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Molecular Cloning of a Gene Encoding α-L-Arabinofuranosidase from Hyperthermophile *Thermotoga maritima* and Characterization of Its Biochemical Properties

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Abstract

α-L-Arabinofuranosidase (α-L-AFase, EC 3.2.1.55) was isolated from hyperthermophilic microorganism, *Thermotoga maritima*. The open reading frame (ORF) of α-L-AFase gene is 1,455 bp long and encodes 484 amino acid residues with a molecular weight of 55,265 Da. The ORF of α-L-AFase gene was introduced into the *E. coli* expression vector, pRSET-B, and overexpressed in *E. coli* BL21. The purified recombinant α-L-AFase showed the highest activity at 100℃ and pH 5.5. The purified enzyme appeared to have no metal cofactor requirement. The *K*m and specific activity values of the recombinant enzyme were 0.99 mM and 1,200 U/mg on *p*-nitrophenyl-α-L-arabinofuranoside. It released only L-arabinose from sugar beet arabinan, sugar beet debranched arabinan and oat spelts arabinoxylan but had no activity on arabinogalactan and gum arabic. This result suggests that L-arabinose could be produced from natural polysaccharides using this enzyme. Mutant enzymes which Glu26, Glu172 and Glu281 residues were replaced to alanine, aspartic acid or glutamine caused *K*cat to decrease by a factor of between 10^3 and 10^4. Glu172 and Glu281 residues of α-L-AFase are seemed to be the acid/base and nucleophile in catalytic reaction, respectively, and Glu26 is supposed to play a key role in substrate binding.

Introduction

L-Arabinose selectively inhibits intestinal sucrase activity in an uncompetitive manner and reduces the glycemic response in animals following sucrose ingestion (1, 2, 3). L-Arabinose can be used as a physiologically functional sugar possessing inhibitory activity against sucrose digestion (4, 5). L-Arabinosyl residues in the furanose form are widely distributed in plant cell wall heteropolysaccharides such as arabinans, arabinoxylans, and arabinogalactans. These polysaccharides are decorated at C-2 and/or C-3 with arabinofuranose molecules as side chains. These side chains may restrict the enzymic hydrolysis of respected polysaccharides. The α-L-AFase (exo-(1→5)-α-L-arabinofuranosidase, EC.3.2.1.55) cleaves the L-arabinose side chains, allowing endo-arabinase (endo-(1→5)-α-L-arabinofuranosidase, EC 3.2.1.99)

to attack the arabinan backbone. These enzymes act synergistically in degrading branched arabinan to generate L-arabinose (6).

To develop an effective process for L-arabinose production from hemicellulose, we have attempted isolation of bacterial thermostable enzyme degrading arabinan. α-L-AFase gene from the thermophilic organism *Thermotoga maritima* was cloned and overexpressed in recombinant *E. coli* BL21 using a T7 expression vector. The purified enzymes were subsequently characterized in terms of optimum pH and temperature, kinetic parameters (*K*m and *V*max) and substrate specificity. Also, we have determined the essential catalytic residues of α-L-AFase by the site-directed mutagenesis experiment.

Results and Discussion

Isolation and expression of a gene for α-L-AFase

To isolate the gene encoding α-L-AFase (ACCESSION AE001710) from *T. maritima*, PCR was carried out using α-L-AFase-specific primers, which were designed according to the GenBank analysis. The PCR product was introduced into the pRSET-B vector to produce pRBTAF. It was determined that this DNA originated from the *abf*B gene of *T. maritima* since it codes for the peptides derived from the native α -L-AFase. We found that a single nucleotide has been altered (T→C) in the *abf*B gene amplified form *T. maritima* (ATCC 43589D) when compared with the nucleotide sequence of *T. maritima* MSB8 obtained from the GenBank. The sequence variance didn't cause any changing in the amino acid sequence. The ORF of the α-L-AFase gene is 1,455 bp and long and encodes 484 amino acid residues with a molecular weight of 55,265 Da. To analyze the enzymatic activity and the substrate specificities of α-L-AFase, the pRBTAF was transformed, overexpressed in *E. coli* BL21, and then analyzed through a discontinuous SDS-PAGE. As shown in Fig. 1, the α-L-AFase was overexpressed in *E. coli* BL21 as soluble form, and purified by heat treatment at 80℃ for 20 min.

