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Co-expression of the *Thermotoga neapolitana aglB* gene with an upstream 3′-coding fragment of the *malG* gene improves enzymatic characteristics of recombinant AglB cyclomaltodextrinase

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Abstract

A cluster of *Thermotoga neapolitana* genes participating in starch degradation includes the *malG* gene of sugar transport protein and the *aglB* gene of cyclomaltodextrinase. The start and stop codons of these genes share a common overlapping sequence, a**TGA**tg. Here, we compared properties of expression products of three different constructs with *aglB* from *T. neapolitana*. The first expression vector contained the *aglB* gene linked to an upstream 90-bp 3′-terminal region of the *malG* gene with the stop codon overlapping with the start codon of *aglB*. The second construct included the isolated coding sequence of *aglB* with two tandem potential start codons. The expression product of this construct in *Escherichia coli* had two tandem Met residues at its N terminus and was characterized by low thermostability and high tendency to aggregate. In contrast, co-expression of *aglB* and the 3′-terminal region of *malG* (the first construct) resulted in AglB with only one N-terminal Met residue and a much higher specific activity of cyclomaltodextrinase. Moreover, the enzyme expressed by such a construct was more thermostable and less prone to aggregation. The third construct was the same as the second one except that it contained only one ATG start codon. The product of its expression had kinetic and other properties similar to those of the enzyme with only one N-terminal Met residue.
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Earlier, we studied a *Thermotoga neapolitana*[1] gene cluster involved in the utilization of cyclic and linear maltodextrins, intermediate products of starch catabolism [1,2]. This cluster included the *malG* gene (transport protein), the *aglB* gene (cyclomaltodextrinase), and the *aglA* gene (α-glucosidase). In this cluster, the stop codon of *malG* overlaps with the tandem ATG codons of *aglB* (A**TGA**TG) [1]. The amino acid sequences of AglB and cyclomaltodex-

trinase from a closely related *Thermotoga maritima* are 92% identical [1]. According to the Henrissat classification [3], both enzymes belong to family 13 of glycosyl hydrolases (GH13). Cyclomaltodextrins (CDs) are a group of non-reducing oligosugars resulting from starch hydrolysis with cyclodextrin glycosyltransferases [4] and hydrolyzed by cyclomaltodextrinases (EC 3.2.1.54) [5]. The most widespread α-, β-, and γ-cyclomaltodextrins consist of 6, 7, and 8 glucopyranose units, respectively, that are linked in a ring.

The *aglB* gene from *T. neapolitana* and the corresponding gene from *T. maritima* were expressed in *Escherichia coli* cells [2,6,7]. In the case of *T. maritima*, the expression vector contained the isolated *aglB* sequence with two

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[1] *Abbreviations used: T. neapolitana, Thermotoga neapolitana;* CDs, Cyclomaltodextrins; GH, glycosyl hydrolases; PMSF, phenylmethylsulfonyl fluoride; PAGE, polyacrylamide gel electrophoresis.
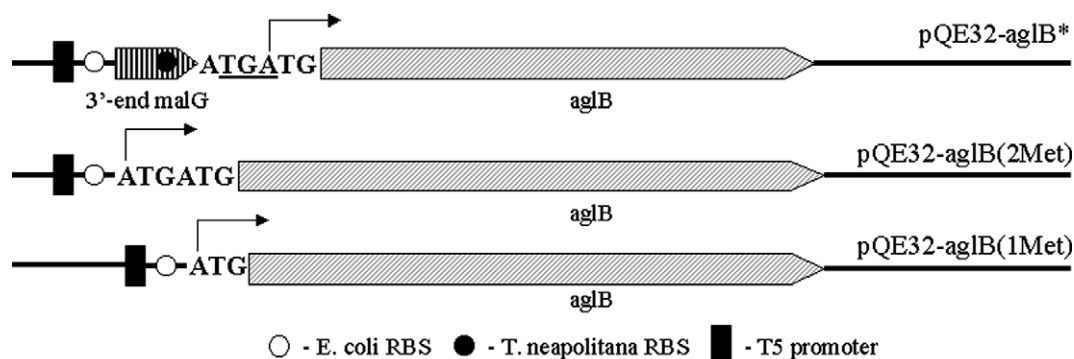
Fig. 1. Scheme of the *agl*B gene expression vectors used. Broken arrows denote translational start sites for *agl*B*, *agl*B(2Met), and *agl*B(1Met). Thick straight line, pQE32 plasmid DNA. T5, optimized promoter of a PQE plasmid (Qiagen).

tandem ATG potential start codons [6,7], while *agl*B from *T. neapolitana* was cloned as part of a genomic fragment where it was linked to an upstream 90-bp 3′-terminal region of the *mal*G gene [2]. Despite highly similar primary structures, properties of the recombinant cyclomaltodextrinases from *T. maritima* and *T. neapolitana* were markedly different. In particular, AglB from *T. maritima* was more aggregated and less active. We assumed that these differences in properties of closely related recombinant enzymes might be due to differences in the expression constructs used. To exclude possible effects of minor genetic heterogeneity between the two species of the *Thermotoga* genus, we tested this suggestion using *agl*B from *T. neapolitana*.

