Biotechnol Lett (2013) 35:1061–1065
DOI 10.1007/s10529-013-1175-9

ORIGINAL RESEARCH PAPER

Identification of a halophilic α-amylase gene from *Escherichia coli* JM109 and characterization of the recombinant enzyme

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Received: 17 November 2012 / Accepted: 27 February 2013 / Published online: 12 March 2013

Abstract A halophilic α-amylase (EAMY) gene from *Escherichia coli* JM109 was overexpressed in *E. coli* XL10-Gold and the recombinant protein was purified and characterized. The activity of the EAMY depended on the presence of both Na^+ and Cl^-, and had maximum activity in 2 M NaCl at 55 °C and pH 7.0. When 2 % (w/v) soluble starch was used as substrate, the specific activity was about 1,090 U mg^{-1} protein. This is the first report on identifying a halophilic α-amylase with high specific activity from non-halophilic bacteria.

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Electronic supplementary material The online version of this article (doi:10.1007/s10529-013-1175-9) contains supplementary material, which is available to authorized users.

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Keywords *Escherichia coli* · Halophilic α-amylase · Halophilic bacteria · Halophilic enzyme

Introduction

α-Amylase (1,4-α-D-glucan-glucanhydrolase, EC.3.2.1.1) belongs to family 13 (GH13) of the glycosyl hydrolases. This enzyme catalyzes the hydrolysis of α-1,4 glycosidic linkage from glycogen, starch, or related polysaccharides to produce glucose, oligosaccharides and dextrins (Gupta et al. 2003). It is also widely used in food, textile, and pharmaceutical industries (Hashim et al. 2004).

Halophilic α-amylases have received considerable interest because of their ability to maintain activity in high salt concentrations and to withstand extreme conditions in industrialized productions (Mevarech et al. 2000). Halophilic microorganisms normally

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thrive in the environments containing high salt concentrations and are considered to be the best sources of halophilic enzymes (Amoozegar et al. 2003; Li et al. 2012). For example, halophilic α-amylases have been obtained from halophilic microorganisms, such as extreme halophilic archaea (Hutcheon et al. 2005; Fukushima et al. 2005) or moderate halophilic bacteria (Coronado et al. 2000; Rui et al. 2011; Mijts and Patel 2002). But this work, we report an unexpected result: a halophilic α-amylase from a non-halophilic microorganism, *Escherichia coli* JM109.

Materials and methods

Strains, plasmids and culture conditions

Escherichia coli JM109 (Promega, USA) was used for EAMY gene cloning. Vector pSE380 (Invitrogen, USA) and *E. coli* XL10-Gold were used as the expression vector and expression host respectively for the EAMY gene. *E. coli* and recombinant strains were grown at 37 °C and 200 rpm either in LB medium alone or with 100 μg ampicillin/ml.

Construction of recombinant expression vector

The EAMY gene was amplified from the genomic DNA of *E. coli* JM109 with a pair of primers (forward: 5′-CCGGAATTCATGCACCATCATCATCATCATCGTAATCCCACGCTG-3′, reverse: 5′-CCCAAGCTTTTAAATCACCTCTTC-3′), which were designed according to the ORF sequence in the databank (GenBank no. gil1736595). The primer sequences contained the *Eco*RI and *Hin*dIII (underlined) restriction sites, and nucleotides coding for a hexa-histidine tag were also included in the forward primer. The PCR fragment was double-digested with *Eco*RI and *Hin*dIII, and then cloned into the expression vector pSE380 with the same restriction enzymes. The inserted DNA fragment was confirmed by sequencing. Finally, the resulting plasmid pSE380-EAMY was transformed into *E. coli* XL10-God using the standard protocol for gene expression.

Expression and purification of EAMY

When the cell density (OD_{600}) reached 0.6, IPTG was added to 1 mM to induce gene expression. After further 10 h incubation at 37 °C, the cells were harvested by centrifugation at 8,000×*g* for 10 min, washed twice with 100 mM potassium phosphate buffer (pH 7.0), resuspended in the same buffer, and disrupted by ultrasonication on ice. Cell debris was removed by centrifugation at 12,000×*g* for 20 min. The recombinant protein was purified using Ni–NTA (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Protein was determined using the Bradford method, with bovine serum albumin as standard. Purified EAMY was analyzed by both SDS-PAGE and native PAGE.

