Effect of Carbon Source on Production of Thermostable α -Amylase, Pullulanase and α -Glucosidase by *Clostridium thermohydrosulfuricum*

By HANNES MELASNIEMI

Research Laboratories of the Finnish State Alcohol Company (Alko Ltd), POB 350,
SF-00101 Helsinki, Finland

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The anaerobic thermophile *Clostridium thermohydrosulfuricum* produced thermostable α -amylase, pullulanase and α -glucosidase activities during growth on starch, pullulan, dextrin or maltose. Synthesis of α -amylase and pullulanase was partially repressed by glucose, whereas α -glucosidase synthesis was not. Fructose completely repressed the synthesis of α -amylase and pullulanase but only partially that of α -glucosidase. α -Amylase and pullulanase activities were predominantly located extracellularly. However, during growth on low amounts of 'soluble' starches (2%, w/v) virtually all activity was cell-associated. Under most conditions examined 75% or more of the α -glucosidase activity was cell-bound. The combined action of these activities produced glucose as the final end-product from amylose and pullulan digestions.

INTRODUCTION

Amylases of microbial and plant origin are nowadays used in the starch processing industries in large quantities (Norman, 1979; Fogarty & Kelly, 1980; Fogarty, 1983; Reichelt, 1983). Most widely used are the α -amylases produced by *Bacillus amyloliquefaciens* and *Bacillus licheniformis*, the glucoamylases of *Aspergillus niger* and *Rhizopus* sp. and the β -amylase of malt. In recent years debranching enzymes, for example the pullulanases of *Klebsiella pneumoniae* (*Enterobacter aerogenes*) (Ohba & Ueda, 1982) and *Bacillus* sp. (Norman, 1982), have gained increasing interest. Good activity and stability at high temperatures are features required for an ideal amylolytic enzyme, therefore thermophilic organisms are often first choice sources when looking for new enzymes (Buonocore *et al.*, 1976; Uchino, 1982; Sakano *et al.*, 1982; Suzuki & Imai, 1985). Those amylases which have been described and used in industry are predominantly produced by aerobic organisms. There are only a few reports on amylases of anaerobic origin (Hockenhull & Herbert, 1945; French & Knapp, 1950; Whelan & Nasr, 1951; Scott & Hedrick, 1952; Hobson & MacPherson, 1952; Ensley *et al.*, 1975). The production of pullulanase and glucoamylase by the anaerobic thermophile *Clostridium thermohydrosulfuricum* and β -amylase by another thermoanaerobe, *Clostridium thermosulfurigenes*, has been recently described (Hyun & Zeikus, 1985*a,b,c*). In this paper the production of α -amylase (EC 3.2.1.1), pullulanase (EC 3.2.1.41) and α -glucosidase activities by *C. thermohydrosulfuricum* is reported and the effect of the carbon source on the synthesis and localization of these enzymes is examined.

METHODS

Organism and growth conditions. *Clostridium thermohydrosulfuricum* E 101-69 was obtained from F. Hollaus (Sugar Research Institute, Fuchsenbigl, Austria). The growth medium contained (g l⁻¹): carbon source, as indicated; yeast extract (Difco), 5; tryptone (Difco), 10; meat extract (Oxoid, Lab-Lemco), 5; KH₂PO₄, 6.8; K₂HPO₄·3H₂O, 11.4; FeSO₄·7H₂O, 0.02; MgSO₄·7H₂O, 0.1; CaCl₂·2H₂O, 0.1; resazurin (BDH), 0.001; pH 6.8. The medium was autoclaved at 121 °C for 20 min (mono- and disaccharides separately) and thioglycolic acid (200 µl l⁻¹; Merck) added. NaAc/NaCl buffer contained: 100 mM-sodium acetate, 2 mM-CaCl₂, 0.1 mM-EDTA, 50 mM-NaCl, pH 5.6. Cell densities were measured with a Klett-Summerson colorimeter and converted to dry weights by using a calibration curve.

Kinetics of enzyme production. This was followed by growing the organism in a water-jacketed 2 l fermenter (Biostat M, B. Braun) at 68 °C in 1.7 l medium containing Zulkowsky starch (2%, w/v; a small glucose polymer) as carbon source, and sometimes glucose or fructose (1%, w/v) as repressors. The culture was stirred at 100 r.p.m. and oxygen-free nitrogen containing 3% hydrogen was bubbled through it at a rate of 10 l h⁻¹. No pH or redox regulation was used. The fermenter was inoculated with 100 ml of a culture grown overnight on glucose (1%). Samples were taken anaerobically. Enzyme activities were assayed in the culture supernatants and in the intact cells, washed with NaAc/NaCl buffer.

