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ORIGINAL PAPER

Cloning and characterization of a maltotriose-producing α-amylase gene from *Thermobifida fusca*

Chao-Hsun Yang · Wen-Hsiung Liu

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Abstract The gene (*tfa*), encoding a maltotriose-producing α-amylase from *Thermobifida fusca* NTU22, was cloned, sequenced and expressed in *Escherichia coli*. The gene consists of 1,815 base pairs and encodes a protein of 605 amino acids. The base composition of the *tfa* coding sequence is 69% G+C and the protein has a predicted p*I* value of 5.5. The deduced amino acid sequence of the *tfa* amylase exhibited a high degree of similarity with amylases from *Thermomonospora curvata* and *Streptomyces* amylases. The purified amylase could be detected as a single band of about 65 kDa by SDS-polyacrylamide gel electrophoresis and this agrees with the predicted size based on the nucleotide sequence. The optimal pH and temperature of the purified amylase were 7.0 and 60°C, respectively. The properties of purified amylase from the *E. coli* transformant are similar to that of an amylase purified from the original *T. fusca* NTU22.

Keywords *Thermobifida fusca* · *Escherichia coli* · Maltotriose-producing α-amylase · Raw starch · Gene cloning

C.-H. Yang · W.-H. Liu (✉)
Institute of Microbiology and Biochemistry,
National Taiwan University, No. 1, Sec. 4,
Roosevelt Rd., Taipei 106, Taiwan
e-mail: whliu@ntu.edu.tw

Present Address:
C.-H. Yang
Department of Cosmetic Science,
Providence University, Taichung, Taiwan

Introduction

Amylase (EC 3.2.1.1, α-1,4-D-glucanohydrolyase) is capable of catalyzing the production of high yields of specific maltooligosaccharides on degrading starch and is of considerable commercial interest [1]. Most α-amylases produce glucose or maltose as the major product from starch. However, amylases that specifically produce malto-oligosaccharides from starch have been reported. These include the maltohexaose producing amylases of *Aerobacter* and *Bacillus* [2–4], a maltopentaose-producing amylase from *Bacillus* [5], the maltotetraose-producing amylases from *Pseudomonas* [6], *Bacillus* [7] and *Chloroflexus* [8] and the maltotriose producing amylases from *Streptomyces* [9], *Bacillus* [10], *Microbacterium* [11], *Natronoccus* [12] and *Streptococcus* [13,14].

Maltotriose has many excellent properties when used in food processing. These include a mild sweetness, ability to store well in moisture, and the prevention of retrogradation of starch in foodstuffs [15]. It has been reported that the fatty acid maltotriose esters may act as antitumor agents [16]. The maltotriose-producing amylase from *Microbacterium imperiale* [11] is now produced on an industrial scale and used to prepare a high-maltotriose containing syrup.

To produce enzymes for the development of enzymatic degradation of renewable lignocellulose, we have isolated a potent extracellular lignocellulolytic enzyme-producing thermophilic actinomycete, *Thermobifida fusca* NTU22, from compost soils collected in Taiwan [17]. It has been reported that a thermophilic actinomycete, *Thermomonospora fusca*, is able to produce extracellular α-amylase that can generate maltotriose as its major end product from soluble starch [18]. Interestingly, the newly isolated strain studied here also

produces an extracellular amylase that releases maltotriose as the major end product from either soluble starch or from raw starch granules. Since amylases that produce maltotriose as their major end product from raw starch granules are relatively rare, optimization of the cultivation conditions for the production of this extracellular amylase by *T. fusca* NTU22 was investigated [19]. Recently, we reported the purification and some properties of the maltotriose-producing α-amylase from *T fusca* NTU22. The molecular weight of the purified enzyme was estimated by SDS-PAGE and gel-filtration on Sepharose CL-6B to be 64 and 60 kDa, respectively. The optimum pH and temperature for the purified enzyme were 7.0 and 60°C, respectively [20].

The lignocellulolytic enzymes and trehalose synthase genes from *Thermobifida fusca* have been cloned and expressed in *E. coli*, *Streptomyces lividans* and *Pichia pastoris* [21, 22]. However, the gene encoding α-amylase has not been cloned. The present paper is concerned with the cloning and characterization of the α-amylase from *T. fusca* NTU22. Some properties of the purified amylase are also determined.