Enzyme assay

The standard reaction was carried out by adding 126 μg purified EAMY to 500 μl reaction mixture containing 100 mM MOPS/NaOH (pH 7.0), 2 % (w/v) soluble starch, and 2 M NaCl followed by incubation at 55 °C for 5 min. Amylase activity was determined by measuring released reducing sugars using the 3,5-dinitrosalicylic acid method (Bernfeld 1955). One unit of amylase activity (U) was defined as the amount of enzyme that produced 1 μmol reducing sugar as maltose per min under the assay conditions. The kinetic parameters were obtained using a Lineweaver–Burk plot with various concentrations of soluble starch under the optimal reaction conditions.

Product analysis

The hydrolysis products of starch by EAMY were analyzed by HPLC at 30 °C using a column (Alltima Amino 100A 5u, 250 × 4.6 mm) and an evaporative light scattering detector. Acetonitrile/water (80:20 v/v) was used as solvent at 1 ml min^{-1}.

Results and discussion

Expression and purification of EAMY

Gene sequencing indicated the sequence of EAMY gene is consistent with the published results of GenBank. EAMY was expressed, purified, and finally analyzed by SDS-PAGE. After SDS-PAGE, a single protein band around 55 kDa was shown (Fig. 1a). This purified enzyme was used in subsequent experiments. The protein band was further identified using peptide

mass fingerprint (PMF) based on matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF-MS). The amino acid sequence was consistent with α-amylases from *E. coli* strains (Supplementary Fig. 1). The protein in native gel exhibited starch-digesting activity (see Fig. 1b).

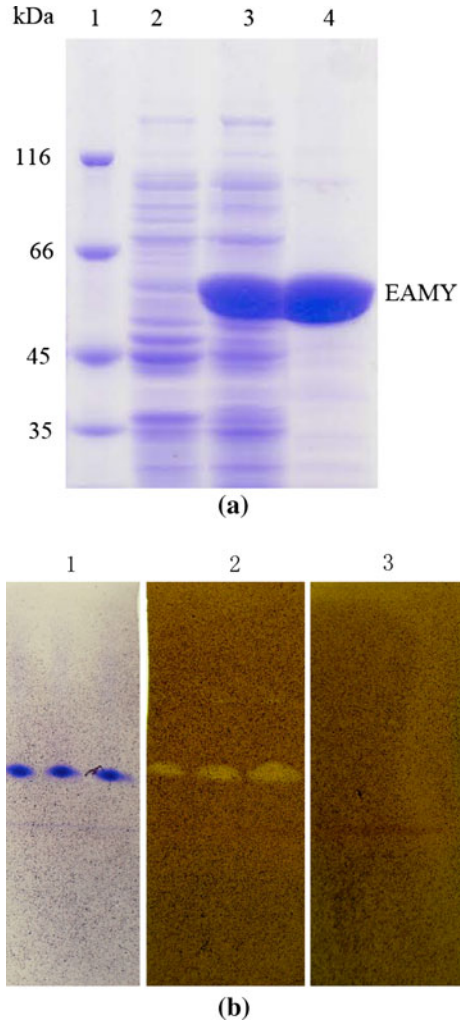

Fig. 1 PAGE analysis of EAMY. **a** SDS-PAGE analysis using 10 % denaturing acrylamide separating gel and 4 % stacking gel with SDS. *Lane 1* protein molecular weight markers, *lane 2* the crude extract of recombinant strain *E. coli* XL10-Gold with pSE380 as control, *lane 3* the crude extract of recombinant strain *E. coli* XL10-Gold with pSE380-EAMY, *lane 4* purified EAMY. **b** Native PAGE analysis of EAMY using 10 % native gradient acrylamide separating gel and 4 % stacking gel without SDS, but with 1 % (w/v) soluble starch at 4 °C. The native gel after electrophoresis at 4 °C was incubated with 100 mM Tris/HCl buffer, pH 7.0, containing 4 M NaCl at 37 °C for 30 min The native gel was divided into three parts and individually stained. The first part was strained with coomasssie blue. The second part was incubated in 100 mM MOPS/NaOH buffer (pH 7.0) containing 2 M NaCl at 55 °C for 30 min and then stained with I2/KI solution. The third part was directly stained with I2/KI solution as control