Enzyme production on different carbon sources. Three replicate serum bottles containing 112 ml medium with different poly- oligo- and monosaccharides (2%, w/v) under oxygen-free nitrogen containing 3% hydrogen were inoculated anaerobically at 68 °C with 1 ml of a culture grown overnight on glucose (1%) and incubated statically at 68 °C for 24 h. The gases formed escaped through a hypodermic needle (0.9 × 40 mm) in the septum.

Cells (16 mg) grown on soluble starch (Merck) in a serum bottle were washed and then disintegrated in 5 ml NaAc/NaCl buffer by three passages through an X-Press (X-5; Biox, Sweden) at a maximum pressure of about 200 MPa. The disintegrated cells were centrifuged for 10 min at 15000 g. The pellet (cell walls and membranes) was washed with NaAc/NaCl buffer and then suspended in the original volume of 20 mM-sodium acetate pH 5.6. The supernatant was centrifuged for 1 h at 100000 g to obtain a soluble fraction and a small pellet, which was resuspended in the same volume of 20 mM-sodium acetate pH 5.6.

To obtain a crude enzyme mixture, (NH₄)₂SO₄ (505 g l⁻¹) was added to the cell-free supernatant from a culture grown for 30 h at 68 °C in an unstirred conical flask in 2 l medium containing Zulkowsky starch (2%). Material precipitated in 2 d at 4 °C was collected and dissolved in and then dialysed against 20 mM-sodium acetate pH 5.6. It contained in 50 ml: 0.42 g protein, 600 kU α-amylase, 1850 kU pullulanase, and 13 kU α-glucosidase. The enzymes were stable for at least 1 year at -20 °C.

Sugar determinations. Reducing sugar was determined by the Somogyi-Nelson method (Nelson, 1944; Somogyi, 1952). Glucose and fructose were measured enzymically using UV tests kits (Boehringer-Mannheim) nos 716251 and 139106 respectively.

Enzyme assays. α-Amylase and pullulanase activities were assayed by measuring the reducing sugar released from pure amylose and pullulan, respectively. The reaction was started by adding enzyme (25 µl) to NaAc/NaCl buffer (1 ml) containing amylose or pullulan (0.5%), and stopped after 15 min at 85 °C by transfer to an ice water bath. Samples were centrifuged (15000 g, 5 min) before diluting them and reading the absorbance at 520 nm. One unit (U) of α-amylase or pullulanase activity was defined as the amount of enzyme releasing 1 nmol reducing sugar min⁻¹ in the above assay, using anhydrous glucose as a standard. The amylose substrate was brought into solution by 1 M-NaOH and finally filtered through a membrane filter (Millipore RAWP 1.2 µm).

α-Glucosidase activity was assayed by measuring glucose release from maltose. Enzyme solution (100 µl) and sodium acetate, pH 5, containing 1.25% (w/v) maltose monohydrate (400 µl) were mixed in an Eppendorf tube. After incubation at 70 °C for 1 h, the tubes were transferred to an ice water bath and the glucose formed was determined. One unit (U) of α-glucosidase activity was defined as the amount of enzyme releasing 2 nmol glucose min⁻¹ in the above assay.

Reaction rates were linear for at least 45 min for α-amylase and pullulanase and for 2 h for α-glucosidase.

Percentage hydrolysis of amylose and pullulan after enzymic digestion was calculated by comparing the amount of reducing sugar formed to the amount released from the same digest by acid hydrolysis (0.5 M-HCl, 100 °C, 3 h).

The iodine staining capacity of amylose during enzymic digestion was determined by pipetting samples (50 µl) into acidic iodine solution (4 ml; 0.2 mM-I₂ and 3 mM-KI in 0.1 M-HCl), mixing vigorously and reading the absorbance at 660 nm.

Hydrolysis products from amylose and pullulan digests were analysed by TLC as described by Hansen (1975) but using 2-propanol, acetone and water (2:2:1, by vol.) as the eluent. Samples were deproteinized with Carrez's reagent (Henninger & Bergmeyer, 1983), desalted by a small column of Amberlite MB-3 resin (BDH) and concentrated by lyophilization.

Protein was determined by the method of Bradford (1976).

