A novel type of thermostable α -D-glucosidase from *Thermoanaerobacter thermohydrosulfuricus* exhibiting maltodextrinohydrolase activity

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An α -glucosidase with the ability to attack polymeric substrates was purified to homogeneity from culture supernatants of *Thermoanaerobacter thermohydrosulfuricus* DSM 567. The enzyme is apparently a glycoprotein with a molecular mass of 160 kDa. Maximal activity is observed between pH 5 and 7 at 75 °C. The α -glucosidase is active towards *p*-nitrophenyl- α -D-glucoside, maltose, malto-oligosaccharides, starch and pullulan. Highest activity is displayed towards the disaccharide maltose. In

addition to glucose, maltohexaose and maltoheptaose can be detected as the initial products of starch hydrolysis. After short incubations of pullulan, glucose is found as the only product. At high substrate concentrations, maltose and malto-oligosaccharide, but not glucose, are used as acceptors for glucosyl-transfer. These findings indicate that the *T. thermohydrosulfuricus* enzyme represents a novel type of α -glucosidase exhibiting maltase, glucohydrolase and 'maltodextrinohydrolase' activity.

INTRODUCTION

Thermophilic anaerobic bacteria belonging to the *Clostridium* group [1] have received considerable interest because of their ability to convert a wide range of carbohydrate substrates from surplus agricultural crops and industrial waste into solvents and fuels. The amylolytic enzyme systems of several bacterial strains which have recently been assigned to the closely related genera *Thermoanaerobacter* and *Thermoanaerobacterium* [1,2] have been analysed in detail. It was found that a novel type of enzyme, exhibiting both α -amylase and pullulanase activities, plays the most important role in starch degradation by these organisms [3–5]. In addition to these endo-acting amylopullulanases, exo-acting amylolytic enzymes have also been detected in the thermoanaerobes. In *Thermoanaerobacterium thermosaccharolyticum*, a glucoamylase (EC 3.2.2.3) releasing β -D-glucose units consecutively from the non-reducing ends of the polymer chains takes part in starch degradation [6]. Glucoamylases attack polysaccharides more rapidly than oligosaccharides. The short-chain degradation intermediates formed by the action of amylopullulanases are the preferred substrates of α -glucosidases. α -Glucosidase (EC 3.2.1.20) effects the hydrolysis of terminal, non-reducing 1,4-linked α -D-glucose residues with release of α -D-glucose. The presence of α -glucosidase activity has been reported for several members of the genera *Thermoanaerobacter* and *Thermoanaerobacterium* [5,7], but the enzyme has been isolated and characterized only from *Thermoanaerobacter ethanolicus* 39E [8].

This paper describes the purification and some properties of the α -glucosidase produced by *Thermoanaerobacter thermohydrosulfuricus*, and presents evidence that this enzyme represents a novel type of α -glucosidase which releases maltodextrins, in addition to glucose, from starch.

MATERIALS AND METHODS

Organism and culture conditions

T. thermohydrosulfuricus DSM 567 was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen

(DSMZ, Braunschweig, Germany). It was grown for 42 h under anaerobic conditions at 65 °C in a complex medium, adjusted to pH 7.2, containing 0.5 % (w/v) Zulkowsky starch (Merck) as carbon source and (per litre): tryptone 10 g, yeast extract 3 g, meat extract 10.0 g, NaCl 5 g, and Mops buffer 10 g.

Enzyme purification

The supernatant was obtained after centrifugation of 12 litres of *T. thermohydrosulfuricus* culture at 5000 g for 20 min. The culture supernatant was concentrated to 110 ml by ultrafiltration employing a Minisette tangential flow system (Filtron) with a Nova 30 membrane filter.

The chromatographic steps of the purification were performed with a Pharmacia FPLC system. A 54 ml volume of the concentrated culture supernatant was applied to a Pharmacia XK 26/20 column filled with 50 ml of Pharmacia chelating Sepharose fast flow, charged with copper and equilibrated with 50 mM potassium phosphate buffer/1 mM imidazole/0.8 M NaCl, pH 7.0 [Cu²⁺-immobilized metal-ion affinity chromatography (IMAC)]. Elution was performed with a 900 ml linear gradient (1–30 mM imidazole) in 50 mM potassium phosphate buffer/0.8 M NaCl, pH 7.0, at a flow rate of 8 ml/min. Fractions (10 ml) were collected and assayed for enzymic activity. α -Glucosidase-containing fractions (12–37) were pooled and dialysed against 20 mM Tris/HCl/1 M NaCl, pH 7.0.