Materials and methods

Microorganisms and vectors

A thermophilic actinomycete, *Thermobifida fusca* NTU22, which was isolated from compost soils collected in Taiwan, was used in this study [17]. For cloning of the amylase gene from *T. fusca* NTU22, *Escherichia coli* DH5α and pUC18 (Boehringer Mannheim, Germany) were used as the host–vector system.

Materials

Czapek-dox powder, yeast extract, casamino acids, tryptone and agar were purchased from Difco (Detroit, MI, USA). Restriction endonucleases and T4 DNA ligation kit were purchased from Roche (Mannheim, Germany). Sepharose CL-6B, DEAE–Sepharose CL-6B and the low molecular weight electrophoresis calibration kit were supplied by GE Healthcare (Little Chalfont, UK). The protein assay kit was obtained from Bio-Rad Laboratories (Hercules, CA, USA). Inorganic salts and all other chemicals were purchased from Sigma (St Louis, MO, USA).

Cloning of the *tfa* gene from a *T. fusca* NTU22 genomic DNA library

The genomic DNA of *T. fusca* NTU22 was isolated based on the methods described by Hopwood et al. [23]. Genomic DNA from *T. fusca* NTU22 was partially digested with *Bam*HI, and fragments of 2–10 kb were isolated by agarose gel electrophoresis and cloned into pUC18/ *E. coli* DH5α to construct the genomic DNA library. The transformants were screened on LB agar plates, which contained 100 μg/ml ampicillin, 40 μg/ml X-gal and 40 μg/ml IPTG at 30°C for 16 h. The transformants were tested for amylase activity by replicating the white colonies onto the LB agar plates, which contained 100 μg/ml ampicillin, 40 μg/ml IPTG and 10 mg/ml soluble starch. After incubation at 30°C for 24 h, the plates were incubated at 60°C for 12 h and stained with iodine solution. Colonies with a clear zone resulting from hydrolysis of starch were selected for further analysis. To identify the amylase activity produced by the *E. coli* transformants, the positive colonies were cultivated aerobically in 500 ml Hinton flasks containing 50 ml LB medium with 100 μg/ml ampicillin, 40 μg/ml IPTG and incubated at 37°C and 125 rpm for 24 h. The *E. coli* cells were harvested and disrupted by sonication. The cell lysate was then centrifuged at 10,000×*g* for 30 min to obtain a supernatant liquid that was designated as the cell free extract for amylase activity analysis. The recombinant plasmid from the *E. coli* transformant giving the highest amylase activity was sequenced in an Applied Biosystems 3730 DNA analyzer (Applied Biosystems, CA, USA). The resulting sequence data was uploaded to the NCBI (National Center for Biotechnology Information) website to search for similarity to known DNA and protein sequences.

Amylase activity assay

Amylase activity was determined by measuring the release of reducing sugar from soluble starch. The reaction mixture contained 0.1 ml of appropriately diluted crude enzyme and 0.9 ml of 100 mM sodium phosphate buffer (pH 7.0) containing 1% (w/v) of soluble starch. After incubating at 60°C for 15 min, the amount of reducing sugar released in the mixture was determined by the dinitrosalicylic acid method [19]. The absorbance of the mixture was measured at 540 nm, and D-glucose was used to create a standard curve. One unit of enzyme activity was defined as the amount of enzyme releasing 1 μmol glucose per minute under the assay condition. All analytical measurements were performed at least in triplicates.

Preparation of cell-free extract

The *E. coli* transformant giving the highest expression of amylase activity was selected and cultivated in a 5 l jar fermentor (M-205, Hotech, Taiwan). The seed culture

