Monomeric and dimeric cyclomaltodextrinases reveal different modes of substrate degradation

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Abstract: Two cyclomaltodextrinases (CDase) of thermophilic origin were investigated for their action on cyclodextrins. Although most CDases known today are made up of at least homodimers, one of the enzymes studied was shown to be a monomer in solution, while the other one was a dimer. Interestingly, the dimeric enzyme had a much superior selectivity for a cyclodextrin substrate compared to its monomeric homologue, with a specific activity on α-cyclodextrin around 100 times higher than for the polymeric substrates starch and pullulan. Moreover, the monomeric CDase had a 10 times higher activity on those polymers than the dimer. The degradation pattern on cyclodextrins was examined by high-performance anion-exchange chromatography in combination with microdialysis. The final products were almost exclusively maltose and glucose in an approximate molar ratio of 2:1. However, the intermediate product ratios were quite different for the two enzymes, revealing that the monomeric CDase had a more random distribution of transitional products. Moreover, the dimeric CDase accumulated maltotriose, which is believed to be due to transglycosylation. The oligomeric state of the enzymes is thought to be a key factor for exhibiting high cyclodextrinase as well as transglycosylation activity.

Key words: Cyclomaltodextrinase, glycoside hydrolase family 13, *Anoxybacillus*, *Laceyella*, oligomerization, cyclodextrin degradation, transglycosylation.

Abbreviations: CD, cyclomaltodextrin; CDase, cyclomaltodextrinase; HPAEC-PAD, high-performance anion exchange chromatography with pulsed amperometric detection; HPTLC, high-performance thin-layer chromatography; MAase, maltogenic amylase; NPase, neopullulanase; *p*NP, *p*-nitrophenyl; *p*NPG2, *p*-nitrophenyl-α-D-maltoside; *p*NPG5, *p*-nitrophenol-α-D-maltopentaoside.

Introduction

Cyclomaltodextrinases (CDase, EC 3.2.1.54) are enzymes capable of degrading cyclomaltodextrins (CDs), which are cyclic, non-reducing oligosaccharides, built up from six, seven, or eight glucopyranose units (α-, β- and γ-CD). Other enzymes also reported to degrade CDs are neopullulanases (NPase, EC 3.2.1.135) and maltogenic amylases (MAase, EC 3.2.1.133). These three different enzymes are all members of the α-amylase family of enzymes, classified based on sequence and structure similarity in glycoside hydrolase family 13, GH13 (Coutinho & Henrissat, 1999). The main function of the enzymes in family 13 is to modify α-linked glucans (starch or starch-like molecules) by hydrolysis or transglycosylation with retention of the α-anomeric configuration (MacGregor et al., 2001).

We have previously used the conserved sequence regions of amylase enzymes (Yamamoto, 1995; Kuriki & Imanaka, 1999) to construct primers based on the CODEHOP consensus primer strategy (Rose et al., 1998) to amplify amylolytic genes from both isolated strains and from enrichment samples. One example of the outcome of this effort was two enzymes, classified by us as cyclomaltodextrinases, amplified from strains closely related to *Anoxybacillus flavithermus* and *Laceyella sacchari*, respectively (Turner et al., 2005b). These enzymes are named *Af*Cda13 and *Ls*Cda13 in agreement with the classification proposed by Henrissat et al. (1998).

The two enzymes are composed of four domains analogous to other CDases. The catalytic module is formed by the A domain, which is a $(\beta/\alpha)_8$-barrel, and the B domain protruding from the A domain. There is also a C domain which is present in most α-amylase family enzymes and believed to stabilize the catalytic

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module by shielding hydrophobic residues of domain A from the solvent (MacGregor et al., 2001). It may also take part in substrate binding (Dauter et al., 1999; Robert et al., 2003). In addition to these, there is an N domain preceding the $(\beta/\alpha)_8$-barrel, not present in regular α-amylases but reported to exist in most CDases, NPases and MAases (Park, 2001). The N-terminal domain is thought to interact with the catalytic domain of another monomer, aiding in the formation of a dimer, and thereby creating a deep and narrow cleft that can host a cyclodextrin molecule (Kamitori et al., 1999; Kim et al., 1999; Park et al., 2000; Kim et al., 2001). The shape of the active site cleft when examining the monomeric unit of a neopullulanase has been shown to be wider and shallower than in other α-amylase enzymes (Hondoh et al., 2002).

Most enzymes reported with high CDase activity are either dimers or larger (tetramer/dodecamer) (Park et al., 2000; Park, 2001; Lee et al., 2005). Park (2001) has shown that a dimeric CD-degrading enzyme (ThMA) exhibits higher catalytic activity on cyclodextrins than a monomer of the same enzyme. Likewise, the activity towards starch increases when the same enzyme converts from a dimer to a monomer. The enzymes studied here also exhibit different rates on cyclodextrin and starch hydrolysis. Both *Af*Cda13 and *Ls*Cda13 contain the extra N-terminal domain proposed as being the key component for oligomerization and high cyclodextrinase activity. Yet, *Ls*Cda13 is a monomer in solution and the preference for cyclodextrins is much inferior.

The main aim of the present study was to investigate the difference in the mode of substrate hydrolysis of *Af*Cda13 and *Ls*Cda13. We focused on the hydrolysis of cyclodextrins since both enzymes were primarily active on these substrates. The hydrolysis pathways of α-, β- and γ-CD were analyzed and the basis for the difference is discussed.

Material and methods

Chemicals

All chemicals were pro-analysis from Merck Eurolabs (Darmstadt, Germany) unless otherwise stated.

Strains and plasmids

The genes encoding the CDases were PCR-amplified from genomic DNA of bacterial strains of the genera *Anoxybacillus* and *Laceyella* isolated in Iceland. These strains are closely related to *Anoxybacillus flavithermus* and *Laceyella sacchari*, respectively, as determined by 16S rRNA sequencing. Forward and reverse primers for the first amplification were designed according to the CODEHOP strategy (Rose et al., 1998), and were constructed to complement the DNA coding sequences of two of the conserved sequence regions I and II in GH13 (Yamamoto, 1995). The upstream and downstream flanking regions were then amplified from the corresponding genomic DNA in a series of nested PCR using one gene specific, 5'-biotin labeled primer, and one arbitrary primer targeting the unknown flanking sequence until the complete genes were obtained (Turner, et al., 2005b). Finally the complete genes were PCR-amplified for insertion into the expression vector pET-22b(+) (Novagen, Madison, WI) incorporating the C-terminal hexa-histidine tag. The gene from *A. flavithermus* (*Afcda13*) was inserted in the *Nco*I and *Not*I sites, while the other gene (*Lscda13*) was inserted in the *Nde*I and *Xho*I sites. Ligation was performed by T4 DNA ligase (Invitrogen Life Technologies, Frederick, MD). The resulting plasmids were transformed into *E. coli* Nova Blue cells (Novagen).