Enzymatic characterization of EAMY

Amylase activity of EAMY was observed in citric acid/HCl/NaOH buffer (pH 6.5), but not in Tris/HCl or MOPS/NaOH buffers (7.0). However, in the latter case the enzyme activity could be detected when NaCl was added, and was surprisingly the highest in MOPS/

Table 1 Effect of various salt solutions at 1 M on EAMY activity

Saline solutions or metal ions	Relative activity (%)
NaCl	100 ± 2.4[a]
KCl	74 ± 3.2
Sodium citrate	73 ± 3.8
K_2SO_4	73 ± 2.8
Sodium cetate	24 ± 3.3
Potassium acetate	19 ± 2.8
$NaNO_3$	4 ± 0.6
KNO_3	3 ± 0.4

The relative activity of EAMY was measured using soluble starch as substrate at 2 % (w/v) containing different salts in 100 mM MOPS/NaOH buffer (pH 7.0, adjusted with NaOH or HCl). The other conditions were the same as standard reaction. 1 M of Na_3PO_4, NH_4Cl, Na_3SO_3 and 10 mM of $CaCl_2$, $MgCl_2$, $CuCl_2$, $ZnCl_2$, $MnCl_2$, $PbCl_2$, $NiCl_2$ were also tested in this kind of experiments but they all could not increase the activity of EAMY

[a] 100 % = 717 U/mg

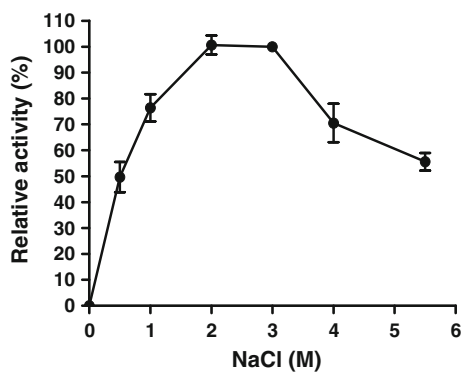

Fig. 2 Effect of NaCl on EAMY activity. EAMY was assayed activity under standard conditions. 100 % = 988 U/mg. *Error bars* represent SD

NaOH buffer. This indicated that both Na^+ and Cl^- were required for EAMY activity. Other salt solutions could also enhance the activity of EAMY in MOPS/NaOH buffer (see Table 1). The highest activity, however, was with NaCl. The effect of NaCl on EAMY activity is shown in Fig. 2. It can be seen from this data that the highest activity was with 2–3 M NaCl.