The α -glucosidase pool of the Cu²⁺-IMAC was applied to a Pharmacia Phenyl-Sepharose HP HiLoad 16/10 column for hydrophobic-interaction chromatography. The column was equilibrated with 20 mM Tris/HCl/1 M NaCl, pH 7.0. Elution was performed with a 220 ml linear gradient (1–0 M NaCl) in 20 mM Tris/HCl, pH 7.0, followed by 130 ml of 20 mM Tris/HCl, pH 7.0, at a flow rate of 3 ml/min. Fractions (5 ml) were collected and assayed for enzymic activity. Fractions containing α -glucosidase (43–54) were pooled and dialysed against 20 mM Tris/HCl, pH 6.5.

The α -glucosidase pool of the hydrophobic-interaction chromatography was applied to a Pharmacia Mono Q HR 5/5 column equilibrated with 20 mM Tris/HCl, pH 6.5. Elution was per-

Abbreviations used: CITase, cycloisomalto-oligosaccharide glucanotransferase; IMAC, immobilized metal-ion affinity chromatography.

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formed with a 20 ml linear gradient (0–0.4 M NaCl), followed by a 3 ml linear gradient (0.4–1 M NaCl) and 5 ml of 1 M NaCl/20 mM Tris/HCl, pH 6.5, at a flow rate of 1 ml/min. Fractions (0.5 ml) were collected and assayed for enzymic activity.

Enzyme assays

α -Glucosidase activity was determined by measuring the release of *p*-nitrophenol from *p*-nitrophenyl α -D-glucoside. Assay mixtures (0.5 ml) containing 2 mM substrate in 0.1 M sodium succinate, pH 6.0, were incubated at 65 °C. Reactions were stopped by addition of two volumes 1 M Na₂CO₃. The absorbance of the liberated *p*-nitrophenol was measured at 395 nm. One unit of activity is defined as the amount of enzyme liberating 1 μ mol of *p*-nitrophenol per min.

The activity of α -glucosidase on maltose, starch and pullulan (Sigma) was determined by measuring the release of glucose. Two types of starch were used: partly soluble starch from potato (Sigma) and fully soluble starch prehydrolysed by the Zulkowsky method (Merck). Assay mixtures (0.5 ml) containing 0.5 % (w/v) substrate in 0.1 M sodium-succinate, pH 6.0, were incubated at 65 °C. Reactions were stopped by heating for 10 min in a boiling-water bath. Glucose was assayed with the glucose oxidase reagent from a glucose assay kit (Sigma Diagnostics no. 510). One unit of activity is defined as the amount of enzyme liberating 1 μ mol of glucose per min.

Pullulanase and amylase activities were determined by measuring the release of reducing sugars from pullulan and starch. Assay mixtures (0.5 ml) containing 0.5 % (w/v) substrate in 0.1 M sodium-succinate, pH 6.0, were incubated at 65 °C. Reducing sugars were determined with the 3,5-dinitrosalicylic acid reagent [9]. One unit of activity is defined as the amount of enzyme liberating 1 μ mol of reducing sugars per min.

Buffers used for the determination of the pH profile were 100 mM sodium-acetate (pH 3.5–6.0), 100 mM Mes (pH 5.0–6.5), 100 mM Mops (pH 6.0–8.0) and 100 mM {3-[(1,1-dimethyl-2-hydroxyethyl)amino]-2-hydroxy-propanesulphonic acid} (pH 8.0–9.0). The pH values of buffers were adjusted at the temperatures of use.

Analytical methods

Protein concentrations were measured by the method of Sedmak and Grossberg [10]. SDS/PAGE was performed in 7.5 % (w/v) polyacrylamide slab gels in the presence of 0.1 % SDS according to the method of Laemmli [11]. Proteins were detected with the Pharmacia silver-stain kit. α -Glucosidase activity was identified by fluorescence upon incubating the polyacrylamide gels at 65 °C with 1 mM 4-methylumbelliferyl- α -D-glucoside in 20 mM Tris/HCl, pH 6.0. Protein glycosylation was analysed with a GlykoTrackKit (Oxford GlykoSystems). The release of oligosaccharides during the degradation of starch, pullulan and maltose was analysed by HPLC (Beckman System Gold) at 85 °C on an Animex HPX-42 A column (Bio-Rad) with water as eluent. N-terminal amino acid sequences were determined by Edman degradation using a gas-phase amino acid Sequenator (model 477A; Applied Biosystems).