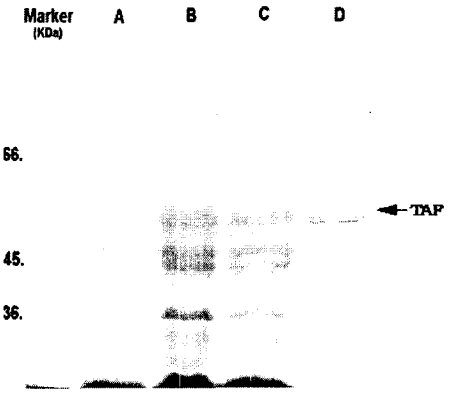

Fig. 1. SDS-PAGE analysis of recombinant α-L-AFase in *E. coli*
Marker: Molecular weight marker (kDa)
Lane A: Total extract of *E. coli* harboring pRSET plasmid only
Lane B: Total extract of *E. coli* harboring pRBTAF
Lane C: Soluble protein of *E. coli* harboring pRBTAF
Land D: Purified recombinant α-L-AFase

Biochemical properties of recombinant α-L-AFase

The effects of temperature and pH on the activity of the recombinant α-L-AFase are shown in Fig. 2. A 2.5 mM sample of *p*NPAF was incubated for 5 min with 0.1 U of purified recombinant α-L-AFase at various temperatures and pHs, and the color developed as a result of *p*NP liberation was measured at 405 nm. The optimal temperature for the activity was observed at 100℃ (Fig. 2A). This is the highest temperature for optimal activity among known α-L-AFase; the α-L-AFase of *Geobacillus stearothermophilus* T-6 (7) and *Thermobacillus xylanilyticus* (8) are reported to be highly active at 70 and 75℃, respectively. The effects of pH on the activity of the recombinant α-L-AFase were tested at 100℃ in the pH range 3.0 to 10.0. The recombinant α-L-AFase showed the highest activity at pH 5.5 (Fig. 2B).

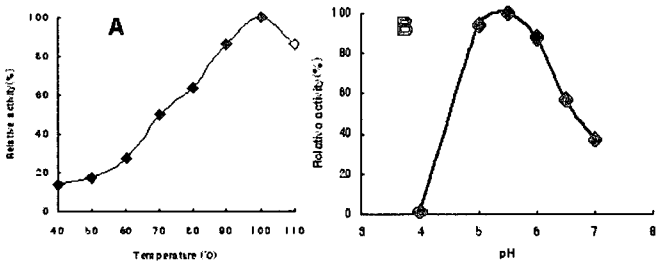

Fig. 2. Effect of temperature and pH on the activity of recombinant α-L-AFase.

Recombinant α-L-AFase produces L-arabinose from arabinoxylan and arabinan

The L-arabinose producing activity of the purified recombinant α-L-AFase was tested using arabinogalactan, gum arabic, sugar beet arabinan and oat spelts arabinoxylan as a substrate. 1% (w/v) of each of the substrates were reacted with 27.1 U of purified recombinant α-L-AFase at 80℃ for 1 h. The enzyme showed activity on arabinan, debranched arabinan and arabinoxylan but had no activity on arabinogalactan and gum arabic (Fig. 3). It was concluded that α-L-AFase hydrolyzes α-1,3-arabinose linkage as well as α -1,5-linkage of arabinan to release L-arabinose from the nonreducing terminus. These results demonstrated that L-arabinose could be produced from natural polysaccharides using recombinant α-L-AFase. This confirmed that recombinant α-L-AFase enzyme is applicable for producing L-arabinose from a biomass resource, such as sugar beet pulp and rice bran. Since one of the objectives of this study is to develop an efficient system for the production of L-arabinose from biomass resources, accelerating the rate of arabinan degradation by α-L-AFase is very important. In addition, the thermostability of the enzymes is also an important consideration. A biochemical reaction at high temperature is favorable in terms of not only preventing microbial infection of the reaction mixture, but also because it increases the solubility of the substrate.