Chemicals. Soluble starch (no. 1252), Zulkowsky starch, maltose monohydrate (no. 5912), anhydrous glucose and fructose (no. 5323) were from Merck; amylose (A-0512), pullulan and dextrin (D-2006) were from Sigma; and soluble starch (Anal aR) from BDH. Maltotriose, -tetraose, -pentaose, -hexaose and -heptaose were from Boehringer-Mannheim and panose was from Koch-Light. Isopanose was purified by preparative TLC from a pullulan hydrolysate digested with partially purified isopullulanase of *Aspergillus niger* CBS 246.65 (ATCC 9642).

RESULTS

Enzyme production

Fig. 1 shows the growth curve (a) and the kinetics of α-amylase, pullulanase and α-glucosidase production (d) by *C. thermohydrosulfuricum* grown in a fermenter with 2% Zulkowsky starch as

Fig. 1. Growth (a–c) and production of α -amylase, pullulanase and α -glucosidase activities (d–f) by *C. thermohydrosulfuricum* grown in a fermenter. Carbon source: (a, d) Zulkowsky starch 2%; (b, e) Zulkowsky starch 2% and glucose 1%; (c, f) Zulkowsky starch 2% and fructose 1%. □, α -amylase; ○, pullulanase and ▽, α -glucosidase in medium. ■, α -Amylase; ●, pullulanase and ▼, α -glucosidase associated with cells. ▲, Growth yield; ◇, glucose and ◆, fructose. (Because of the high reducing sugar background in the media to which glucose or fructose was added, the α -amylase and pullulanase activity in the medium was determined in these cases after diluting the samples 1:2 with buffer and by doubling the incubation time to 30 min.)

the carbon source. All these activities were produced only during the active phase of growth. Almost all of the α -amylase and pullulanase activity was found in the medium whereas little remained cell-bound. On the other hand, the bulk of the α -glucosidase activity was cell-associated and only a minor fraction was extracellular. When the medium was supplemented with 1% glucose, the final yield of bacteria increased (Fig. 1b) but the amount of α -amylase and pullulanase in the culture was only about 25% of that obtained without glucose (Fig. 1e). The amount of α -glucosidase, however, was not lowered by the presence of glucose; on the contrary, the level increased proportionally with the increase in biomass (Fig. 1e). Neither α -amylase nor pullulanase was detected if the medium was supplemented with 1% fructose (Fig. 1f). The amount of α -glucosidase was also lowered to about one third of that obtained without fructose (Fig. 1f).

From the carbon sources tested (Table 1), the highest total activity of α -amylase and pullulanase was obtained on the readily soluble small glucose polymers, i.e. dextrin and Zulkowsky starch. Total activity of α -glucosidase on the other hand was highest on the less soluble 'soluble' starches. Growth on maltose was slow but after 96 h the yields of cells and of total α -amylase and pullulanase activities were similar to those after 24 h growth on the soluble starches.

Table 1. *Effect of carbon source on the production of α -amylase, pullulanase and α -glucosidase activities by *C. thermohydrosulfuricum* and their localization (medium vs washed cells)*

C. thermohydrosulfuricum was grown statically in serum bottles for 24 h with 2% carbon source. The results are means of three replicate cultures.

Carbon source	Enzyme activity (U ml ⁻¹)						Growth yield (mg dry wt ml ⁻¹)
	α -Amylase		Pullulanase		α -Glucosidase		
	Medium	Cells	Medium	Cells	Medium	Cells	
Dextrin	700	40	2050	90	45	40	1.7
Zulkowsky starch	600	70	1830	240	20	55	1.4
Pullulan	640	50	1670	140	25	65	1.8
BDH soluble starch	0	490	10	1550	25	80	2.1
Merck soluble starch	0	430	10	1240	15	85	1.6
Maltose	50	30	110	100	0	5	0.2
Maltose (96 h)	380	20	1210	30	25	25	1.6
Glucose	10	0	10	0	5	10	1.7

Distribution of enzymes between cells and media

Depending on the carbon source used, the α -amylase, pullulanase and α -glucosidase produced were either located extracellularly or remained in varying amounts associated with the cells (Table 1). With the less soluble 'soluble' starches virtually all of the α -amylase and pullulanase activity remained cell-associated. After 24 h growth not even small starch molecules or dextrans could be detected by staining samples of the cultures with iodine (i.e. mixing 200 μ l culture and 20 μ l iodine solution containing 20 mM-I₂ and 0.3 M-KI gave no blue or reddish brown colour). This makes adhesion of the enzymes to any large starch molecules and subsequent pelleting with these during centrifugation an unattractive explanation for the association of the activities with the cells. With the truly soluble carbon sources the α -amylase and pullulanase activities were found in the medium. The ratio of the α -amylase activity to that of the pullulanase remained constant at about 1:3 within the limits of experimental error for all of the carbon sources tested, irrespective of whether the bulk of the activity was in the medium or cell-associated (Table 1). The distribution of α -glucosidase activity between cells and medium was less variable than for the other two activities, about 75% of the activity being cell-associated except after growth on dextrin or for 96 h on maltose.