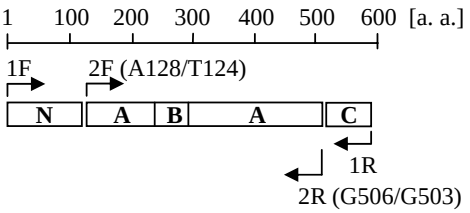

Fig. 1. The domain organization of *Ls*Cda13 and *Af*Cda13. The N-terminal domain (N) contains approximately 120 residues, and is followed by the catalytic module (approximately 470 residues), composed of domain A and B (50 residues), and finally the 80-residue long C-domain (C). The primers used to clone the wild-type genes, as well as to construct domain deletion-mutants, are marked. The notes given in parenthesis point out the targeted position (for *Af*Cda13 and *Ls*Cda13, respectively) in the amino acid sequence, which for primer 2F corresponds to the N-terminal amino acid and for 2R corresponds to the C-terminal amino acid.

Domain deletion mutants were created by amplifying the *Afcda13* and *Lscda13* genes without the N and/or the C domain. Primers were used to target different positions in the gene sequence (Fig. 1). Primers 1F and 2R for either gene were used to construct ΔC mutants. Moreover, ΔN mutants were generated using primer 2F in combination with 1R. Finally mutants (ΔN ΔC) were produced lacking both the N and the C domains by utilizing primers 2F and 2R.

Inserts from positive clones were fully sequenced using the T7 forward and T7 reverse primers and the Big Dye Terminator DNA Sequencing Kit v2.0 (PE Applied Biosystems, Foster City, CA) on an ABI 3100 sequencer (PE Applied Biosystems).

Primary structure analysis and three-dimensional models

BLAST-searches (Altschul et al., 1990) using the amino acid sequences of the two enzymes (GenBank accession numbers AY937388 and AY937387) were performed in order to find the closest homologues. Domain boundaries were obtained by aligning the sequences to homologues with known three-dimensional structures and verified with models of the target enzymes. Three-dimensional models of the two studied enzymes were made by SWISS-MODEL in Swiss-PdbViewer 3.7 (Schwede et al., 2003) using the 3-D structures of TVAII from *Thermoactinomyces vulgaris* (PDB code 1JI2; Kamitori et al., 2002) and NPase from *Geobacillus stearothermophilus* (PDB code 1J0H; Hondoh et al., 2003). Energy minimization was performed on the constructed model and side-chains with high energy were fixed manually or by the exhaustive search option.

Expression and purification

The plasmids were transformed into the *E. coli* expression strains BL21(DE3) and Tuner(DE3). Inducer con-

centration optimization in Tuner(DE3) was performed according to the method described elsewhere (Turner et al., 2005a). The enzymes were produced in a lab-scale of 2 L by fed-batch cultivation using a defined medium (Ramchuran et al., 2002). After 2-3 h of induction the cells were harvested by centrifugation at 6,000 × *g*, 4 °C, 5 min.

The cell pellets obtained from the cultivations were dissolved in binding buffer (20 mM Tris-HCl, 20 mM imidazole, 0.75 M NaCl, pH 7.5) and ultrasonicated by a titanium probe with 14 mm diameter (Dr. Hielscher, Teltow, Germany) using a sound intensity of 60% and a cycle of 0.5. The pellets were generally sonicated for 5 × 5 min and lysis was checked by microscopy.

Soluble proteins were separated from insoluble proteins and cell-debris by centrifugation at 40,000 × *g* for 40 min at 4 °C. The supernatant (crude extract) was passed through a 0.45 μm Minisart high-flow filter (Sartorius, Göttingen, Germany) and purified by immobilized metal ion affinity chromatography (IMAC) using an ÄKTA prime system (Amersham Biosciences, Uppsala, Sweden) with a HiTrap Chelating 1 mL column. The gel matrix was first washed with deionized water before 5 mg/mL copper sulphate (5 vol) was loaded. The column was then washed with deionized water before equilibration with 10 mL binding buffer. Crude extract was loaded through a sample loop (5 mL). Unbound proteins were washed off by running through 10 mL binding buffer. Elution was performed by a gradient ranging from 20 to 500 mM imidazole in 20 mM Tris-HCl, 0.75 M NaCl, pH 7.5. Twenty fractions of 1 mL were collected. The fractions containing protein as observed in the chromatogram (by 280 nm absorbance) were analyzed as described in the following section.

Protein analysis

Enzyme activity. Hydrolyzing activity was measured by the DNS (3,5-dinitrosalicylic acid) assay for reducing sugars (Miller, 1959), modified to fit microtitre plates. To 80 μL of 1.25% β-cyclodextrin (Pfanstiehl Laboratories, Waukegan, IL) dissolved in 20 mM buffer, 20 μL of enzyme solution/buffer/standard was added and incubated at 55 °C for 5–15 min in a Biometra T Gradient thermal cycler (Nordic BioSite, Täby, Sweden). Linearity of activity was ensured throughout the entire incubation. As a standard, D-glucose was used in concentrations ranging from 2 to 15 μmol/mL. The enzymatic reaction was stopped by adding 100 μL DNS solution and heating the samples for 5 min at 99 °C. Individual enzyme blanks were prepared by adding the enzyme solution after DNS had been added and then heating at 99 °C. To a microtitre plate containing 150 μL water, 150 μL of the sample was transferred and the absorbance was read at 550 nm in an ASYS Hitech DigiScan plate reader (ASYS Hitech, Eugendorf, Austria). One unit (1 U) corresponds to the amount of enzyme that will release 1 μmol of reducing sugar equivalents (expressed as glucose) per minute.

Purity and protein concentration. The purity was estimated by SDS-PAGE according to Laemmli (1970) using a 10% running gel and staining for 1 h in 0.2% Coomassie brilliant blue, 10% acetic acid, 40% methanol. The gels were destained for 2–3 h in a solution containing 10% acetic acid and 40% methanol. The total protein concentration was determined by the BCA-copper method (Sigma, Steinheim, Germany) using bovine serum albumin as standard.

Gel filtration. The native molecular weights were estimated by gel filtration using Superdex 200 Highload 16/60 in 50 mM Tris-HCl, 150 mM NaCl, pH 7.4 with 1 mL/min flow, using ovalbumin (43 kDa), chymotrypsin (25 kDa), β-amylase (200 kDa) and BSA (66 kDa) as standards.

