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Purification and characterization of a novel thermostable α-L-arabinofuranosidase (α-L-AFase) from *Chaetomium* sp.

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ABSTRACT

The purification and characterization of an extracellular α-L-arabinofuranosidase (α-L-AFase) from *Chaetomium* sp. was investigated in this report. The α-L-AFase was purified to homogeneity with a purification fold of 1030. The purified α-L-AFase had a specific activity of 20.6 U mg^{-1}. The molecular mass of the enzyme was estimated to be 52.9 kDa and 51.6 kDa by SDS–PAGE and gel filtration, respectively. The optimal pH and temperature of the enzyme were pH 5.0 and 70 °C, respectively. The enzyme was stable over a broad pH range of 4.0–10.0 and also exhibited excellent thermostability, i.e., the residual activities reached 75% after treatment at 60 °C for 1 h. The enzyme showed strict substrate specificity for the α-L-arabinofuranosyl linkage. The K_m and V_{max} values for *p*-nitrophenyl (*p*NP)-α-L-arabinofuranoside were calculated to be 1.43 mM and 68.3 μmol min^{-1} mg^{-1} protein, respectively. Furthermore, the gene encoding α-L-AFase was cloned and sequenced and found to contain a catalytic domain belonging to the glycoside hydrolase (GH) family 43 α-L-AFase. The deduced amino acid sequence of the gene showed the highest identity (67%) to the putative α-L-AFase from *Neurospora crassa*. This is the first report on the purification, characterization and gene sequence of an α-L-AFase from *Chaetomium* sp.

1. Introduction

After cellulose, hemicellulose is the second most abundant polysaccharide in plant cell walls, representing approximately 20–35% of lignocellulosic biomass [1]. L-Arabinosyl residues are widely distributed in hemicelluloses constituting the monomeric or oligomeric side chains on the β-(1,4)-linked xylose or galactose backbones in arabinan, arabinoxylan, and arabinogalactan [2]. The presence of arabinose and other branches distinctly restricts the hydrolysis of hemicelluloses [3]. Hence, the complete biodegradation of hemicelluloses requires a wide variety of hydrolytic enzymes. A number of accessory enzymes, such as α-L-arabinofuranosidases, a-glucuronidases, acetylxylan esterases, and phenolic acid esterases are responsible for the cleavage of the side chains [4]. The enzyme α-L-arabinofuranosidase (α-L-AFase, EC 3.2.1.55) is capable of hydrolyzing α-L-arabinofuranosides, such as aryl α-L-arabinofuranosides and non-reducing α-L-arabinofuranosyl residues, from arabinofuranose-containing substrates [5]. Based on the amino acid sequence similarity, α-L-arabinofuranosidases have been classified in the glycoside hydrolase (GH) families 43, 51, 54 and 62 [6].

The α-L-AFases have recently received much attention for their potential application in agro-industrial processes, such as functional L-arabinose sugar production, improvement of feed digestibility, bioconversion of biomass to fuel, and clarification of juices [7,8]. Many α-L-AFases have been isolated and characterized from various fungi [9–13], bacteria [14–16], and plants [17,18]. Most of the reported fungal α-L-AFases have focused on mesophilic fungi, such as *Trichoderma* [19,20], *Aspergillus* [21–24] and *Penicillium* [5,12,25,26]. The enzymes from thermophilic fungi are generally more attractive options for industrial use because they have higher optimal temperatures and thermostability compared with enzymes from mesophilic fungi. There are few reports on the α-L-AFases from thermophilic fungi, such as *Talaromyces emersonii* [9] and *Talaromyces thermophilus* [27]. Moreover, no reports are available on the purification and properties of α-L-AFases from the thermophilic *Chaetomium* species.