If the organism was grown on mixtures of Merck soluble and Zulkowsky starch as the carbon source (2%), the proportion of α -amylase and pullulanase activity in the medium increased gradually as the relative amount of Zulkowsky starch was increased. With 'soluble' starch (Merck) the amount of carbon provided seemed also to have a pronounced effect on the localization of the α -amylase and pullulanase activities. When the amount of 'soluble' starch was increased from 2% to 8%, the location of both activities changed from cell-associated (~90%) to medium (~90%), while 4% 'soluble' starch gave intermediate values. In a similar experiment with the Zulkowsky starch, both activities were located in the medium (~90%) irrespective of the amount of the carbon source provided (data not shown).

The α -amylase, pullulanase and α -glucosidase activities associated with the cells were tightly associated and could not be removed by washing the cells with NaAc/NaCl buffer. These cell-associated activities were most active when assayed from intact cells. All of the α -amylase and pullulanase activity recovered from cell extracts and almost all of the α -glucosidase activity recovered was located in the low-speed pellet of crushed cells representing the cell wall/membrane fraction. The following high-speed pellet showed no amylolytic activity, the rest of the recovered α -glucosidase activity (<5%) being located in the soluble fraction.

Hydrolysis products

The thin-layer chromatogram in Fig. 2 shows the hydrolysis products released from amylose and from pullulan by a crude mixture of *C. thermohydrosulfuricum* α -amylase and pullulanase

Fig. 2. A thin-layer chromatogram showing the products released from amylose and pullulan by *C. thermohydrosulfuricum* crude enzyme mixture after 15 min incubation at 85 °C. 1, Standards: glucose, maltose, maltotriose, -tetraose, -pentaose, -hexaose and -heptaose (G1–G7); 2, amylose plus crude enzyme mixture; 3, amylose; 4, pullulan; 5, pullulan plus crude enzyme mixture; 6, maltotriose; 7, panose; 8, isopanose. To 2 ml of a 0.5% solution of amylose (a) and pullulan (b) in NaAc/NaCl buffer, 50 μ l of diluted crude enzyme mixture was added to give 170 U α -amylase ml^{-1} (a) and 90 U pullulanase ml^{-1} (b).

(see Methods) with a relatively low enzyme concentration and a short incubation time at 85 °C. Besides dextrans not resolved, the α -amylase produced from amylose a series of malto-oligosaccharides ranging from maltose to malto-octaose, with maltopentaose as the most prominent species. Notable is the absence of glucose. The pullulanase on the other hand degraded pullulan to maltotriose; a bigger oligosaccharide, possibly an intermediate made of two maltotriose units, is also seen.

The time course of hydrolysis of amylose and pullulan by a crude mixture of *C. thermohydrosulfuricum* α -amylase, pullulanase and α -glucosidase in a prolonged hydrolysis at 65 °C with a higher enzyme concentration showed, with both substrates, initially a phase of rapid release of reducing sugar to a level of about 50% hydrolysis during the first hour and then a much slower phase of degradation approaching 100% hydrolysis at 48 h. The iodine-staining capacity of amylose disappeared rapidly at a level of about 20% hydrolysis. The release of reducing sugar from pullulan was about three times faster than that from amylose at the beginning of the incubation, but after about 20 min the rate of hydrolysis of both substrates was the same. Reducing sugar determinations after 48 h of enzymic digestion gave a hydrolysis percentage of 101.8% with amylose and 100.5% with pullulan as compared to mild acid hydrolysed samples. Corresponding glucose determinations gave 99.6% glucose from amylose and 99.3% from pullulan (data not shown).

Hydrolysis products from the prolonged hydrolysis of amylose (Fig. 3) show that amylose was rapidly hydrolysed to a level containing only maltose, maltotriose, glucose and maltotetraose as the main products, and that the following much slower phase represented the hydrolysis of maltose to glucose.