Analysis of hydrolysis/transglycosylation products

High-performance thin-layer chromatography (HPTLC). HPTLC was performed on a CAMAG system based on an Automatic TLC sampler III version 2.13, TLC Scanner 3 and an Automatic Development Chamber (ADC) controlled by WinCats version 1.1.1.0 software (CAMAG, Muttenz, Switzerland). TLC 10 × 20 cm Silica gel 60-plates were used (Merck Eurolabs). Enzyme reactions were performed using 1–40 μg enzyme and 6–30 mM substrate in 20 mM Tris-HCl pH 7.9. The substrates used were maltopentaose, *p*-nitrophenyl-α-D-maltopentaoside (*p*NPG5), *p*-nitrophenyl-α-D-maltoside (*p*NPG2), *p*-nitrophenyl-α-D-glucoside, maltose (Sigma) and glucose (Merck Eurolabs). The reactions were carried out at 40 °C in a total volume of 500 μL where enzyme and substrates were dissolved in 20 mM Tris-HCl, pH 7.9. At fixed time intervals, a 50 μL sample was withdrawn and added to 950 μL of deionized water, before placed at −20 °C until analysis. A volume of 5–10 μL of the sample was loaded on the HPTLC-plate. A standard consisting of glucose (Merck Eurolabs), maltose, maltotriose, maltotetraose and maltopentaose (Sigma) each in a concentration of 3 mM was prepared. A volume of 1.5–2 μL of standard was loaded on the plate. The plate was developed by two different mobile phases, the first one consisting of isopropanol, ethyl acetate and water in the ratio 3:1:1 (once to the height of 50 mm) and the second one of n-butanol, acetic acid and water, 3:1:1 (twice to the height of 75 mm) in the ADC unit. The products and substrates were visualized by dipping the plate in a vanillin solution (1 g of vanillin, 25 mL of ethanol, 25 mL of water and 35 mL of 85% phosphoric acid) (Chahid et al., 1992) and heating to 190 °C for 6 min (Andersson & Adlercreutz, 2001).

High-performance anion-exchange chromatography (HPAEC) and microdialysis. Microdialysis with automated sampling using an in-laboratory fabricated microdialysis probe (Laurell & Buttler, 1995; Torto & Gorton, 1999) was used as described previously (Torto et al., 1998). The effective dialysis length was set to 10 mm. The enzymatic reactions and simultaneous microdialysis were performed at 40 °C in 5 mL vials housed in a heating and stirring module (Pierce, Rockford, IL). The enzymatic reactions were performed with a substrate concentration of 50 μM in a total volume of 3.5 mL and the amount of enzyme added was 1–2 μg. The membrane was perfused at 2 μL/min using a CMA/100 syringe pump (CMA microdialysis, Solna, Sweden) and sample injections took place every 30 min with an injection volume of 10 μL. Calibration curves of the sugar substrates as well as the transfusion over the membrane were obtained by injecting the sugars at different concentrations with and without using the microdialysis set-up.

A chromatographic system, HPAEC with pulsed amperometric detection (HPAEC-PAD), with CarboPac PA-100 pre- and analytical columns, GP 40 gradient pump and ED40, all from Dionex Corporation (Sunnyvale, CA) was used for separation and detection of the hydrolysis products. The electrochemical detection was used with the following waveform: $E_1 = 0.10$ V ($t_d = 0.20$ s, $t_1 = 0.20$ s), $E_2 = -2.00$ V ($t_2 = 0.02$ s), $E_3 = 0.60$ V ($t_3 = 0.01$ s) and E_4

= −0.10 V (t_4 = 0.06 s) vs. a Ag/AgCl reference electrode and a gold working electrode. The system was controlled by the PeakNet software from Dionex. The flow through the column was 1 mL/min and the temperature of the column was 30 °C. A gradient programme was used for the elution of the saccharides with 150 mM NaOH (eluent A) and 250 mM NaOAc prepared in 150 mM NaOH (eluent B). From 0-2 min, eluent A was kept at 80% and from 2–4 min eluent A decreased to 60%. From 4–7 min, eluent A was kept at 60% and from 7–10.5 min eluent A decreased to 0%. From 10.5–14 min, eluent A was kept at 0% and between 14 and 15 min it was readjusted to 80%.

Results

Enzyme structure

The domain organization of *Af*Cda13 and *Ls*Cda13 is typical of CDases with the N domain, the $(\beta/\alpha)_8$-barrel (domain A) with the B domain protruding from it and finally the C domain (Fig. 2A). The catalytic modules were most conserved, but the N-domains of the respective enzyme also showed significant sequence conservation, while the C-domains generally were less well conserved. No signal peptide could be identified in either of the two enzymes. The individual domains were compared to homologous enzymes, whose three-dimensional structures had been determined and domain functions assigned. The closest homologue to the respective enzyme was used to construct a three-dimensional model. Domain boundaries were hence identified, which were utilized to construct domain deletion mutants (Fig. 1). For *Af*Cda13 the NPase from *Geobacillus stearothermophilus* (Hondoh et al., 2003) was used as template (69% identity). Like most CDases, both the *G. stearothermophilus* NPase and *Af*Cda13 are homodimers (see below). Therefore, a dimeric model of *Af*Cda13 was constructed using SWISS-MODEL in Swiss-PdbViewer 3.7 (Fig. 2B). The model of *Ls*Cda13 was constructed using the neopullulanase TVAII from *Thermoactinomyces vulgaris* (Kamitori et al., 2002) as a template (65% identity). These two enzymes however differ in that TVAII is a dimer, while *Ls*Cda13 is a monomer in solution, and the model was constructed by using only chain A of TVAII (Fig. 2C).

The *Af*Cda13-catalytic module was 77% identical to the module of the NPase of *G. stearothermophilus*, and the *Ls*Cda-module showed 73% identity to the catalytic module of TVAII. The level of identity (55%) between *Af* and *Ls*Cda13, was lower, but still the second highest found so far for *Ls*Cda13. The catalytic residues were hence identified in both enzymes (D338, E357 and D424, *Af*Cda13-numbering), as well as conserved residues previously identified to be of importance for substrate binding (Fig. 2D). Calcium ions have been shown to affect the activity and thermal stability of *Ls*Cda13, though calcium ion dependence could not be detected for *Af*Cda13 (data not shown). Comparison with the homologous sequences, however, showed that the ligands of a single Ca^{2+} -binding site (Kamitori et al., 2002; Hondoh et al., 2003), situated in the A-domain, are conserved in both the *Af*Cda13 and *Ls*Cda13 sequences (Fig. 2 A,C).