was produced in a 500 ml Hinton flask containing 100 ml LB-Ap-IPTG medium consisting of tryptone 10.0 g, yeast extract 5.0 g, sodium chloride 7.5 g , ampicillin 100 mg and IPTG 40 mg/l of distilled water (pH 7.3). The culture was inoculated with cells of the *E. coli* transformant scraped from a maintenance agar plate cultured at 37°C and shaken (150 rpm) for 24 h. One hundred and fifty milliliter aliquots of seed culture were inoculated into a 5 l fermentor loaded with 3 l of LB-Ap-IPTG medium. Cultivation was carried out at 300 rpm (revolutions per minute), 1 vvm (air volume/culture volume, min) and 37°C for 16 h. The cells were harvested by centrifugation at 10,000×*g* for 30 min and washed with 2 volumes of 100 mM sodium phosphate buffer (pH 7.0). The washed cells were stored at −20°C until use. Wet cells (30 g) were resuspended in 200 ml of 100 mM phosphate buffer (pH 7.0). The suspension was disrupted with a Soniprep 150 sonicator (MSE Sci. Inc., Leicestershire, England) at 50 W for 5 min with ice-water cooling. The debris was removed by centrifugation at 12,000×*g* for 20 min. The supernatant liquid was used as a cell-free extract.

Enzyme purification

All purification procedures were done at 4°C in 50 mM phosphate buffer (pH 7.0) unless otherwise stated. The cell free extract was heated at 50°C for 2 h. Next the enzyme solution was centrifuged at 12,000×*g* for 30 min at 4°C to remove the precipitate. The supernatant liquid was brought to 40% saturation with ammonium sulfate. The precipitate was removed by centrifugation at 12,000×*g* for 30 min; then, ammonium sulfate was added to the supernatant liquid to 60% saturation. The precipitate was collected, dissolved in buffer and applied to a Sepharose CL-6B column (1.6 × 80 cm) pre-equilibrated with the same buffer. The eluted enzymatically active fractions were pooled and applied to a DEAE-Sepharose CL-6B column (2.6 × 10 cm) pre-equilibrated with the same buffer. This was washed with the same buffer until no further elution of protein could be detected in the eluate. The adsorbed enzyme was then eluted with a linear gradient of the same buffer containing sodium chloride from 0 to 1.0 M. The eluted active fractions were pooled and used as purified enzyme.

Results

Cloning of the maltotriose-producing α-amylase gene (*tfa*) from *T. fusca* NTU22

After partial digestion of genomic DNA using *Bam*HI, a genomic library of *T. fusca* NTU22 was created using the *E. coli* DH5α-pUC18 host–vector system. About 8,000 white transformants on the LB-Ap-IPTG-X-gal agar plates were isolated, and screened for their amylase activity on the LB-Ap-IPTG-starch agar plates. Three positive transformants were selected and the expression of amylase activity by these strains was tested using 50 mL shaken flask cultures. A plasmid containing a 10 kb insert was selected because it expressed the highest amylase activity and was denoted as pAMY13. The plasmid pAMY13 was further subcloned by using pUC18 as vector. One transformant was selected and named pAMY13H8 as it contained a 3.0 kb *Bam*HI-*Hind* III inserted fragment, which possessed the highest amylase activity.

Sequence analysis of the *tfa* gene from *T. fusca* NTU22

The nucleotide sequence of the 3.0 kb *Bam*HI–*Hin*dIII inserted fragment was determined. The DNA sequence revealed an open reading frame of 1,815 base pairs, with an ATG initiation codon at position 291 and a TGA termination codon at position 2,106. The initiation codon is 5-bases after a potential ribosome-binding site (Shine-Dalgarno sequence), GGAG. Furthermore, the amino acid sequence deduced from this open reading frame corresponds to a single polypeptide of 605 amino acids, with a calculated molecular weight of 65 kDa. This open reading frame was named *tfa* (Accession No: DQ473479). The base composition of the *tfa* coding sequence is 69% G + C and the pI value was predicted to be 5.5. Alignment of the nucleotide sequence with NCBI database resulted in a 96.5% similarity to the *tam* from *Thermomonospora curvata* CCM3352 [24].

Purification of amylase from the *E. coli* transformant

Purification of the amylase was described previously in materials and methods. Some protein was denatured after heating at 50°C for 2 h, but almost all the amylase activity remained (98.5%). The results of the purification are summarized in Table 1. The purified enzyme obtained exhibited 42% of the total initial activity and there was a 153-fold increase in specific activity compared with the cell free extract.