The goal of this work was to compare expression products of vectors carrying (i) the *agl*B gene as part of a *T. neapolitana* genomic fragment where it was linked to the upstream 90-bp fragment of *mal*G, (ii) the isolated *agl*B sequence from *T. neapolitana* with two tandem ATG potential start codons, and (iii) the same sequence with only one ATG start codon. The vector inserts are schematized in Fig. 1 and designated as *agl*B*, *agl*B(2Met), and *agl*B(1Met), respectively. Accordingly, AglB*, AglB(2Met), and AglB(1Met) denote the corresponding expression products (recombinant enzymes) of these constructs.

Materials and methods

Strains and vectors

An *E. coli* XL1-Blue strain (Stratagene, USA), a recombinant pQE32-*agl*B* plasmid [2] and a pQE30 expression vector (Qiagen, Germany) were used.

Escherichia coli cells were grown at 37 °C in Luria-Bertani broth with oxytetracycline (12 μg/ml) and with or without ampicillin (100 μg/ml). For preparation of crude extracts, recombinant *E. coli* cells were grown under antibiotic selection at 37 °C in LB, collected by centrifugation, washed in 50 mM Tris–HCl (pH 8.0), and resuspended in the same buffer supplemented with 2 mM phenylmethylsulfonyl fluoride (PMSF, Sigma, USA). The cell suspension was transferred to ice, sonicated to

transparency, heated at 75 °C for 30 min, chilled on ice, and centrifuged. The supernatant was used to measure enzymatic activity.

Recombinant DNA techniques

Isolation of plasmid DNA, digestion with restriction endonucleases, DNA ligation, agarose gel electrophoresis, transformation etc., were carried out using standard protocols [8,9]. In a pQE32-*agl*B* plasmid (see Fig. 1), a 1581-bp *Pvu*II–*Asu*II fragment of *T. neapolitana* genomic DNA was inserted so that the 90-bp 3′-end coding region of the *T. neapolitana mal*G gene and the *agl*B coding sequence were expressed using different ribosome binding sites (the *E. coli* RBS and *T. neapolitana* RBS, respectively) [2].

The vector pQE32-*agl*B(2Met) for expression of the isolated *agl*B coding sequence with two tandem ATG tandem start codons (see Fig. 1) was constructed in two stages. First, a 738-bp 5′-terminal region of *agl*B was PCR-amplified with forward 5′ CACACA<u>CAATTG</u>ATTAAA<u>GA GG</u>AGAAATTAACC<u>ATGATG</u>TATCCCATACCAAGT TGGGTATAC 3′ and reverse 5′ ATACTTTCCGGAAG AGAATTCCTTTACTTTCATG 3′ primers and the pQE32-*agl*B* plasmid as template. The *Mfe*I site and the RBS in the forward primer sequence are underlined. The PCR mixture (50 μl) included 800 μM each of dNTPs, 20 pmol each of primers, 100 ng DNA template, 2.5 U *Taq* DNA polymerase (Sintol, Russia) and 1× *Taq* buffer. Then, the 738-bp amplification product carrying *Mfe*I and *Kpn*2-I sites was cloned into an *Eco*RI + *Kpn*2-I digested expression vector pQE30. A *Bgl*I–*Eco*RI fragment of the resulting intermediate plasmid pQE30-(5′-end_*agl*B) containing the 738-bp 5′-terminal region of *agl*B was cloned into an *Bgl*I + *Eco*RI digested pQE32-*agl*B that contained the rest of *agl*B. The resulting recombinant plasmid pQE32-*agl*B carried the coding sequence of the cyclomaltodextrinase gene (*agl*B) from *T. neapolitana* under the control of the T5 phage promoter.

The vector pQE32-*agl*B(1Met) for expression of the isolated *agl*B coding sequence with only one ATG start codon (see Fig. 1) was prepared as above but using the forward primer lacking one ATG start codon.