The N-domain of the respective enzyme (residues 1-113 in *Ls*Cda13 and 1-118 in *Af*Cda13) had a slightly lower degree of sequence conservation, but the most similar domains were in the same enzymes as the most similar catalytic modules. Despite the difference in oligomeric state, this domain from *Ls*Cda13 was 58% identical to the N-domain of TVAII, which was the domain showing highest sequence identity to *Ls*Cda13. The N-domain of *Af*Cda13 showed 68% identity to the corresponding domain of the NPase from *G. stearothermophilus*, while the identity to the N-domain of *Ls*Cda13 was only 30.5%. Y45 (*Af*Cda13-numbering), which is involved in recognizing the hydrophobic cavity of a cyclodextrin in dimeric enzymes (Fig. 2D), was conserved in both the dimeric and monomeric enzyme. This is not the case for TVAI, an extracellular monomeric enzyme, composed of domains N, A, B, and C. In this enzyme, two different sugar binding sites, (N and NA) have been identified in the N-domain (Abe et al., 2004), and the domain has been suggested to have a starch binding function. However, the TVAI N-domain is merely 19 and 20% identical to the N-domains of *Af*Cda13 and *Ls*Cda13, respectively, and the residues identified to interact with the bound oligosaccharides were not conserved in *Af*Cda13 and *Ls*Cda13, rendering sugar-binding predictions impossible. Moreover, both CDases are here reported to be intracellular enzymes, making it unlikely that the N-domains of these enzymes would play a role in starch binding.

Cloning, expression and purification

The complete genes *Afcda13* and *Lscda13* and a series of domain deletion mutants of *Afcda13* (Fig. 1) were cloned in the expression vector pET-22b(+) (Novagen). Only the wild-type enzymes could be expressed in active form in the *E. coli* expression system. Although even the wild-type enzymes were very difficult to express in active soluble form, a reasonable amount of the target enzymes were obtained in the soluble fraction using the conditions found after optimization of the expression of *Afcda13* by inducer-tuning in the *E. coli* strain Tuner(DE3) (Turner et al., 2005a). Both enzymes were hence produced utilizing a concentration of 0.05-0.1 mM IPTG in fed-batch cultivations using either substrate-limited (Ramchuran et al., 2002) or temperature-limited (de Maré et al., 2004) feeding-control strategies. Fed-batch technology in combination with the inducer-tuning strategy resulted in 300 mg of *Af*Cda13/L culture volume. Both enzymes had a hexaHis-tag expressed in their C-terminal end and were purified selectively by immobilized metal ion affinity chromatography using a copper ligand (results not shown).

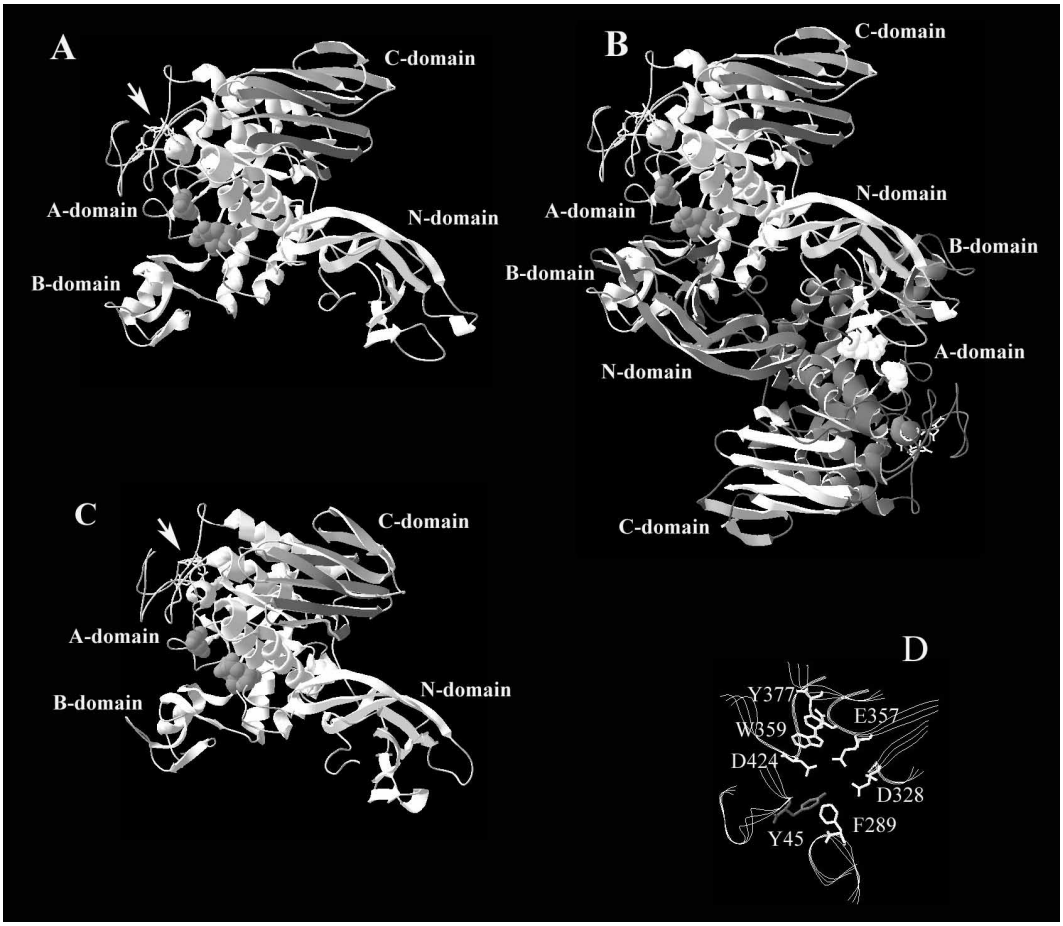

Fig. 2. Models of *Af*Cda13 in its monomeric (A) and its native dimeric (B) state, and of the native monomeric *Ls*Cda13(C). The domain composition is similar for both enzymes, and the individual domains are indicated. The position of the conserved residues, corresponding to the calcium binding ligands (side-chains shown as ball and stick model) are indicated by an arrow on the monomeric form of the respective enzyme (A, C). The side chains of the three catalytic residues (D328, E357, D424 and D325, E354, D421 for *Af*Cda13 and *Ls*Cda13, respectively) are shown as space-fill models in the respective enzyme (A, B, C) and in addition a close up of the area surrounding these residues is shown using the model of *Af*Cda13 (D). Two additional conserved residues of the A-domain are shown (W359, Y377), involved in interactions with β-CD and γ-CD (Ohtaki et al., 2004). The residues F289 from the B-domain, and Y45 from the N-domain of the other monomer, are also conserved, and identified as important for recognition of the hydrophobic cavity of CDs.

Oligomeric state and substrate specificity

In order to determine the oligomeric state of the enzymes, they were run through a gel filtration column. The experiments revealed that *Af*Cda13 exists mainly as a dimer at optimum pH 6.5 (native Mr of 148 kDa), whereas *Ls*Cda13 gave a native Mr of 74 kDa at the pH optimum of 6, corresponding to a monomer in solution.