In our previous studies, a newly isolated *Chaetomium* sp. CQ31 was identified as a species of moderately thermophilic fungus; the strain can secrete thermostable xylanases when grown in a medium that uses corncob as a carbon source [28]. In further investigations

Abbreviations: CAPS, (cyclohexylamino)-1-propanesulphonic acid; CHES, 2-(cyclohexylamino)ethanesulfonic acid; GH, glycoside hydrolase; MES, 2-(N-morpholino)ethanesulfonic acid; MOPS, 3-(N-morpholino)-propanesulfonic acid; PDA, potato dextrose-agar; *p*NP, *p*-nitrophenyl; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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using the same medium, the strain also produced α-L-AFase. Hence, the objective of the present study was to purify and characterize extracellular α-L-AFase from *Chaetomium* sp. CQ31. In addition, the α-L-AFase gene was cloned and sequenced.

2. Materials and methods

2.1. Materials

TRIzol (Invitrogen, Carlsbad, USA) and Oligotex mRNA Midi Kit (Qiagen, Hilden, Germany) were used for total RNA extraction and mRNA purification. SMARTer™ RACE cDNA Amplification was purchased from Clontech (Palo Alto, California, USA). The pMD18-T vector and LA Taq DNA polymerase were purchased from TaKaRa (Takara, Dalian, China). The *p*-nitrophenyl (*p*NP)-β-D-fucopyranoside was purchased from Biosynth (Staad, Switzerland); *p*NP-β-D-xylopyranoside, *p*NP-α-L-arabinopyranoside, *p*NP-β-D-glucopyranoside, *p*NP-α-L-arabinofuranoside, *p*NP-α-D-galactopyranoside and *p*NP-β-D-galactopyranoside were purchased from Sigma–Aldrich (St. Louis, Missouri, USA). Corncobs were obtained locally, cut into small pieces, and ground in a hammer mill. Q-Sepharose Fast Flow and Sephacryl-100 HR gels were purchased from Pharmacia (Uppsala, Sweden). All other chemicals used were analytical grade unless otherwise stated.

2.2. Fungal strain and growth conditions

Chaetomium sp. CQ31 was preserved in the China General Microbiological Culture Collection Center (CGMCC, accession no. 3341). It was maintained on potato dextrose-agar (PDA) medium at 4 °C and transferred to fresh medium every 6–7 weeks. The PDA plates were incubated at 37 °C for 5 d and then stored at 4 °C until use.

For α-L-AFase production, the fermentation medium contains corncob 40 g l^{-1}, beef peptone 12 g l^{-1}, $MgSO_4{\cdot}7H_2O$ 0.3 g l^{-1}, $CaCl_2$ 0.3 g l^{-1}, $(NH_4)_2SO_4$ 0.3 g l^{-1}, $FeSO_4$ 0.3 g $l^{-1}$and Tween 80 5 g l^{-1}. The initial pH of the culture medium was adjusted to pH 7.0 and not controlled during the fermentation process. A 1-cm^2 piece of agar medium covered with 5-d-old mycelia was transferred into a 250-ml Erlenmeyer flask containing 50 ml of fermentation medium and incubated at 35 °C on a rotary shaker at 200 rpm for 7 d. Following incubation, the broths were centrifuged at 10,000 × *g* for 10 min, and the supernatant was used as crude enzyme for subsequent analysis.

2.3. Enzyme assay and protein determination

The activity of α-L-AFase was assayed using *p*NP-α-L-arabinofuranoside as the substrate, according to the method of Chacon et al. [11] with slight modifications. Each reaction contained 180 μl of 1 mM *p*NP-α-L-arabinofuranoside and 20 μl of enzyme in 50 mM citrate buffer (pH 6.0). After incubation at 50 °C for 10 min, the reaction was quenched by adding 100 μl of 1 M Na_2CO_3, and the amount of *p*NP released was measured at 410 nm. One unit of α-L-AFase activity was defined as the amount of the enzyme required to release 1 μmol of *p*NP from *p*NP-α-L-arabinofuranoside per minute under the above conditions. The specific activity was expressed as units per milligram of protein.

Protein concentrations were measured by the Lowry method [29] using BSA (bovine serum albumin) as the standard.

