Biosci. Biotechnol. Biochem., **72** (8), 2203–2205, 2008

Note

Cloning and Functional Expression of an α-Galactosidase from *Yersinia pestis biovar Microtus* str. 91001

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Received March 7, 2008; Accepted May 18, 2008; Online Publication, August 7, 2008
[doi:10.1271/bbb.80145]

A gene encoding a glycoside hydrolase (GH) family 36 α-galactosidase was cloned from *Yersinia pestis biovar Microtus* str. 91001 and expressed in *Escherichia coli*. The purified recombinant α-galactosidase (Aga-Y) was optimally active at 37 °C and pH 6.8. The features of temperature profile, thermoliability, kinetics, and amino acid composition indicated that Aga-Y had properties of a cold-adapted enzyme.

Key words: α-galactosidase; glycoside hydrolase; *Yersinia pestis biovar Microtus* str. 91001; characterization

α-Galactosidase (α-D-galactoside galactohydrolase; EC 3.2.1.22) is widely distributed in nature[1)] and has been utilized in many industries, and in medical treatment and scientific research.[2–5)] It is also involved in cell-wall modification during tissue development.[6)] Based on amino acid sequence similarities, α-galactosidases have been classified into glycoside hydrolase (GH) families 4, 27, 36, and 57 in the CAZy database (http://www.cazy.org/fam/acc_GH.html).[4)] Most α-galactosidases of GH-27 come from eukaryotes and those of GH-36 from prokaryotes. These two families contain the majority of known α-galactosidases.

Yersinia pestis biovar Microtus str. 91001, a non-plague bacterium optimally growing at 28–30 °C, has unique features in carbohydrate utilization: arabinose negative, and rhamnose and melibiose positive.[7)] Some enzymes of this strain studied in our laboratory showed properties adapted to the intestinal temperature of the animal. Hence, the aim of this study was the cloning and functional expression of an α-galactosidase from this strain that has similar temperature properties and potential application in the feed industry.

A fragment of 2.4 kb containing the α-galactosidase gene (Genbank accession no. BK006342), *aga-y*, was amplified from the genomic DNA of *Y. pestis* str. 91001 and sequenced according to the putative α-galactosidase gene (Genbank accession no. AE017042). SD sequence (AGGAG) and protein analysis showed that *aga-y* has a full length of 2,127 bp, encoding 708 amino acid residues with a calculated molecular mass of 80.37 kDa and a calculated pI of 5.99. There was no signal peptide at the *N*-terminus of the encoded protein, Aga-Y. The identities between Aga-Y and α-galactosidases from *Escherichia coli* E24377A (ABV20016), *Erwinia carotovora* subsp. *Atroseptica* SCRI1043 (YP_048866), *Vibrio shilonii* AK1 (ZP_01867825), *Aspergillus clavatus* NRRL 1 (XP_001275168) and *Penicillium* sp. F63 (ABC70181) were 59, 57, 54, 28, and 25% respectively, suggesting that Aga-Y belongs to the GH-36 family and is more closely identical to the α-galactosidases from bacteria than from fungi.

For heterogeneous expression, the gene *aga-y* was amplified using primers P1 (5′-GCTCCATGGTTATGAATACTACCTTTG-3′, *Nco*I site under lined) and P2 (5′-GATCTCGAGGCGACCTTCTATATTTACGGCTGAAAGCT-3′, *Xho*I site under lined), linked pET-22b(+) vector, and then transformed into *E. coli* BL21 (DE3; Novagen, Darmstadt, Germany). The positive clone was grown in LB medium containing 100 μg/ml of ampicillin at 18 °C for 12 h after induction with 1 mM IPTG, but no enzyme activity was be detected after induction at 37 °C. It is similar to some recombinant psychrophilic enzymes that frequently are not expressed or are quickly inactivated at 37 °C.[8)] The cells were harvested, sonicated, and centrifuged. Two milliliters of the supernatant (crude enzyme), was loaded onto a HiTrap Chelating column

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Abbreviations: IPTG, isopropyl-β-D-thiogalactoside; *p*NP, *p*-nitrophenyl; *p*NP-α-Gal, *p*-nitrophenyl-α-D-galactopyranoside; SDS–PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis

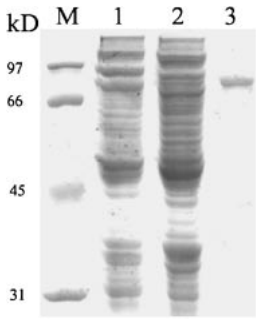

Fig. 1. Expression and Purification of Recombinant Aga-Y from *E. coli*.

