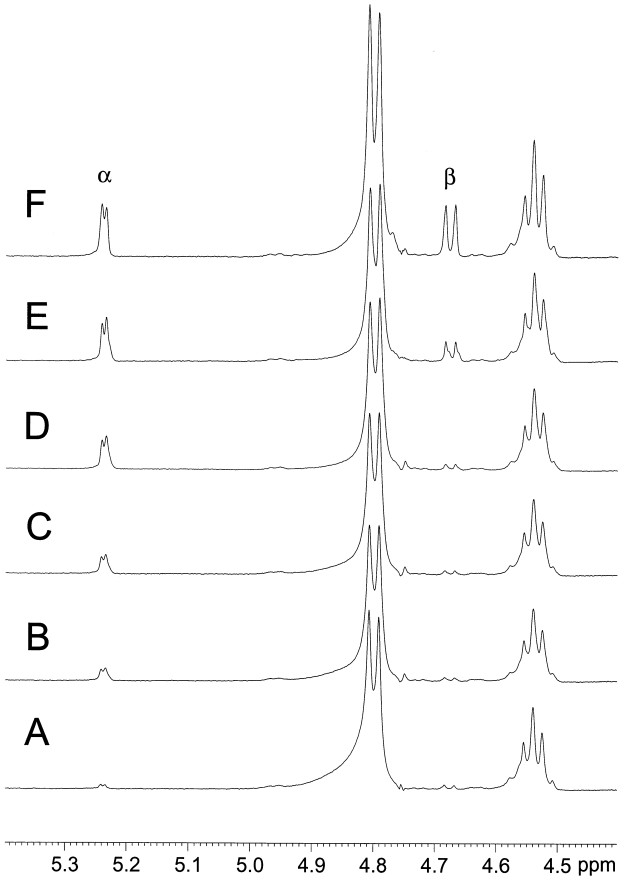

Fig. 3. ^{1}H-NMR spectra during the hydrolysis of laminarin by LPHase. A: initial substrate; B–F: same as A but 6 min, 12 min, 30 min, 150 min and 72 h, respectively, after the addition of the enzyme. The signals corresponding to the α- and β-anomers of free H-1 are indicated.

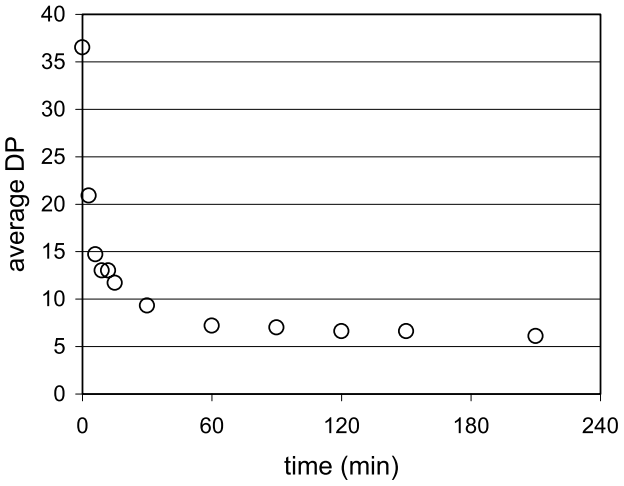

Fig. 4. Evolution of the average degree of polymerisation of laminarin during the course of hydrolysis by LPHase.

The degree of polymerisation (DP) of laminarin could be estimated by dividing the intensity of the signals at 4.75 ppm corresponding to H-1 protons of β-1,3-linked glucose units by the intensity of the signals of reducing H-1 (sum of the signals at 5.24 and 4.67 ppm). This approximation gave an average DP of 36.5 for the initial laminarin substrate. Upon addition of LPHase, the DP fell down rapidly to reach a plateau approaching the value of 5 (Fig. 4), in perfect agreement with the reported product specificity of the enzyme [6].

This is the first determination of the stereochemistry of hydrolysis in family GH-64. Interestingly, while all β-1,3-glucanases previously studied were 'retaining' enzymes, LPHase is the first inverting β-1,3-glucanase characterised. The inverting mechanism implies that the molecular mechanism of hydrolysis by LPHase does not involve the formation of a covalent glycosyl–enzyme intermediate. Since the molecular mechanism has been shown to be conserved within the families of glycoside hydrolases [11], the present demonstration is sufficient to conclude that all family GH-64 glycoside hydrolases operate by an inverting mechanism.

Three-dimensional structures have been determined for β-1,3-glucanases of families GH-5, GH-16 and GH-17 [17–19]. Since LPHase does not display any sequence similarities with these β-1,3-glucanases and since LPHase operates by an inverting mechanism while enzymes of families GH-5, GH-16 and GH-17 operate by retention of the anomeric configuration, one can expect LPHase to display considerable differences in its active site compared to that of members of families GH-5, GH-16 and GH-17. The efficient preparation and purification of a hexahistidine-tagged form of LPHase reported here now allows us to initiate a structural study of the enzyme. Such a study should also reveal the particular structural features which give rise to the unusual and narrow product specificity of LPHase.

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