Properties of amylase from *E. coli* transformant

As shown in Figs. 1 and 2, the purified enzyme showed an apparent single protein band either on native-PAGE (10% gel) or SDS-PAGE (10% gel). The subunit size of the single protein band was estimated to be 65 kDa from its mobility relative to standard proteins

Table 1 Summary of the purification of amylase from *E. coli* transformant

Step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Cell free extract	1018.0	782	0.78	1.0	100.0
Heat treatment	410.0	770	1.88	2.4	98.0
$(NH_4)_2SO_4$ fractionation	128.6	506	3.93	5.0	64.0
Sepharose CL-6B	17.2	405	23.59	30.0	51.0
DEAE–Sepharose CL-6B	2.8	335	120.00	153.0	42.0

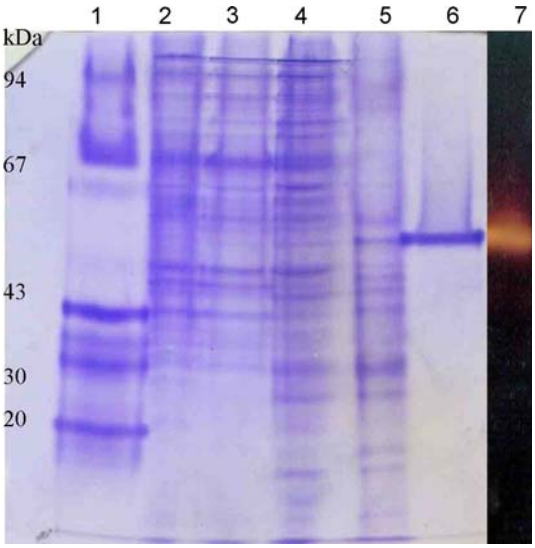

Fig. 1 Native PAGE of the purified amylase from *E. coli* DH5α (pAMY13H8). *Lane 1* low molecular mass standard protein: phosphorylase b (94 kDa), albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20 kDa). *Lane 2* cell free extract. *Lane 3* heat treated cell free extract. *Lane 4* ammonium sulfate fractionation. *Lane 5* Sepharose CL-6B column fraction. *Lane 6* DEAE–Sepharose CL-6B column fraction. *Lane 7* activity staining after DEAE–Sepharose CL-6B column fraction. Acrylamide concentration: 10%; electrophoresis conditions: 150 V, 1 h

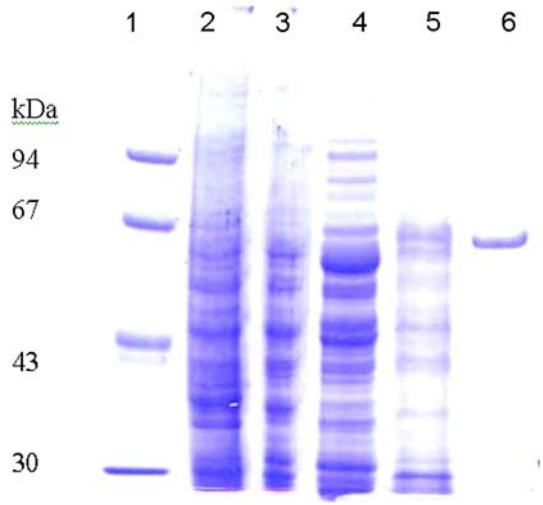

Fig. 2 SDS-PAGE of the purified amylase from *E. coli* DH5α (pAMY13H8). *Lane 1* low molecular weight standard protein-phosphorylase b (94 kDa), albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa). *Lane 2* cell free extract. *Lane 3* heat treated cell free extract. *Lane 4* ammonium sulfate fractionation. *Lane 5* Sepharose CL-6B column fraction. *Lane 6* DEAE-Sepharose CL-6B column fraction. Acrylamide concentration: 10%; electrophoresis conditions: 150 V, 1 h