RESULTS

Enzyme purification

An α -glucosidase with the ability to attack polymeric substrates was purified from concentrated culture supernatant of *T. thermohydrosulfuricus* DSM 567 grown on 0.5 % (w/v) Zulkowsky

Table 1 Purification of α -glucosidase from crude extract of *T. thermohydrosulfuricus*

Activity was determined by measuring the release of *p*-nitrophenol from *p*-nitrophenyl- α -D-glucoside.

Purification step	Volume (ml)	Protein (mg)	Activity (m-units)	Specific activity (m-units/mg)	Purification (-fold)
Concentrated supernatant	54	462.80	1402	3.0	1.0
Cu ²⁺ -IMAC	260	231.40	833	3.6	1.2
Phenyl-Sepharose	55	8.30	432	52.1	17.4
Mono Q	0.5	0.24	74	308.3	101.8

starch (Table 1). During the purification it was important to separate the α -glucosidase from other starch- and pullulan-hydrolysing enzymes present in the supernatant (Table 2). The predominant amylolytic enzyme exhibiting both amylase and pullulanase activity was identified as an amylopullulanase. Separation of the α -glucosidase from the amylopullulanase was achieved by the second purification step, i.e. hydrophobic interaction chromatography on Phenyl-Sepharose HP (Figure 1). Both enzymes could be detected and distinguished by measuring the release of reducing sugars from starch and pullulan (Figure 1A). The amylopullulanase exhibits higher activity towards pullulan than towards starch, whereas the α -glucosidase prefers starch as substrate. Under the conditions chosen, a significant part of the amylopullulanase activity was detected in the flow-through of the column, while the residual activity eluted during the descending sodium chloride gradient. α -Glucosidase-containing fractions, in which the release of reducing sugars from starch was higher than from pullulan, were not found before the end of the salt gradient. Figure 1(B) demonstrates that the activities releasing glucose from starch, pullulan and maltose showed the same elution pattern. Moreover, the *p*-nitrophenyl- α -D-glucoside-hydrolysing activity was found in the same fractions (Figure 1C). On the basis of these results, fractions 43–54 were combined to form an α -glucosidase pool from which most of the amylopullulanase had been removed. With subsequent anion-exchange chromatography on Mono Q, a pure preparation of the α -glucosidase was obtained (Figure 2).

Properties of the enzyme

The apparent molecular mass of the α -glucosidase, as determined by SDS/PAGE (Figure 2), is 160 kDa. Identification of the single protein band detected in the purified preparation with the α -glucosidase was confirmed by affinity staining with 4-methylumbelliferyl- α -D-glucoside (results not shown). Owing to retardation during gel filtration on both a Superdex 200 and a Sephacryl S-200 column, the size of the enzyme under non-denaturing conditions could not be determined. The α -glucosidase stained positively with a glycoprotein detection kit. The N-terminal amino acid sequence of the enzyme was identified by Edman degradation (Figure 3).

Effect of pH and temperature on enzyme activity

The α -glucosidase exhibited a broad pH optimum between 5.0 and 7.0, with a rapid decline of the activity at higher or lower pH values. When incubated for 1 h with starch as substrate, the purified enzyme (10 μ g/ml) displayed optimal activity at 75 °C under standard assay procedures. At this temperature the α -

Table 2 Enzyme activities related to starch and pullulan degradation in concentrated culture supernatant of *T. thermohydrosulfuricus*

Activities towards pullulan and starch were determined by measuring the release of reducing sugars, as described in the Materials and methods section.

Substrate	Activity (units)	Specific activity (m-units/mg)
Pullulan	2211.3	4780
Starch	1294.4	2800
<i>p</i> -Nitrophenyl- α -D-glucoside	1.4	3

Figure 1 Separation of the amylopullulanase from the α -glucosidase of *T. thermohydrosulfuricus* by FPLC hydrophobic-interaction chromatography on a Phenyl-Sepharose HP column

Fractions were assayed for (A) the release of reducing sugars from starch (○) and pullulan (●); (B) the release of glucose from starch (○), pullulan (●) and maltose (□); (C) the release of *p*-nitrophenol from *p*-nitrophenyl- α -D-glucoside (○) and protein content (■).

glucosidase was stable for at least 7 h. In the temperature range 65–85 °C, the activity amounted to 70 % of the maximum. Above 85 °C the activity decreased rapidly, and below 45 °C less than 15 % of the maximal activity was observed (results not shown).