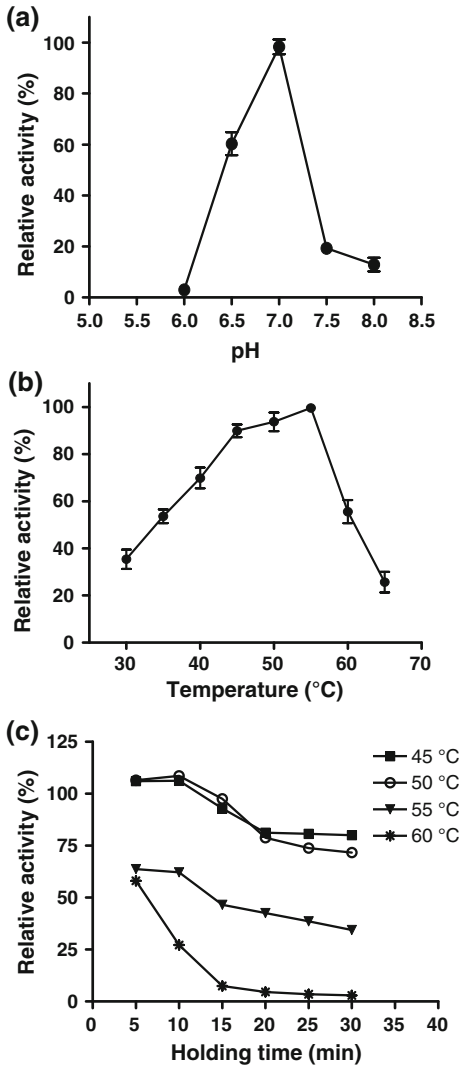

Fig. 3 Characterization of EAMY. **a** Reactions using 2 % (w/v) soluble starch as substrate were assayed at 40 °C with different pH values (6.0–8.0) for 5 min in other standard reaction conditions. The maximum value was set as 100 %. 100 % = 1,007 U/mg. **b** Reactions were set at different temperatures, ranging from 30 to 65 °C to detect the effect of temperature on enzyme activity with pH 7.0. Other reaction conditions were the same as in (**a**). 100 % = 1,087 U/mg. **c** Thermostability of the recombinant amylase. EAMY was respectively incubated in 100 mM MOPS/NaOH buffer (pH 7.0) without substrate for different time at 45, 50, 55 and 60 °C, and then measured the residual activities at 55 °C for 5 min. 100 % = 1,005 U/mg. *Error bars* represent SD

Effects of pH and temperature on EAMY activity

The optimum pH for EAMY activity was 7.0 (Fig. 3a), and the optimum temperature was 55 °C (Fig. 3b). EAMY showed a quite poor thermostability in terms of ordinary bacterial α-amylase (Fig. 3c) and it would be totally inactivated when incubated for 20 min at 60 °C.

Substrate specificity and product analysis

EAMY could hydrolyze soluble starch, maltohexaose, maltopentaose, and maltotetraose (Table 2). The starch hydrolysis products were maltose, maltotriose and small amounts of glucose (see Supplementary Fig. 2).

Kinetic analysis of EAMY

The protein content of EAMY was 2.5 mg/ml, and its specific activity was 1,087 U/mg. As calculated from the Lineweaver–Burk plot, the K_m and K_{cat} values were 4.3 mg/ml and 825/s, respectively (see Supplementary Fig. 3). Compared with other halophilic α-amylases from different sources, EAMY had superiority of specific activity over the so-far-reported halophilic α-amylases from moderate halophilic bacteria and extremely halophilic archaea (Table 3).

Table 2 Substrate specificities of EAMY

Substrates	Activity (U/mg)
Soluble starch	1,004 ± 20.4
Maltohexaose	424 ± 10.7
Maltopentaose	260 ± 4.1
Maltotetraose	84 ± 2.4

The different polysaccharides (oligosaccharides) were used as substrate at 2 % (w/v) to detect activity of EAMY under standard conditions. The reaction samples were analyzed by HPLC. The sample concentration was diluted to ~0.1 %, and 20 µl was loaded onto the column. No activity was found towards maltose, maltotriose, isomaltose, isomalto-ogligosaccharide, sucrose, α-cyclodextrin, β-cyclodextrin and pullulan after incubation at 55 °C for 12 h

Table 3 Enzymatic characterization of halophilic α-amylases from different sources

Enzyme source	Specific activity (U/mg)	Optimal pH	Optimal temperature (°C)	Optimal NaCl concentration (M)	K_m for soluble starch (mg/ml)	Reference
Escherichia coli non-halophilic	1,087	7.0	50	2–3	4.27	This study
Nesterenkonia sp. moderate alophile	181	7.5	45	0.5	4.5	Rui et al. (2011)
Halothermothrix oreni moderately halophilic	22	7.5	65	0.86	Not reported	Mijts and Patel (2002)
Halomonas meridiana moderate alophile	Not reported	7.0	37	0.17	Not reported	Coronado et al. (2000)
Haloarcula hispanica halophilic archaea	Not reported	6.5	50	4	Not reported	Hutcheon et al. (2005)
Haloarcula sp. halophilic archaea	Not reported	7.0	50	4.3	Not reported	Fukushima et al. (2005)

Conclusions

The EAMY is activated by NaCl and retains high activity in high concentrations of NaCl, shows good salt-tolerant ability, making it potentially to be used in the processing of seafood, saline food, and other fermented food.

Acknowledgments This work was supported by the National Natural Science Foundation of China (Grant no. 31160311), Science and Technology Development Project of Guangxi (Contract no. 11107008-3) and the Natural Science Foundation of Guangxi (Contract no. 2012GXNSFAA053051).

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