The substrate specificities were determined using the DNS assay for reducing sugars. The experiments were performed at 55 ℃ at pH 6.4 and 7.5 for *Ls*Cda13 and *Af*Cda13, respectively, and linearity of activity was ensured during the entire incubation (5–15 min). The outcome demonstrated that the dimeric enzyme *Af*Cda13 has a high activity on cyclodextrins, whereas the activity on starch and pullulan was only about one-hundredth of the CDase activity (Fig. 3). Moreover, the highest activity of *Af*Cda13 was obtained for the smallest cyclodextrin (α-CD) and decreased gradually with increasing ring size. The monomeric *Ls*Cda13 was much less selective for a certain substrate. The specific activity of *Ls*Cda13 on α-CD was 10 times lower than that of *Af*Cda13, while it was only 4 times lower for β-CD and twice lower for γ-CD. In contrast, the activity on both starch and pullulan was higher than that of *Af*Cda13, although a slight preference for cyclodextrin sugars was shown, which justified the classification as a CDase (Fig. 3).

Hydrolysis/transglycosylation products

The hydrolysis products of*Af*Cda13 and *Ls*Cda13 on polymeric soluble starch were mainly maltose and some glucose (data not shown). The polymer pullulan was hydrolyzed to mainly panose, but reaction with *Af*Cda13 also yielded some maltose and glucose (data not shown).

Products from a set of oligomeric and CD-substrates were analyzed by HPTLC and HPAEC-PAD. HPTLC-analysis after incubation of *Af*Cda13 and

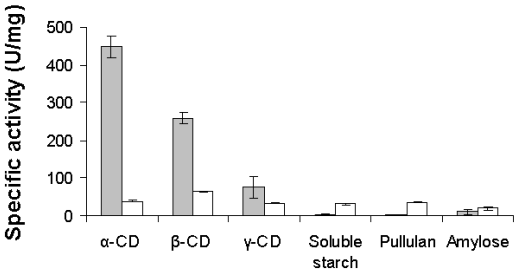

Fig. 3. Specific activities of *Ls*Cda13 (white bars) and *Af*Cda13 (grey bars) determined at 55 ℃ for 5–15 min at pH 6.4 (*Ls*Cda13) and 7.5 (*Af*Cda13). All substrate concentrations were 1%, except for starch, which had a concentration of 2%. No activity was detected on amylopectin. Each value is the mean of at least three determinations ± the standard deviation.

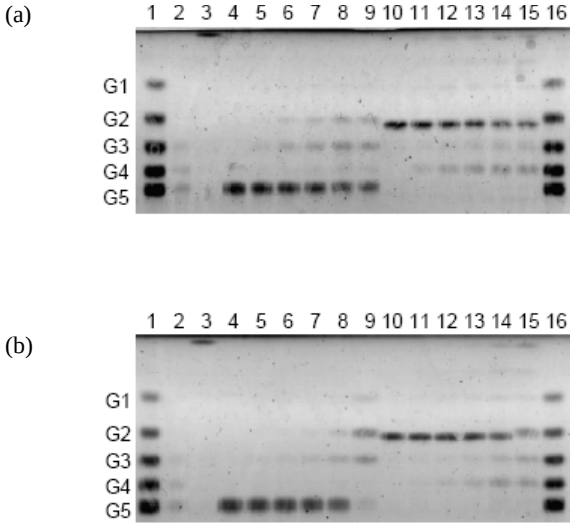

Fig. 4. HPTLC analysis of products formed by *Af*Cda13 (a) and *Ls*Cda13 (b) after incubation with maltopentaose and *p*NPG5. Reaction mixtures containing 6 mM substrate and 1 μg enzyme in 20 mM Tris-HCl buffer, pH 7.9 were incubated for 0.5 to 20 h at 40 ℃ and diluted 20 times before being analyzed. Lanes 1 and 16, 2 μL standard glucose (G1) through maltopentaose (G5); lane 2, *p*NP; lane 3, *p*-nitrophenyl-α-D-glucoside; lane 4, G5; lanes 5–9, G5 + enzyme incubated for 0.5, 1, 2, 6 and 20 h; lane 10, *p*NPG5; lanes 11–15, *p*NPG5 + enzyme incubated for 0.5, 1, 2, 6 and 20 h.

*Ls*Cda13 (1 μg) with 6 mM maltopentaose showed that maltotriose and maltose were initially generated (Fig. 4, lanes 5–9), while reactions with *p*NPG5, yielded the initial products maltotetraose and *p*NP-glucoside (Fig. 4, lanes 12–15). After a prolonged incubation with *p*NPG5, maltotriose and maltose were also formed, and additional faint bands (possibly corresponding to *p*NPG2 and *p*-nitrophenyl-α-D-maltotrioside) were observed (Fig. 4, lane 15). At higher enzyme load (40 μg) only maltose and glucose remained after 22 h of incubation with maltopentaose, and similarly, maltose, glucose and *p*NP-glucoside remained after incubation with *p*NPG5 (data not shown). Based on these results the reactions appear to give hydrolysis products similar to endo-acting enzymes, although an initial preference for

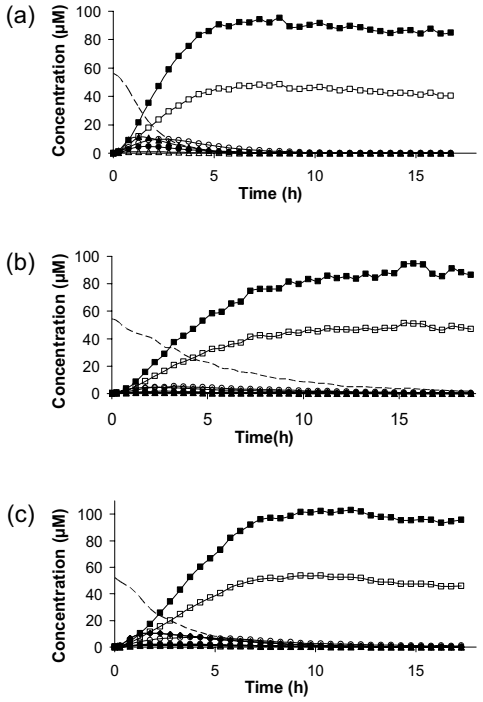

Fig. 5. Hydrolysis pattern of α-cyclodextrin (a), β-cyclodextrin (b), and γ-cyclodextrin (c) by *Ls*Cda13 analyzed by HPAEC-PAD. The reactions were performed at 40 ℃ in 20 mM MES buffer, pH 6 using 50 μM cyclodextrin and approximately 1 μg enzyme. Broken line, (a) α-CD / (b) β-CD / (c) γ-CD; open squares, G1; filled squares, G2; open circles, G3; filled circles, G4; open triangles, G5; filled triangles, G6; open rombs, G7; filled rombs, G8.

attack on the reducing end is seen yielding a product with a DP (degree of polymerization) of 2.