2.4. Purification of α-L-AFase

The crude enzyme was subjected to 40–50% ammonium sulfate saturation. After stirring at 4 °C for 1 h, the precipitated proteins were collected by centrifuging at 10,000 × *g* for 10 min. The proteins were then dissolved in 20 mM Tris–HCl (pH 9.0) buffer and dialyzed against the same buffer overnight. The dialyzed sample was then loaded onto a Q-Sepharose Fast Flow (10 cm × 1.0 cm) ion exchange column pre-equilibrated with 20 mM Tris–HCl (pH 9.0) buffer. The bound proteins were eluted with a gradient of 0–125 mM NaCl at a flow rate of 3.0 ml min^{-1}; the fractions with α-L-AFase activity were collected and concentrated. A 0.5-ml concentrated sample was loaded onto a Sephacryl S-100 HR (100 cm × 1.0 cm) gel filtration column, which was pre-equilibrated with 50 mM Tris–HCl buffer (pH 7.0). The protein was eluted at a flow rate of 0.3 ml min^{-1}. The purity of α-L-AFase in the purification process was analyzed by SDS–PAGE. All purification steps were carried out at 4 °C unless otherwise stated.

2.5. SDS–PAGE and native molecular mass determination

The homogeneity of the purified α-L-AFase was determined by SDS–PAGE using a 12.5% acrylamide gel as described by Laemmli [30]. Protein bands were visualized by staining with Coomassie brilliant blue R-250. The molecular weight standards (Pharmacia) used for the calibration of the molecular mass of α-L-AFase were phosphorylase b (97.0 kDa), albumin (66.0 kDa), ovalbumin (45.0 kDa), carbonic anhydrase (30.0 kDa), trypsin inhibitor (20.1 kDa) and a-lactalbumin (14.4 kDa).

Native molecular mass of the purified α-L-AFase was determined by gel filtration chromatography on a Superdex-75 column (40 cm × 1.0 cm) previously equilibrated with 50 mM Tris–HCl (pH 7.0) buffer. The protein sample was injected and eluted at a flow rate of 0.35 ml min^{-1} with the same buffer. Molecular weight standards from Sigma used to calibrate the column were phosphorylase b (97.2 kDa), bovine serum albumin (68.0 kDa), albumin (45.0 kDa), and chymotrypsinogen a (25.7 kDa) and cytochrome c (12.3 kDa).

2.6. Identification of internal peptide sequences

To determine the partial amino acid sequence, purified α-L-AFase was digested by trypsin and submitted to the National Center of Biomedical Analysis (China) for amino acid sequencing using high-performance liquid chromatography–electrospray ionization tandem mass spectrometry (HPLC–ESI-MS/MS). Mass spectral sequencing was performed using a Q-TOF II mass analyzer (Q-TOF2, Micromass Ltd., Manchester, UK). Peptide sequencing was performed using a palladium-coated borosilicate electrospray needle (Protana, Denmark). The mass spectrometer was used in positive ion mode with a source temperature of 80 °C and a potential of 800 V was applied to the Nanospray probe. MS/MS spectra were transformed using MaxEnt3 software (MassLynx, Micromass) and amino acid sequences were interpreted manually using PepSeq software (BioLynx, Micromass).

2.7. Enzymatic properties

The optimal pH was determined by measuring the enzyme activities in different buffers of 50 mM concentration with the pH ranging from 3.0 to 11.0. These buffers were citrate buffer (pH 3.0–6.5), acetate buffer (pH 4.0–5.5), 2-(N-morpholino) ethanesulfonic acid (MES) buffer (pH 5.0–7.0), 3-(N-morpholino)-propanesulfonic acid (MOPS) buffer (pH 6.5–8.5), 2-(cyclohexylamino)ethanesulfonic acid (CHES) buffer (pH 8.0–11.0) and (cyclohexylamino)-1-propanesulphonic acid (CAPS) buffer (pH 9.0–11.0). To determine the pH stability of α-L-AFase, the enzyme was incubated in the above buffers at 50 °C for 30 min before the residual α-L-AFase activities were measured according to the standard assay.

