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Analysis of the catalytic center of cyclomaltodextrinase from *Thermoanaerobacter ethanolicus* 39E

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The amino acid sequences of cyclomaltodextrinase (CDase) from *Thermoanaerobacter ethanolicus* 39E (formerly *Clostridium thermohydrosulfuricum* 39E) and other amylolytic enzymes were compared by using linear alignment and hydrophobic cluster analysis. Two Asp and one Glu residue, which were considered to be the catalytic residues of the compared enzymes according to crystallographic or protein engineering experiments, were also conserved in CDase. Asp^{325}, Asp^{421} and Glu^{354} of the CDase were individually replaced by means of site-directed mutagenesis. The mutant enzymes completely lost activity, suggesting that these residues play an important role in catalysis.

Cyclomaltodextrinase; Active center; Hydrophobic cluster analysis; Site-directed mutagenesis; *Thermoanaerobacter ethanolicus*

1. INTRODUCTION

Cyclomaltodextrinase (EC 3.2.1.54) (CDase) is an enzyme which hydrolyzes cyclodextrins (CDs). The main distinction of CDases is that they can fastly degrade CDs, while the starch-splitting enzymes generally are not able to cleave cyclic oligosaccharides. Linear maltodextrins are also effective substrates for CDases, but the rates of hydrolysis of other polysaccharides are significantly lower. At present the information concerning CDases is not as extensive as for other amylolytic enzymes, and this enzyme has only been purified and characterized from a few sources (for review see [1]).

We have recently reported the properties of the CDase purified from the thermophilic anaerobe, *Thermoanaerobacter ethanolicus* 39E ([2]; the former name *Clostridium thermohydrosulfuricum* was changed in accordance with a new classification [3]). The gene encoding this enzyme has been cloned and sequenced [4]. To our knowledge no other sequences of CDase genes have been published to date, however, sequence comparison of CDase with other amylolytic enzymes showed some strongly conserved regions suggesting that these enzymes may use a similar mechanism of catalysis [4]. In the present study we tentatively identified the catalytic residues of the CDase and analyzed them by means of site-directed mutagenesis.

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Abbreviations: CDase, cyclomaltodextrinase; CD, cyclodextrin; CGTase, cyclodextrin glycosyltransferase; HCA, hydrophobic cluster analysis.

2. MATERIALS AND METHODS

Amino acid sequences were aligned and compared using the Sequence Analysis Package Version 6.2 [5]. Hydrophobic cluster analysis was carried out as described previously [6,7].

Escherichia coli TG-1 [*supE hsdΔ5 thi Δ(lac-proAB)* F′(*traD36 proAB*$^+$ *lacI*^{q}*lacZ*ΔM15)] [8] was used as a host for DNA manipulations. For introduction of the mutations the *Kpn*I–*Sph*I DNA fragment of plasmid pPG22 containing CDase gene [9] was cloned into *Kpn*I–*Sph*I sites of phagemid pUC119 [10]. Cells carrying recombinant phagemid were infected with the helper virus M13KO7 [10], and single-stranded phagemid DNA was purified as described by Sambrook et al. [8]. The oligonucleotides used for mutagenesis were synthesized at the Macromolecular Structure Facility, Department of Biochemistry, Michigan State University, followed by purification by thin-layer chromatography (SurePure Oligonucleotide Purification kit; US Biochemical, Cleveland, OH, USA). The site-directed mutagenesis was performed with an Oligonucleotide-directed in vitro mutagenesis system version 2.1 (Amersham Co., Arlington Heights, IL, USA). The mutations were verified by DNA sequencing with the Sequenase kit (US Biochemical). Primers used for sequencing were synthesized previously for determination of the primary structure of CDase gene [4].

For screening of the CDase activity cells harbouring mutant or intact CDase gene were grown in 5.0 ml of LB media containing 50 μg/ml ampicillin at 37°C for 12 h. Cells were centrifuged, resuspended in 1 ml of 50 mM acetate buffer (pH 6.0) and disrupted by sonication. The cell lysate was centrifuged and the supernatant was used for the determination of enzyme activity. CDase activity was assayed at 65°C as described previously [4]. Protein was determined by the method of Bradford [11]. SDS-PAGE was performed as described by Laemmli [12].