The degradation patterns of the most preferred substrates, α-, β-, and γ-cyclodextrins, were followed in more detail using microdialysis with automatic sample injections to HPAEC-PAD. The final products were in all cases maltose and glucose in the approximate molar ratio of 2:1 (Figs 5, 6). Although the final products were similar, the intermediate products were quite disparate. *Ls*Cda13 was fairly indiscriminate in the generation of intermediate products (Fig. 5), whereas *Af*Cda13 had a much more distinct pattern and produced maltotriose to a high ratio (Fig. 6). The maltotriose even accumulated and was in all cases not hydrolyzed (Fig. 6B), indicating that either product inhibition, transglycosylation or enzyme deactivation was occurring. Deactivation of the enzyme was not considered to be likely since accumulation of maltotriose only happened for one substrate. Thus, to investigate the possibility of product inhibition, reactions with maltotriose with and without maltose and glucose, respectively, were carried out. The results clearly showed that the hydrolysis of maltotriose (30 μM) by *Af*Cda13 was not affected by the addition of maltose (200 μM) (data not shown). Addition of glucose (200 μM) to the reaction resulted in a slightly higher maltotriose concentration at the corresponding time, although complete hydrolysis was still

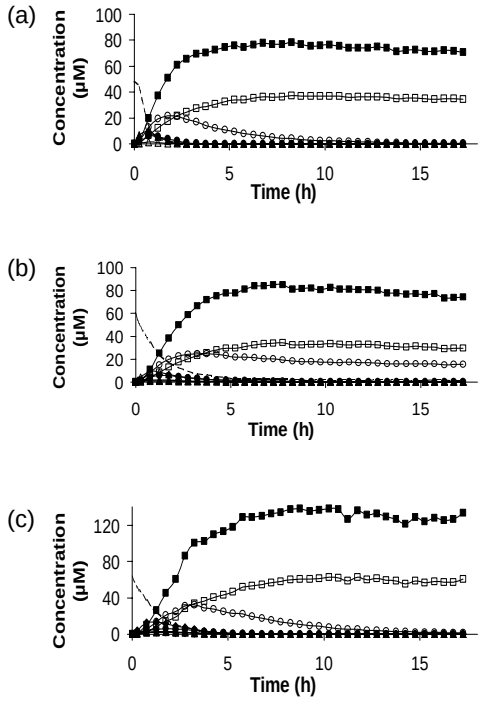

Fig. 6. Hydrolysis pattern of α-cyclodextrin (a), β-cyclodextrin (b), and γ-cyclodextrin (c) by AfCda13 analyzed by HPAEC-PAD. The reactions were performed at 40 °C in 20 mM sodium phosphate buffer, pH 7 using 50 μM cyclodextrin and approximately 2 μg enzyme. Broken line, (a) α-CD / (b) β-CD / (c) γ-CD; open squares, G1; filled squares, G2; open circles, G3; filled circles, G4; open triangles, G5; filled triangles, G6; open rombs, G7; filled rombs, G8.

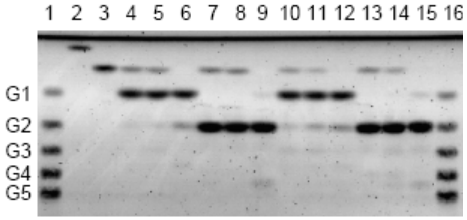

Fig. 7. HPTLC analysis of products formed by AfCda13 and LsCda13 after incubation with pNP-α-D-maltoside (pNPG2) and glucose/maltose. Reaction mixtures containing 6 mM pNPG2, 30 mM glucose and maltose, respectively, and 20–40 μg of enzyme in 20 mM Tris-HCl, pH 7, were incubated for 10 min to 18 h at 40 °C and diluted 20 times before analyzed. Lanes 1 and 16, 1.5 μL standard glucose (G1) through maltopentaose (G5); lane 2, pNPG; lane 3, pNPG2; lane 4, pNPG2 + G1 + 20 μg LsCda13 10 min incubation; lane 5, pNPG2 + G1 + 40 μg LsCda13 10 min incubation; lane 6, pNPG2 + G1 + 40 μg LsCda13 18 h incubation; lane 7, pNPG2 + G2 + 20 μg LsCda13 10 min incubation; lane 8, pNPG2 + G2 + 40 μg LsCda13 10 min incubation; lane 9, pNPG2 + G2 + 40 μg LsCda13 18 h incubation; lane 10, pNPG2 + G1 + 20 μg AfCda13 10 min incubation; lane 11, pNPG2 + G1 + 40 μg AfCda13 10 min incubation; lane 12, pNPG2 + G1 + 40 μg AfCda13 18 h incubation; lane 13, pNPG2 + G2 + 20 μg AfCda13 10 min incubation; lane 14, pNPG2 + G2 + 40 μg AfCda13 10 min incubation; lane 15, pNPG2 + G2 + 40 μg AfCda13 18 h incubation.

observed after 6 h. Maltose was formed, however, more quickly in the presence of glucose, and hence, product inhibition was excluded as the reason for maltotriose accumulation. Rather, the higher amount of maltotriose in the reactions performed in the presence of glucose supports that transglycosylation was taking place. In fact, also when AfCda13 reacted with maltotetraose, although maltose was the main product, 2–3 times more maltotriose than glucose was formed (data not shown). Based on these data a plausible reaction may be the cleavage of maltotetraose into two maltose units whereupon glucose can act as a nucleophile to generate maltotriose. In accordance with our observations, transglycosylation will then lead to that more maltotriose than glucose is present in the reaction mixture.

When performing the same experiment with LsCda13, it could not be established that transglycosylation occurred since maltotriose and glucose were produced in an approximate molar ratio of 1:1 (data not shown).

Further support for transglycosylation reactions catalyzed by AfCda13 was collected using pNPG2, with the good leaving group p-nitrophenol, as glycosyl donor while glycosyl acceptors in the form of glucose and maltose were supplied. After incubations with 6 mM pNPG2 and 30 mM glucose or maltose, for up to 18 h, the samples were diluted 20 times in water and analyzed by HPTLC. pNPG2 was mainly hydrolyzed to maltose as expected, but faint bands of larger products were also present (including maltotriose and some other faint bands corresponding to larger sugars), undoubtedly formed by transglycosylation (Fig. 7). Hence, both the HPTLC and the HPAEC-PAD techniques supported transglycosylation activity by AfCda13.

Discussion

Both AfCda13 and LsCda13 are presumed to be intracellular enzymes, due to the absence of a signal peptide. Their native roles would hence be hydrolysis of cyclodextrins. CDs have been demonstrated intracellularly, and a specific uptake mechanism has also been proposed for the bacterium *Klebsiella oxytoca* (Fiedler et al., 1996; Park et al., 2000). The putative intracellular location of the AfCda13 and LsCda13 makes it unlikely that their physiological role is degradation of polymeric substrates.