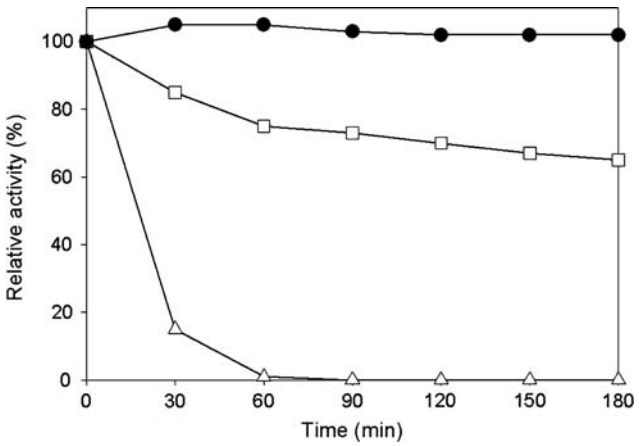

Fig. 3 Thermal stability of the purified amylase from the *E. coli* transformant. The purified enzyme was incubated at various temperatures for 30–120 min, and the residual enzyme activities were determined. *Filled circle* 50°C; *open square* 60°C; *open triangle* 70°C

by SDS-PAGE. The optimal pH and temperature of the purified enzyme (25) were 7.0 and 60°C, respectively. About 70% of the original activity remained at 60°C after 3 h (Fig. 3). The purified enzyme was stable over the pH range of 6.0 to 10.0 at 4°C for 24 h. The major end product that was produced by hydrolysis of sago or soluble starch by the purified enzyme was maltotriose (63%). It is apparent that the properties of purified amylase from the *E. coli* transformant are similar to that of the amylase from *T. fusca* NTU22 [20].

Discussion

The alignment of the amino acid sequence of *tfa* with the deduced amino acid sequences of *T. curvata*, *Streptomyces venezuelae* and *Streptomyces limosus* amylases is shown in Fig. 4. The *T. fusca* amylase shows a remarkable degree of similarity to the *T. curvata* and *Streptomyces* amylase (96.5% identity with *T. curvata*, 48% identity with *S. venezuelae* and 47% identity with

Fig. 4 Alignment of the deduced amino acid sequence of the amylase genes from various microorganisms. The deduced amino acid sequence of the amylase genes from *T. fusca* NTU22 (THF) was compared with those of amylase genes from *T. fusca* YX (TYX) [32], *S. venezuelae* (STV), *S. limosus* (STL), and *T. curvata* CCM3352 (THC). *Arrowheads* mark putative signal peptidase cleavage sites. Regions conserved among amylases from various organisms are shown as shaded boxes

S. limosus) [24–26]. A putative 33 amino acid signal sequence is predicted based on a comparison with *Streptomyces* amylase sequences with a cleavage site after an Ala-His-Ala triplet. The molecular weight of the mature protein so produced would be in agreement with that estimated by SDS-PAGE from *T. fusca* NTU22.

Two regions show major differences between the *T. fusca* enzyme and the *T. curvata* enzyme. One is found at the 326th amino acid and the other occurs in the region between the 570th and the 592nd amino acids. Even with this high similarity, the characteristics of the enzymes are very different. The major end product hydrolyzed from soluble starch by *T. curvata* amylase is maltose [27]. However, the main product hydrolyzed either from soluble starch or raw starch granules by the *T. fusca* amylase is maltotriose. The C-terminal part of the *S. limosus* and *T. fusca* amylase show a homology to the C-terminal of the glucoamylase of *Aspergillus niger* and β-amylase of *Clostridium thermosulfurogenes* [28]. This domain encompasses approximately 100 amino acids and has been suggested to enable binding to granular starch. The presence of this domain in the *T. fusca* amylase may thus explain the ability of this enzyme to hydrolyze raw starch granules.

Computer modeling of the three-dimensional structure with the Swiss-Model program predicts that the *T. fusca* amylase has an $(\alpha/\beta)_8$ barrel [29]. It clearly belongs to glycoside hydrolase family 13 [30]. On the basis of sequence homologies with other family members and the computer-predicted 3D structure of the enzyme, it is suggested that two conserved amino acid residues, Glu253 and Asp219, act as a proton donor and a nucleophile.

In the light of their economical benefits, several thermostable enzymes cloned from thermophilic microorganisms have been expressed in mesophilic microorganisms to reduce the energy needed for cultivation [31]. To the best of our knowledge, there are no reports in the literature concerning the cloning of a thermostable maltotriose-producing α-amylase gene from a thermophilic microorganism.

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