3. RESULTS AND DISCUSSION

To receive information about the residues which might constitute the catalytic center of the CDase we compared its amino acid sequence with those of related enzymes previously investigated in respect to catalytic residues. Among five enzymes chosen for comparison,

		Region 1	Region 2	Region 3	Region 4
CDX	T. et.	238 DAVFNH...320	DGWRL**D**VANE...354	**E**VWH...416	LIGSH**D**
AMY	A. or.	117 DVVANH...201	DGLRIDTVKH...230	**E**VLD...292	FVENH**D**
AMY	po. p.	96 DAVINH...192	AGFRL**D**ASKH...233	EVID...295	FVDNH**D**
CGT	al. B.	135 DFAPNH...224	DGIRV**D**AVKH...268	**E**YHQ...323	FIDNH**D**
CGT	B. ci.	135 DFAPNH...224	DGIRV**D**AVKH...257	**E**WFL...323	FIDNH**D**
NPL	B. st.	242 DAVFNH...323	DGWRL**D**VANE...357	**E**IWH...419	LLGSH**D**

Fig. 1. Sequence similarities between CDase and amylolytic enzymes from various sources. Abbreviations and references of the sequences are as follows: CDX T. et., CDase from *T. ethanolicus* 39E [4]; AMY A. or., α-amylase from *A. oryzae* [23]; AMY po. p., α-amylase from porcine pancrease [24]; CGT al. B., CGTase from alkalophilic *Bacillus* [25]; CGT B. ci., CGTase from *B. circulans* [26]; NPL B. st., neopullulanase from *B. stearothermophillus* [19]. Numbering starts from the first amino acid of the mature protein. Numbering of the regions corresponds to highly conserved regions among α-amylases [18]. Catalytic residues proposed for the enzymes (for the references see text) are in bold.

for α-amylase from *Aspergillus oryzae* (Taka-amylase A), α-amylase from porcine pancreas and CGTase from *Bacilus circulans* these residues were proposed from X-ray diffraction analysis ([13,14,15], respectively), and for neopullulanase from *Bacillus stearothermophilus* and CGTase from alkalophilic *Bacillus* from site-directed mutagenesis experiments ([16,17], respectively).

The first method which we applied for sequence comparison was a commonly used linear alignment. On the whole, amino acid identity of CDase from *T. ethanolicus* 39E and the five amylolytic enzymes ranged from 48% (for neopullulanase) to 16% (for Taka-amylase A). Three highly conserved regions were identified in the CDase and compared enzymes (Fig. 1; regions 1, 2 and 4). Previously four highly conserved regions were detected in α-amylases [18]. These homologous regions were also identified in some other amylolytic enzymes [19]. In region 3 only glutamic acid is strictly conserved in the CDase and the aligned amylolytic enzymes (Fig. 1). The second method used for sequence comparison was the recently developed HCA method [6]. This is based on the two-dimensional representation of the sequences followed by detection and comparison of the shapes, sizes and relative positions of the hydrophobic clusters. HCA plots were built for the CDase and some of the enzymes compared on Fig. 1. The parts of these plots containing the revealed conserved regions are shown on Fig. 2. Since the sequence alignment indicates the short conserved regions, HCA detected that the whole sequences from region 1 to region 4 have an apparent correspondence among the hydrophobic cluster architectures. Since there is a positive correlation between the hydrophobic clusters and the regular secondary structure elements in proteins [20], the enzymes compared on Fig. 2 are likely to share some characteristics of the three-dimensional structures.

Analysis of the crystalline structures revealed that Glu^{230} and Asp^{297} in Taka-amylase A; Asp^{197} and Asp^{300} in porcine pancreatic α-amylase; Asp^{229}, Glu^{257} and Asp^{328} in CGTase from *B. circulans* may play a catalytic role. Two Asp and one Glu residue (shown in bold on Fig. 1) of neopullulanase from *B. stearothermophilus* and CGTase from alkalophilic *Bacillus* were individually modified by site-directed mutagenesis. The mutated enzymes completely lost activity, and it was suggested that these residues have a catalytic function. As can be seen on Fig. 1 the residues identified as catalytic for the enzymes mentioned above are conserved both in these enzymes and in the CDase. Moreover, HCA revealed that these residues are located within well-conserved segments (Fig. 2, segments I–VI). Since catalytic centers are often highly conserved we proposed that Asp^{325}, Glu^{354} and Asp^{421} of the CDase may be implicated in catalysis. In order to test this hypothesis site-directed mutagenesis was performed and the effect of mutations on CDase activity was determined.