Figure 2 SDS/PAGE of α -glucosidase fractions

Three separate SDS/PAGE assays (A, B, C) of the fractions obtained during the purification of α -glucosidase from *T. thermohydrosulfuricus* are shown. Proteins were detected by silver staining. Lanes: 1, crude extract; 2, pooled fractions from the Cu²⁺-IMAC column; 3 and 4, molecular-mass markers (values in kDa on the right); 5, pooled fractions from the Phenyl-Sepharose column; 6, peak fraction from the Mono Q column; 7, molecular-mass markers (values in kDa on the right)

T. thermohydrosulfuricus:

α -glucosidase 1 **A**STGNLIQ**R**VYTDK**A**R**Y**NP 19

B. circulans:

CITase precursor 30 **A**STPGFI**E**RVYTDK**A**R**Y**EP 48

Figure 3 N-terminal amino acid sequence of the *T. thermohydrosulfuricus* α -glucosidase

Identical amino acids are indicated with bold letters.

Table 3 Substrate specificity of the α -glucosidase from *T. thermohydrosulfuricus*

Assays were performed at substrate concentrations of 2 mM *p*-nitrophenyl- α -D-glucoside or 0.5 % (w/v) of the di-, oligo- and polymeric substrates in 0.1 M sodium succinate, pH 6.0, at 65 °C, using an enzyme concentration of 1.08 μ g/ml (purified enzyme from the Mono Q column). Incubations were carried out for 90 min. The release of glucose or *p*-nitrophenol was measured as appropriate.

Substrate	Specific activity (units/mg)
<i>p</i> -Nitrophenyl- α -D-glucoside	0.3
Maltose	4.1
Maltotriose	2.4
Maltotetraose	2.2
Maltopentaose	2.0
Zulkowsky starch	2.0
Potato starch	1.3
Pullulan	0.5

Substrate specificity

The activity of the α -glucosidase of *T. thermohydrosulfuricus* towards various substrates is summarized in Table 3. As observed for microbial α -glucosidases in general, the purified enzyme was capable of cleaving maltose and malto-oligosaccharides, as well as the aryl-substrate *p*-nitrophenyl- α -D-glucoside. Highest ac-

Figure 4 HPLC analysis of the products formed from Zulkowsky starch

Reaction mixtures containing 0.25% (w/v) Zulkowsky starch were incubated at 65 °C for 15 min (A), 1 h (B), 5 h (C) and 18 h (D). G, glucose; G2, maltose; G3, maltotriose; G4, maltotetraose; G5, maltopentaose; G6, maltohexaose; G7, maltoheptaose; S, Zulkowsky starch.

tivity was displayed towards the disaccharide substrate maltose. Under optimal conditions the rate of *p*-nitrophenyl α -D-glucoside cleavage was approximately one order of magnitude lower than that of maltose hydrolysis. Analysis of the activity towards malto-oligosaccharides indicated a preference for short-chain oligomers. Malto-oligosaccharides were hydrolysed at a decreasing rate, concomitant with the increasing number of glucose units of the substrates. Interestingly, the enzyme also displayed activity towards the polymeric substrates starch and pullulan.

Analysis of degradation products

The degradation products formed by α -glucosidase action on two different types of starch, pullulan and maltose were analysed by HPLC. The degradation process of Zulkowsky starch is illustrated by the chromatograms shown in Figure 4. Figure 4(A) shows the initial products released during short incubations (5–15 min) of the substrate. Unexpectedly, maltohexaose and, to a smaller extent, maltoheptaose could be detected in addition to glucose. Upon further incubation (1 h), maltopentaose, maltotetraose, maltotriose and maltose appeared (Figure 4B). As degradation proceeded (Figures 4C and 4D), the relative amount of the longer products declined progressively, whereas the relative amount of the shorter products increased, so that glucose was finally found as the main degradation product. Thus, *T. thermohydrosulfuricus* α -glucosidase was able to effect a complete conversion of Zulkowsky starch into glucose. With potato starch as substrate, the kinetics and the products of degradation were the same, albeit the degradation process was much slower (results not shown).

Pullulan, a polysaccharide that consists of α -1,6-linked maltotriose units, was hydrolysed at a significantly lower rate than Zulkowsky starch or potato starch. Glucose was the only product

which could be detected after short incubations (results not shown). When maltose was used as substrate, maltotriose and maltotetraose were detected in addition to glucose (Figure 5A). Upon further incubation, the amount of glucose increased and even larger malto-oligosaccharides were formed (Figure 5B). Finally, maltose and the intermediate oligomers produced were almost completely degraded to glucose (Figure 5C). The formation of malto-oligosaccharides can be attributed to a trans-glycosylation activity of the purified α -glucosidase. The observed glucosyl transfer could be eliminated by reduction of the maltose concentration to 0.03% (results not shown).