The effect of temperature on α-L-AFase activity was determined at different temperatures (30–90 °C) in 50 mM citrate buffer (pH 5.0). For the determination of thermostability, purified α-L-AFase in 50 mM citrate buffer (pH 5.0) was incubated at different temperatures (30–90 °C) for 30 min. The thermal inactivation of the enzyme was further studied at 55 °C, 60 °C, 65 °C, and 70 °C by incubating the enzyme in 50 mM citrate buffer (pH 5.0) for 5 h. Aliquots were withdrawn at different time intervals. After cooling on ice for 30 min, the remaining enzyme activity was measured according to the described assay. Each assay was carried out in triplicate and the results presented are the average of three trials.

For the determination of effects of metal ions and some reagents on the α-L-AFase activity, the enzyme was incubated in 50 mM citrate buffer (pH 5.0) with 1 mM of individual metal ion or a reagent at 50 °C for 30 min before the residual activities were measured.

2.8. Substrate specificity and kinetic parameters

Substrate specificity analysis was performed at 50 °C for 10 min. Activities were measured by the rate of *p*-nitrophenyl or reducing sugar formed during the hydrolysis of different chromogenic (1 mM) or polysaccharide (1%) substrates in 50 mM citrate buffer (pH 5.0). The tested substrates were *p*NP-β-D-xylopyranoside, *p*NP-α-L-arabinopyranoside, *p*NP-β-D-glucopyranoside, *p*NP-β-D-fucopyranoside, *p*NP-α-D-galactopyranoside, *p*NP-β-D-galactopyranoside, arabinan, arabinoxylan, avicel, carboxymethylcellulose (CMC), birchwood xylan, beechwood xylan and oat spelt xylan. One unit of enzyme activity was defined as the amount of enzyme that releases 1 μmol of *p*-nitrophenyl or reducing sugar per minute under the above conditions.

For kinetics determination, hydrolysis was carried out in 50 mM citrate buffer (pH 5.0) at 50 °C with the substrate concentrations ranging from 0.8 mM to 2.8 mM. The constant kinetic parameters of K_m and V_{max} were estimated using Lineweaver–Burk plots.

2.9. Cloning and sequence analysis of the α-L-AFase gene

The cloning of full-length α-L-AFase cDNA was performed according to Sambrook and Russell [31]. Genomic DNA of *Chaetomium* sp. CQ31 was isolated with a Fungal DNA Midi Kit (Omega Bioteck, Doraville, USA). Total RNA was isolated with the TRIzol reagent, and mRNA was purified using the Oligotex mRNA Midi Kit. The obtained genomic DNA was then used as a template for subsequent polymerase chain reaction (PCR) amplification. To clone the α-L-AFase gene, degenerate primers of CsAraCP1 (AARTGGTAYATHTAYGTNGC) and CsAraCP2 (CCYTCRTTNACYTTRTANCC) were designed based on the conserved sequences (KWYIYVA and IGYKINE) of known fungal α-L-AFases. The PCR product was purified, ligated to pMD18-T vector, and sequenced. The full-length cDNA sequence of the α-L-AFase was obtained by 5′ and 3′ RACE (rapid amplification of cDNA ends) using a SMART RACE cDNA Amplification Kit in accordance with the manufacturer's instructions. PCR was performed with the following primer pairs: CsAraGSP1 (GCCAGCATCAGGCTCGAGTTCT) and Universal Primer A Mix (UPM) for the first PCR, Nested Universal Primer A (NUP, BD Biosciences) and CsAraNGSP1 (CGAGAAGGTGTCCCAGTTGGTC) for 5′ RACE; CsAraGSP2 (CGGACCAACTGGGACACCTTCT) and UPM for the first PCR, NUP and CsAraNGSP2 (GCCGGACGAGAACTCGAGCCTG) for