Despite their common cellular location and similar domain architecture (being 44% identical at the amino acid sequence level) the two enzymes have quite different properties. One of the major differences is the variation in preference for the smaller CD molecule compared to larger polymeric substrates such as starch and pullulan, where AfCda13 has a much better selectivity for CDs than LsCda13. LsCda13, on the other hand, has almost the same hydrolyzing capacity on pullulan and starch as it has on cyclodextrins. Size exclusion chromatography revealed that AfCda13 exists as a homodimer in solution at its optimum pH, analogous to a

great number of the CDases known today (Park et al., 2000). *Ls*Cda13, on the other hand, turned out to be a monomer at its optimum pH. The difference in oligomeric state between *Af*Cda13 and *Ls*Cda13 is likely contributing to the observed difference in specificity.

As *Ls*Cda13 is monomeric, it lacks the deep and narrow cleft, which is formed between the N-domain of one monomer (involving the interacting residue Y45 important for CD-recognition) and the $(\beta/\alpha)_8$-barrel of the other monomer (including the catalytic residues of domain A, and residue F289 of domain B, shown to interact with CD-substrates), and believed to contribute to a high CDase activity and a low starch activity (Kamitori et al., 1999; Kim et al., 1999; Park et al., 2000). Even though the CD-specificity of the monomeric *Ls*Cda13 is inferior to that of *Af*Cda13, these studies demonstrate that a monomeric enzyme with the domain organization similar to that of other CDases can catalyze hydrolysis of cyclodextrins. Furthermore, the intracellular *Ls*Cda13 has activity on all three CD-substrates (α-, β-, and γ-CD), opposed to the monomeric extracellular TVAI with identical domain composition, active only on γ-CD (Abe et al., 2004). Thus, the oligomerization is not necessary for CDase activity, but it is likely a major factor contributing to high CDase specificity. In addition, the domain deletion studies showed that the presence of all the domains in the wild-types is important for enzymatic function, as the deletion mutants failed to produce enzymes, active on any of the substrates such as β-cyclodextrin, starch or pullulan (data not shown). This is in accordance with the results of Tonozuka et al. (2002) who reported that the N domain was essential for the proper folding of TVAI and TVAII.

The distribution of intermediate CD-hydrolysis products of the monomeric enzyme *Ls*Cda13 was much more random than for *Af*Cda13. It was especially obvious that *Af*Cda13 accumulated maltotriose, which was not an effect of product inhibition or enzyme inactivation, but instead a result of transglycosylation. The transglycosylating activity of *Af*Cda13 was also verified by reactions with *p*NPG2 together with either glucose or maltose, but could not be proven for the monomeric *Ls*Cda13. Transglycosylating activity has been revealed for a number of dimeric homologues to *Af*Cda13 (Tonozuka et al., 2002; Hondoh et al., 2003), and – although speculative at this stage – we suggest that the oligomeric state may be a central feature for this. Supporting evidence is so far found in the crystal structure of a closely related enzyme, which revealed an extra sugar-binding space at the bottom of the cleft, formed by the N domain and the $(\beta/\alpha)_8$-barrel, that may accommodate an acceptor sugar taking part in a transglycosylation reaction (Kim et al., 1999).

In conclusion, the oligomeric state of CDases is likely a very important factor for obtaining a high selectivity towards cyclodextrins. Moreover, the experimental data for the two enzymes studied in this paper indicate that it may also play a role in transglycosylation.

Acknowledgements

We thank Camilla Larsson, Frida Nilsson and Martin Sixtensson for analytical work. We also thank Mats Lindstam for helping with constructing the models, and Prof. Patrick Adlercreutz for valuable comments on the manuscript. This work was supported by the European Union (Contract no QLK3-CT-2000-01068), and The Swedish Research Council (VR). Magnus Bergvall Foundation, Bengt Lundqvists Minne, the Royal Physiographic Society in Lund and Bertil Andersson Foundation are thanked for additional support.

References

Abe, A., Tonozuka, T., Sakano, Y. & Kamitori, S. 2004. Complex structures of *Thermoactinomyces vulgaris* R-47 α-amylase 1 with malto-oligosaccharides demonstrate the role of domain N acting as a starch-binding domain. J. Mol. Biol. **335:** 811–822.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W. & Lipman, D.J. 1990. Basic local alignment search tool. J. Mol. Biol. **215:** 403–410.

Andersson, M. & Adlercreutz, P. 2001. A kinetic study of almond-β-glucosidase catalysed synthesis of hexyl-glycosides in low aqueous media. Influence of glycosyl donor and water activity. J. Mol. Catal. B: Enzym. **14:** 69–76.

Chahid, Z., Montet, D., Pina, M. & Graille, J. 1992. Effect of water activity on enzymatic synthesis of alkylglycosides. Biotechnol. Lett. **14:** 281–284.

Coutinho, P.M. & Henrissat, B. 1999. Carbohydrate-active enzymes: an integrated database approach, pp. 3–12. In: Gilbert, H.J., Davies, G.J., Henrissat, B. & Svensson, B. (eds) Recent Advances in Carbohydrate Bioengineering, The Royal Society of Chemistry, Cambridge.

Dauter, Z., Dauter, M., Brzozowski, A.M., Christensen, S., Borchert, T.V., Beier, L., Wilson, K.S. & Davies, G.J. 1999. X-ray structure of Novamyl, the five-domain "maltogenic" α-amylase from *Bacillus stearothermophilus*: maltose and acarbose complexes at 1.7 Å resolution. Biochemistry **38:** 8385–8392.

de Maré, L., Velut, S., Briechle, S., Wennerberg, C., Cimander, C., Ramchuran, S., Turnert, P., Silfversparre, O., Holst, O. & Hagander, P. 2004. Temperature limited fed-batch cultivations with a probing feeding strategy for *Escherichia coli*, ref no. 01. In: 9th International Symposium on Computer Applications in Biotechnology, Nancy.

Fiedler, G., Pajatsch, M. & Böck, A. 1996. Genetics of a novel starch utilisation pathway present in *Klebsiella oxytoca*. J. Mol. Biol. **256:** 279–291.

Henrissat, B., Teeri, T.T. & Warren, R.A.J. 1998. A scheme for designating enzymes that hydrolyse the polysaccharides in the cell walls of plants. FEBS Lett. **425:** 352–354.

Hondoh, H., Kuriki, T. & Matsuura, Y. 2002. Three-dimensional structure of *Bacillus stearothermophilus* neopullulanase. Biologia, Bratislava **57 (Suppl. 11):** 77–82.

Hondoh, H., Kuriki, T. & Matsuura, Y. 2003. Three-dimensional structure and substrate binding of *Bacillus stearothermophilus* neopullulanase. J. Mol. Biol. **326:** 177–188.

Kamitori, S., Abe, A., Ohtaki, A., Kaji, A., Tonozuka, T. & Sakano, Y. 2002. Crystal structure and structural comparison of *Thermoactinomyces vulgaris* R-47 α-amylase 1 (TVAI) at 1.6 Å resolution and α-amylase 2 (TVAII) at 2.3 Å resolution. J. Mol. Biol. **318:** 443–453.