Three oligonucleotides were designed to produce one of the following single substitutions in the CDase gene: Asp^{325} to Asn^{325}, or Asp^{421} to Asn^{421}, or Glu^{354} to Gln^{354} (Table I). As a result of the mutagenesis procedure *E.*

Table I

Mutant CDases constructed by oligonucleotide directed mutagenesis

Phagemid	Oligonucleotide[a]	Mutation	Activity (U/mg)
pPG222	–	wild type	5.5
pPG1.2	AGGCTGAATGTTGCTA	Asp-325 → Asn-325	< 0.01
pPG2.2	GTAGGACACGTTTGCCA	Glu-354 → Gln-354	< 0.01
pPG3.8	GGAAGACATAATACTGAG	Asp-421 → Asn-421	< 0.01

[a] Sequences of oligonucleotides are written from 5′ to 3′. Underlined nucleotides denote differences from the wild type sequence.

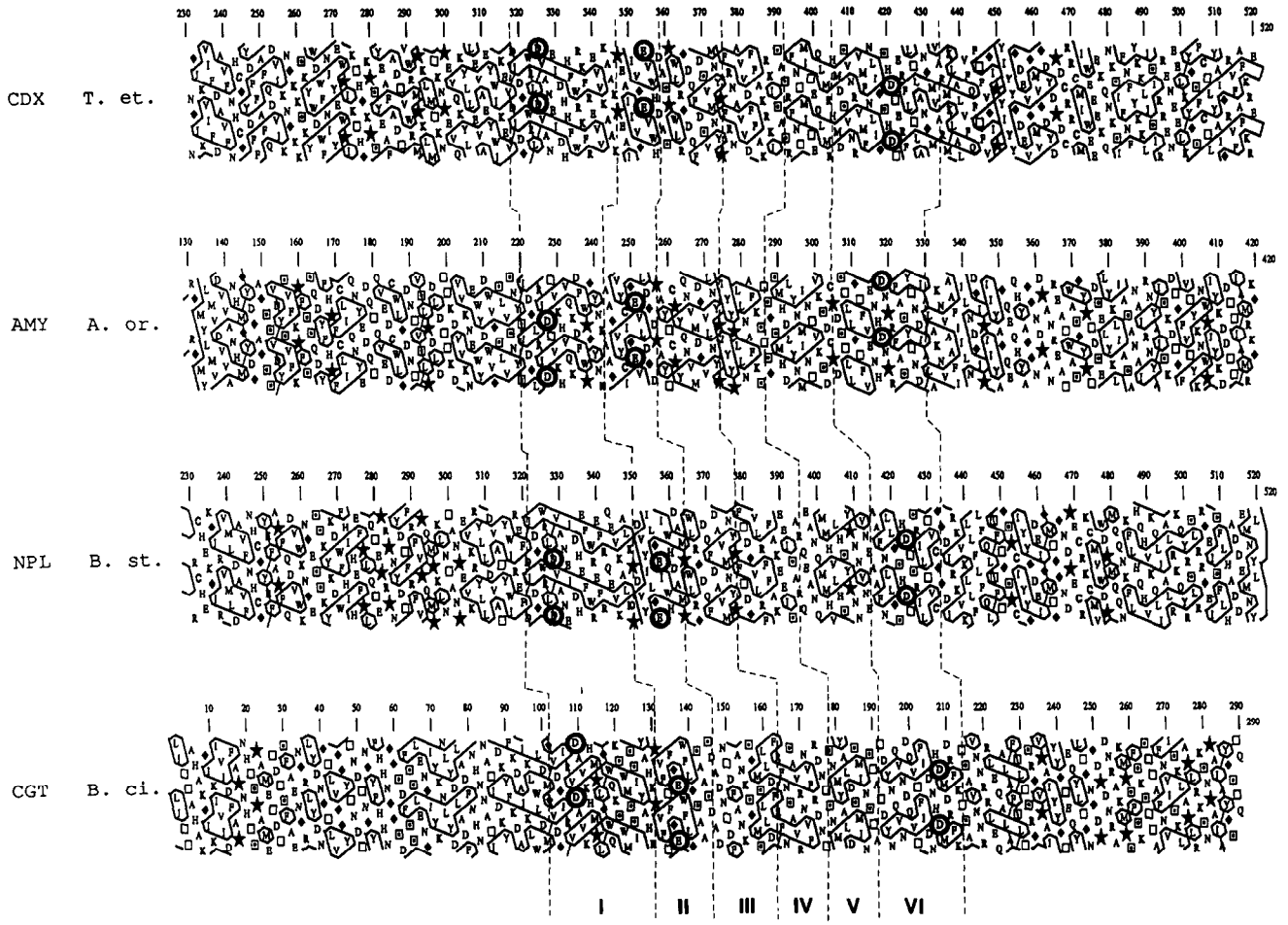

Fig. 2. HCA plots of the CDase from *T. ethanolicus* 39E, Taka-amylase A, neopullulanase from *B. stearothermophilus*, and CGTase from *B. circulans*. Abbreviations of the enzymes are the same as in Fig. 1. Numbering starts from the first amino acid of the mature protein for CDX and NPL. The first amino acid of the plot for AMY (arginine, R) corresponds to the 110th amino acid of the mature protein, and the first amino acid of the plot for CGTase (leucine, L) is 121st amino acid of the mature protein. Proline is symbolized by ★, glycine by ◆, serine by ⊡ and threonine by □. Proposed catalytic residues are ringed with circles. For the parts of the sequences containing catalytic residues the correspondences between hydrophobic clusters (segments I–VI) are shown by vertical lines.

coli TG-1 clones with the desired amino acid substitutions in the CDase gene confirmed by DNA sequencing were selected. The wild-type and the mutant enzymes were prepared as described in section 2 and used for determination of the CDase activity. As shown in Table I no activity was detected for the mutant enzymes. The activity could not be detected even when the assay was performed with 500-times excess of the mutant enzymes in comparison with the assay for the wild-type enzyme. The preparations of the enzymes, which have been used for the CDase assay, were also analyzed by SDS-PAGE. The cell extract of *E. coli* TG-1(pUC119) was used as a control. A new protein with a molecular weight corresponding to the purified CDase [4] was easily visualized both for wild-type and all mutant CDase samples (data not shown). Therefore, cells containing mutant CDase genes are able to synthesize full-size, but inactive enzymes. Since the single substitution of the selected acidic residues to their respective amide form led to such dramatic loss of the enzyme activity we concluded that these residues play a catalytic role.

This is the first report where the catalytic amino acids of CDase, an enzyme hydrolyzing cyclic oligosaccharides consisting of glucopyranose units, have been identified. The results of our study indicate that two conserved Asp and one conserved Glu of the CDase from *T. ethanolicus* 39E constitute a catalytic triad. This finding corresponds to suggestions concerning catalytic residues of the other amylolytic enzymes excluding Taka-amylase A and α-amylase from porcine pancrease (Fig. 1). According to the three-dimensional structures of these enzymes only two from three conserved acidic residues (in different combinations) were proposed as catalytic (for details see Fig. 1). But the recent study of the α-amylase from the other source, *Bacillus stearothermophilus*, by random mutagenesis revealed that the carboxyl groups of two Asp and one Glu play a role in the enzymatic mechanism [21]. Therefore, it is likely

that all three conserved acidic residues of Taka-amylase A and α-amylase from porcine pancrease may take part in catalysis.

Based on the homology data (Fig. 1,2) we suggested that the CDase and the other amylolytic enzymes may have the similar catalytic mechanism. For Taka-amylase A the acid-base catalytic mechanism analogous to that of lysozyme [22] was proposed [13]. Thus, for the CDase we proposed that Glu^{354} and Asp^{421} function as a general base and acid, respectively. The third residues of the catalytic triad, Asp^{325}, may promote donation of a proton by Glu^{354} by stabilizing the ionized carboxylate through hydrogen binding.

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