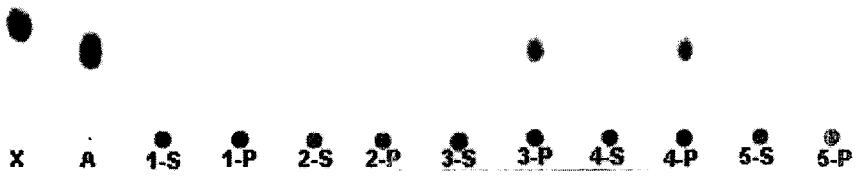

Fig. 3. TLC analysis of arabinose-containing polysaccharides digestion by α-L-AFase.
X; D-xylose, A; L-arabinose, 1-S; arabinogalactan, 2-S; gum arabic, 3-S; sugar beet arabinan, 4-S; debranched arabinan, 5-S; oat spelt xylan, P is product after reaction.

Identification of catalytic residues of recombinant α-L-AFase

Since the alignment reported by Zverlov *et al.* (9) was composed of only seven family 51 α-L-AFase sequences, we generated a new alignment which includes 10 sequences. Analysis of the alignment confirmed the conserved nature of Glu172 and Glu281 and in addition, revealed other invariant acidic residue Glu26. In order to investigate the importance of all three residues, each one were independently substituted by alanine, aspartic acid, and glutamine. After mutagenesis by PCR and each mutants were confirmed by sequencing analysis. Wild-type and 9 mutants were expressed in *E. coli* BL21. The reaction rate of *p*NP liberation from *p*NPAF by the recombinant α-L-AFase was investigated. The kinetic parameters, *K*m and *K*cat, of the purified recombinant α-L-AFase were determined using *p*NPAF at concentrations in the range 0.1-2.5 mM. The *K*m value was determined by analyzing the slope of Lineweaver-Burk plots. A Lineweaver-Burk plot showed a linear response over this concentration range. Kinetic analysis of reactions catalysed by Glu26, Glu172 or Glu281 clearly indicated that all of these residues are important for catalytic activity. Replacement of homologous Glu. residues suppressed the enzymes activities. Overall, the mutation of either of these residues to alanine, aspartic acid or glutamine causes *K*cat to decrease by a factor of between 10^3 and 10^4 (Table 1). Unexpectively, in our study we also revealed a third Glu26 which is not directly involved in catalysis, but which is nevertheless critical for normal hydrolytic activity. Based on our kinetic data, we tentatively propose that Glu26 may be involved in the stabilization of a catalytic transition state. Among the catalytic residues of α-L-AFase, Glu172 and Glu281 are seemed to be the acid/base and nucleophile in catalytic reaction, respectively, and Glu26 is supposed to play a key role in substrate binding.

Table 1. Kinetic parameters for the hydrolysis of pNPAF by wild-type and mutants.

	*K*m (mM)	*K*cat (S^{-1})	*K*cat/*K*m ($mM^{-1}s^{-1}$)
Wild type	0.99	2.17×10^6	2.18×10^6
Glu26Ala	NA	NA	NA
Glu26Asp	0.22	1.6×10^3	7.5×10^3
Glu26Gln	0.27	1.2×10^3	4.3×10^3
Glu172Ala	0.56	2.5×10^3	4.2×10^3
Glu172Asp	0.25	1.2×10^3	4.8×10^3
Glu172Gln	0.43	2.0×10^3	4.6×10^3
Glu281Ala	NA	NA	NA
Glu281Asp	0.40	2.3×10^3	5.8×10^3
Glu281Gln	0.12	3.9×10^2	3.3×10^3

NA; no activity detected

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