Kamitori, S., Kondo, S., Okuyama, K., Yokota, T., Shimura, Y., Tonozuka, T. & Sakano, Y. 1999. Crystal structure of *Thermoactinomyces vulgaris* R-47 α-amylase II (TVAII) hydrolyzing cyclodextrins and pullulan at 2.6 Å resolution. J. Mol. Biol. **287:** 907–921.

Kim, J.S., Cha, S.S., Kim, H.J., Kim, T.J., Ha, N.C., Oh, S.T., Cho, H.S., Cho, M.J., Kim, M.J., Lee, H.S., Kim, J.H., Choi, K.Y., Park, K.H. & Oh, B-H. 1999. Crystal structure of a maltogenic amylase provides insights into a catalytic versatility. J. Biol. Chem. **274:** 26279–26286.

Kim, T.J., Nguyen, V.D., Lee, H.S., Kim, M.J., Cho, H.Y., Kim, Y.W., Moon, T.W., Park, C.S., Kim, J.W., Oh, B.H., Lee, S.B., Svensson, B. & Park, K.H. 2001. Modulation of the multisubstrate specificity of *Thermus* maltogenic amylase by truncation of the monomer/dimer equilibrium. Biochemistry **40:** 14182–14190.

Kuriki, T. & Imanaka, T. 1999. The concept of the α-amylase family: structural similarity and common catalytic mechanism. J. Biosci. Bioeng. **87:** 557–565.

Laemmli, U. 1970. Cleavage of structural proteins during assembly of the head of bacteriophage T4. Nature **227:** 680–685.

Laurell, T. & Buttler, T. 1995. A microdialysis probe offering arbitrary membrane length and an in situ tunable relative recovery. Anal. Method Instrum. **2:** 197–201.

Lee, H.S., Kim, J.S., Shim, K.H., Kim, J.W., Park, C.S. & Park, K.H. 2005. Quaternary structure and enzymatic properties of cyclomaltodextrinase from alkalophilic *Bacillus* sp. I-5. Biologia, Bratislava **60 (Suppl.16):** 73–77.

MacGregor, E.A., Janecek, S. & Svensson, B. 2001. Relationships of sequence and structure to specificity in the α-amylase family of enzymes. Biochim. Biophys. Acta **1546:** 1–20.

Miller, G.L. 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal. Chem. **31:** 426–428.

Ohtaki, A., Mizuno, M., Tonozuka, T., Sakano, Y. & Kamitori, S. 2004. Complex structures of *Thermoactinomyces vulgaris* R-47 α-amylase 2 with acarbose and cyclodextrins demonstrate the multiple substrate recognition mechanism. J. Biol. Chem. **279:** 31033–31040.

Park, K.H. 2001. The multisubstrate specificity and the quaternary structure of cyclodextrin-/pullulan-degrading enzymes. J. Appl. Glycosci. **48:** 293–299.

Park, K.H., Kim, T.J., Cheong, T.K., Kim, J.W., Oh, B.H. & Svensson, B. 2000. Structure, specificity and function of cyclomaltodextrinase, a multispecific enzyme of the α-amylase family. Biochim. Biophys. Acta **1478:** 165–185.

Park, K.H., Lee, H.S., Kim, T.J., Cheong, K.A., Nguyen, V.D., Min, M.J., Cho, H.Y., Kim, Y.W., Park, C.S., Oh, B.H. & Kim, J.W. 2002. N- and C-terminal region mediated oligomerization of the cyclodextrin-/pullulan degrading enzymes. Biologia, Bratislava **57 (Suppl. 11):** 87–92.

Ramchuran, S.O., Nordberg Karlsson, E., Velut, S., Maré de, L., Hagander, P. & Holst, O. 2002. Production of heterologous thermostable glycoside hydrolases and the presence of host-cell proteases in substrate limited fed-batch cultures of *Escherichia coli* BL21(DE3). Appl. Microbiol. Biotechnol. **60:** 408–416.

Robert, X., Haser, R., Gottschalk, T.E., Ratajczak, F., Driguez, H., Svensson, B. & Aghajari, N. 2003. The structure of barley α-amylase isozyme 1 reveals a novel role of domain C in substrate recognition and binding: a pair of sugar tongs. Structure **11:** 973–984.

Rose, T.M., Schultz, E.R., Henikoff, J.G., Pietrokovski, S., McCallum, C.M. & Henikoff, S. 1998. Consensus-degenerate hybrid oligonucleotide primers for amplification of distantly related sequences. Nucleic Acids Res. **26:** 1628–1635.

Schwede, T., Kopp, J., Guex, N. & Peitsch, M.C. 2003. SWISS-MODEL: an automated protein homology-modeling server. Nucleic Acids Res. **31:** 3381–3385.

Tonozuka, T., Yokota, T., Ichikawa, K., Mizuno, M., Kondo, S., Nishikawa, A., Kamitori, S. & Sakano, Y. 2002. Crystal structures and substrate specificities of two α-amylases hydrolyzing cyclodextrins and pullulan from *Thermoactinomyces vulgaris* R-47. Biologia, Bratislava **57 (Suppl. 11):** 71–76.

Torto, N., Bång, J., Richardson, S., Nilsson, G.S., Gorton, L., Laurell, T. & Marko-Varga, G. 1998. Optimal membrane choice for microdialysis sampling of oligosaccharides. J. Chromatogr. **A806:** 265–278.

Torto, N. & Gorton, L. 1999. Technical issues of *in vitro* microdialysis sampling in bioprocess monitoring. Trends Anal. Chem. **18:** 252–260.

Turner, P., Holst, O. & Nordberg Karlsson, E. 2005a. Optimized expression of soluble cyclomaltodextrinase of thermophilic origin in *Escherichia coli* by using a soluble fusion-tag and by tuning of inducer concentration. Protein Expr. Purif. **39:** 54–60.

Turner, P., Labes, A., Fridjonsson, O.H., Hreggvidson, G.O., Schönheit, P., Kristjansson, J.K., Holst, O. & Nordberg Karlsson, E. 2005b. Two novel cyclodextrin degrading enzymes isolated from thermophilic bacteria have similar domain structures but differ in oligomeric state and activity profile. J. Biosci. Bioeng. (in press).

Yamamoto, T. 1995. Enzyme chemistry and molecular biology of amylases, pp. 81–100. In: Yamamoto, T., Kitahata, S., Ciba, S., Hiromi, K., Matsuura, Y., Minamiura, N., Nitta, Y., Shinke, R. & Yamane, K. (eds) Enzyme Chemistry and Molecular Biology of Amylases and Related Enzymes, CRC Press, Boca Raton.

Received November 29, 2004
Accepted March 07, 2005