DISCUSSION

The present study shows that *C. thermohydrosulfuricum* (E 101-69) produces α -amylase, pullulanase and α -glucosidase activities. Hyun & Zeikus (1985b, c) on the other hand reported that pullulanase and glycoamylase were involved in starch degradation by *C. thermohydrosulfuri-*

Fig. 3. A thin-layer chromatogram showing the products released from amylose by the concerted action of *C. thermohydrosulfuricum* α -amylase and α -glucosidase during a prolonged hydrolysis at 65 °C. S, standards: glucose, maltose, maltotriose, -tetraose, -pentaose, -hexaose and -heptaose (G1–G7). To 20 ml of 0.5% amylose in NaAc/NaCl buffer, 2 ml of the crude enzyme mixture was added to give 1100, 3400 and 24 U ml⁻¹ of α -amylase, pullulanase and α -glucosidase respectively.

cum (E39) but that an active α -amylase was not involved. They did not find maltose, maltotriose or maltotetraose in their starch hydrolysate digested with a cell extract. Secondly, they used soluble starch as a substrate for α -amylase, which makes a distinction between α -amylase and pullulanase activities difficult, because pullulanase also produces reducing sugar from soluble starch. However, in the present work, α -amylase activity was assayed using pure amylose as substrate. The presence of α -amylase is evidenced by typical hydrolysis products released from amylose (Fig. 2) and by the rapid disappearance of the iodine-staining capacity of amylose during hydrolysis. Amylose azure is also readily hydrolysed (data not shown). The α -amylase assay used is free from interference from α -glucosidase, because glucose was not detected after a short digestion of amylose at 85 °C liberating reducing sugar in excess of the upper limit of the α -amylase assay.

Obviously the release of large amounts of glucose upon hydrolysis of soluble starch in the assumed absence of an α -amylase made Hyun & Zeikus (1985*b, c*) conclude that *C. thermohydrosulfuricum* (E39) also produces a glucoamylase. In the opinion of the author any decision about whether the glucose-forming activity of *C. thermohydrosulfuricum* is due to a glucoamylase (EC 3.2.1.3) or an α -glucosidase (EC 3.2.1.20) cannot be made at present. I have described the activity produced by the strain (E 101-69) as α -glucosidase because it certainly cleaves α -glycosidic bonds, at least those in maltose. The main activity of this organism responsible for cleaving the α -1,4 bonds in starch is obviously the α -amylase. Whether the α -glucosidase is capable of using starch as a substrate, or only degrades further the products of the α -amylase, is hard to ascertain without purifying the α -glucosidase from the α -amylase activity. The configuration of the glucose liberated should also be determined before a more definite classification of the α -glucosidase activity can be made.

The pullulan-degrading activity of *C. thermohydrosulfuricum* is a pullulanase like those of *K. pneumoniae* (Ohba & Ueda, 1975) and *Bacillus cereus* (Takasaki, 1976) in that it produces maltotriose from pullulan by cleaving the α -1,6 bonds. *Thermoactinomyces vulgaris* (Shimizu *et al.*, 1978) and *Bacillus stearothermophilus* (Suzuki & Imai, 1985) on the other hand are known to produce an α -amylase capable of hydrolysing the α -1,4 bonds of pullulan to produce panose, whereas *A. niger* (ATCC 9642) synthesizes an isopullulanase (EC 3.2.1.57), cleaving the α -1,4 bonds of pullulan to isopanose (Sakano *et al.*, 1972).

An interesting feature of *C. thermohydrosulfuricum* α -amylase, pullulanase and α -glucosidase is the location of the bulk of all these activities in the cell wall/membrane fraction, when the organism is grown on low amounts of high molecular mass starch (Table 1). A significant portion of *Clostridium acetobutylicum* α -amylase and α -glucosidase is also cell-associated (Ensley *et al.*, 1975). These activities can however be removed by washing the cells with buffer unlike *C. thermohydrosulfuricum*. Bacterial α -glucosidases are usually soluble intracellular or extracellular enzymes (Kelly & Fogarty, 1983); however a membrane-bound α -glucosidase has been described in *B. amyloliquefaciens* (Urlaub & Wöber, 1978). The localization of pullulanase in *K. pneumoniae* is known to be strongly affected by the carbon source used. A soluble extracellular pullulanase is produced, when *K. pneumoniae* is grown on maltose, whereas the enzyme remains membrane-bound when growth is on maltose plus glucose (Wallenfels *et al.*, 1966). The results of this study suggest that the formation of the extracellular α -amylase and pullulanase activities by *C. thermohydrosulfuricum* is a result of the presence of considerable amounts of soluble dextrans or malto-oligosaccharides in the culture.

It seems that the production of the α -amylase and pullulanase activities in *C. thermohydrosulfuricum* is co-ordinately regulated. Both activities are synthesized in a constant proportion and repressed in the same manner, and in addition, their localization is identical during growth on different carbon sources. The α -glucosidase on the other hand shows a different pattern of repression and localization.

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