DISCUSSION

Microbial α -glucosidases show great variation in their patterns of substrate specificity and have been classified into two main groups: those with highest activity towards maltose and those with highest activity towards aryl α -D-glucosides [12]. Some α -glucosidases also exhibit 1,4- α -D-glucan glucohydrolase activity and have therefore been designated as exo- α -1,4-glucosidases [12–15]. Glucose is found as the sole product of the successive hydrolysis from the non-reducing chain ends of oligomeric and polymeric substrates.

The α -glucosidase of *T. thermohydrosulfuricus* reported in this paper can be considered as a maltase, since it preferentially hydrolyses maltose and short-chain malto-oligosaccharides. Furthermore, the release of glucose from starch and pullulan shows that the enzyme also displays glucohydrolase activity. Unexpectedly, HPLC analysis of the initial reaction products revealed that not only glucose but also the linear oligosaccharides maltohexaose, and to a minor extent maltoheptaose, were produced from potato starch and Zulkowsky starch. Thus, the enzyme purified from *T. thermohydrosulfuricus* represents a novel

Figure 5 HPLC analysis of the products formed from maltose

Reaction mixtures containing 0.25% (w/v) maltose were incubated at 65 °C for 1 h (A), 5 h (B) and 18 h (C). G, glucose; G2, maltose; G3, maltotriose; G4, maltotetraose; G5, maltopentaose; G6, maltohexaose.

type of α -glucosidase exhibiting 'maltodextrinohydrolase' activity (Figure 6). The formation of hexaose (and heptaose) reflects the helical conformation of unbranched α -glucan chains with at least six D-glucopyranosyl residues per helical turn [16]. The enzyme acts predominantly as a processive glucohydrolase, releasing single glucose units from the non-reducing end of an α -glucan chain, although occasionally the postulated maltodextrinohydrolase activity effects the removal of one complete helix turn consisting of six (or seven) residues. While the enzyme continues with the hydrolysis of the respective chains, the released oligosaccharides accumulate before they are hydrolysed further in a secondary reaction. In addition to the hydrolase activity, the enzyme is able to exhibit glucanosyltransferase activity. However, this activity cannot account for the formation of maltohexaose and maltoheptaose, since only maltose and malto-oligosaccharides, but not glucose, are used as acceptors for glucanosyl transfer.

The α -glucosidase which has previously been partially purified from the closely related bacterial species *T. ethanolicus* [7,8] differs from the *T. thermohydrosulfuricus* α -glucosidase/maltodextrinohydrolase in substrate specificity. The activity of the *T. ethanolicus* enzyme towards starch and pullulan amounts to no more than 5% of the maltose-hydrolysing activity, whereas

Figure 6 Proposed model for the hydrolysis of starch by the α -glucosidase/maltodextrinohydrolase of *T. thermohydrosulfuricus*

the *T. thermohydrosulfuricus* enzyme exhibits significantly higher activities towards these polymeric substrates. The pattern of reaction products released from the polysaccharide substrates has not been elucidated for the *T. ethanolicus* α -glucosidase. Noteworthy is the high sub-unit molecular mass of both *Thermoanaerobacter* enzymes (approximately 160 kDa), which compares only roughly with the approximately 125 kDa α -glucosidases of *Pyrococcus furiosus* [17] and *Bifidobacterium pseudolongum* [18] and of the fungi *Aspergillus awamori*, *Mucor javanicus* and *Penicillium purpurogenum* [12]. The N-terminal amino acid sequences of these enzymes have not been reported.

α -Glucosidases with the ability to release glucose from starch have been described for several thermophilic *Bacillus* species [13,14]. These enzymes, formerly named exo- α -1,4-glucosidases, are significantly smaller (approximately 65 kDa) than the two *Thermoanaerobacter* α -glucosidases. Moreover, the N-terminus of the *T. thermohydrosulfuricus* α -glucosidase/maltodextrinohydrolase shows no sequence similarity to these enzymes, nor to the 540×10^3 kDa hexameric α -glucosidase from *Bacillus thermoamyloliquefaciens* described recently [19]. Instead, similarity could be detected to a *Bacillus circulans* cycloisomalto-oligosaccharide glucanotransferase (CITase) (Figure 3). This CITase produces cyclic isomalto-oligosaccharides from the α -1,6-glucan dextran and has a molecular mass of about 104 kDa (GenBank accession number D88360). The formation of cyclic malto-oligosaccharides could be excluded for the *T. thermohydrosulfuricus* α -glucosidase because significantly longer retention times were determined for α - and β -cyclodextrin.

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