Lane M, low molecular weight markers; lane 1, extract of *E. coli* clone harboring pET-22b(+) with IPTG induction; lane 2, crude enzyme extract of *E. coli* clone harboring pET-22b(+)/*aga-y* with IPTG induction; lane 3, Aga-Y after purification using a HiTrap Chelating column.

(Amersham Pharmacia, Uppsala, Sweden) for purification with a linear imidazole gradient of 10 to 500 mM in phosphate buffer (20 mM phosphate, 500 mM NaCl, pH 7.4). The enzyme activity was assayed by a modified *p*NP-α-Gal method[2] and loaded into SDS–PAGE (12% w/v, pH 8.3) (Fig. 1). One unit of α-galactosidase activity was defined as the amount of enzyme that released 1 µmol of *p*NP from *p*NP-α-Gal (Sigma, St. Louis, Mo, USA) per min at 37 °C. The α-galalctosidase activity in the crude enzyme was 0.19 U/ml, higher than that of α-Gal from *Thermus* sp. T2, but lower than α-Gal II or α-Gal III from rice.[4,9] The recombinant Aga-Y was purified 111.4-fold with a specific activity of 21.2 U/mg. The relative molecular weight was calculated to be 80 kDa, corresponding to the estimated value.

The purified recombinant Aga-Y was optimally active at pH 6.8, and retained > 60% activity at pH 6.5–7.5 (Fig. 2A). In the test of pH stability, the enzyme had stability in a narrow range around pH 7.0 (Fig. 2B). The optimum temperature of Aga-Y was 37 °C (Fig. 2C), close to that of the AgaA from *Carnobacterium piscicola* BA (32–37 °C)[10] but lower than most GH-36 α-galactosidases from bacteria.[3] After incubation at 50 °C for 2, 5, and 10 min, about 63, 10, and 3% of activity was retained respectively (Fig. 2D). Metal ions Ag^+ and Hg^{2+} inactivated Aga-Y completely. This suggests that the Cys residue is located at the catalytic site, since these ions have been reported to attack Cys residues at active sites in GH-36 α-galactosidases.[3,9] Kinetic studies were performed with *p*NP-α-Gal as substrate at 37 °C for 2 min. The Michaelis constant (K_m) and catalytic constant (k_{cat}) were 0.51 mM and 3,279/s. The K_m for melibiose as substrate was 1.35 µM by the GOD-POD method, and the values for raffinose and stachyose could not be determined by the DNS method. A hydrolyzing experiment was performed,[2] and it showed the hydrolysis ability of melibiose (11.68%) > stachyose (11.27%) > rafffinose (10.63%).

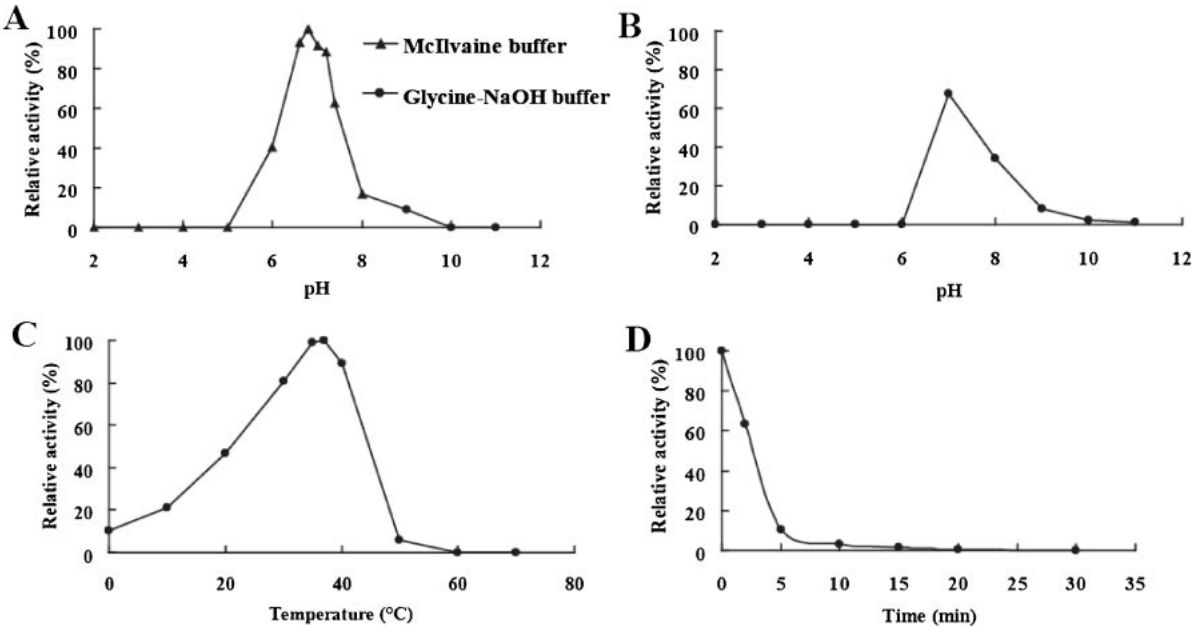

Fig. 2. Effects of pH and Temperature on Aga-Y Activity.

A, Effect of pH on Aga-Y activity. The effect of pH on the activity of Aga-Y was tested in 0.1 M McIlvaine buffers at pH 2.2–8.0 and Glycine-NaOH at pH 9.0–11.0. B, Effect of pH on the stability of Aga-Y. pH stability was determined by measuring the residual activity after incubation in the same buffers of various pHs at a 1:10 ratio (v/v) at 37 °C for 30 min. C, Effect of temperature on Aga-Y activity. The effect of temperature on the activity of Aga-Y was detected in 0.1 M McIlvaine buffer, pH 6.8, from 0 to 70 °C. D, Effect of temperature on the stability of Aga-Y. Thermal stability was determined by measuring residual activity after pre-incubation at 50 °C for 2, 5, 10, 15, 20, and 30 min. 100% relative value was 21.1 U/mg.

Table 1. Optimum Temperature, Kinetic Parameters and Amino Acid Composition of Aga-Y and Four Other GH-36 α-Galactosidases*

Property	Aga-Y	Agl1	MelA	Aga36A
Optimum Temperature (°C)	37	40	55	70
Kinetics				
K_m (mmol)	0.51	1.40	ND	4.30
k_{cat} (/s)	3,279	2,123	ND	608
K_m/k_{cat} (mmol/s)	6,429	1,500	ND	141
Amino acid Composition				
Cys (%)	1.27	0.27	0.54	0.54
Gly (%)	7.20	9.13	6.08	6.66
Met (%)	2.82	1.77	2.97	2.04
Gln (%)	4.52	2.86	4.59	2.72
Trp+Tyr+Phe (%)	9.32	9.94	11.21	12.77
Arg/Lys ratio	1.87	1.46	1.22	0.71

ND, Not detected in the original reports.
*Aga-Y: from *Y. pestis* str. 91001, BK006342; Agl1: from *Penicillium* sp. F63 CGMCC 1669, ABC70181; MelA: from *Lactobacillus fermentum* CRL 722, AY612895; Aga36A: from *C. stercorarium*, AB089353

Comparisons of temperature properties, kinetic, and amino acid composition with α-galactosidases of about 80 kDa are summarized in Table 1. Aga-Y had a lower optimum temperature, close to some known cold-adapted enzymes,[11] weak thermostability (lost activity at 50 °C for 10 min) as compared with mesophilic Aga-F78 (stable at 50 °C for 10 min)[2] and thermophilic Aga36A from *Clostridium stercorarium* (stable at 70 °C for 10 min).[12] Aga-Y had a lower K_m and a higher k_{cat} value, resulting in higher k_{cat}/K_m than any other α-galalctosidase studied (Table 1). A comparison of amino acid compositions showed that Aga-Y had higher contents of Gly, Met, Gln, and a higher Arg/Lys ratio and lower total contents of Trp+Tyr+Phe (Table 1).[11] All these characteristics were similar to the specific properties of cold-adapted enzymes.[11] The results suggest that Aga-Y might be a cold-adapted enzyme.

Acknowledgment

This work was supported by the Chinese National High Technology Research and Development Program (863 Program, Grants no. 2007AA100601 and no. 2006AA02Z220). We thank Professors R. F. Yang and X. Y. Wang of the Chinese Academy of Military Medical Sciences for kindly providing *Y. pestis* str. 91001 genome DNA.

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