IDENTIFICATION OF ISOAMYLASE, A GLYCOGEN-DEBRANCHING ENZYME, FROM *BACILLUS AMYLOLIQUEFACIENS*

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1. Introduction

Bacillus amyloliquefaciens is well known for its production of an extracellular α-amylase. The enzyme is secreted into the culture fluid during the exponential phase of growth when the bacteria are grown with an amylaceous carbon source, e.g. maltose, maltodextrins, soluble starch, or glycogen. α-Amylase has been purified, crystallized and investigated in depth with regard to its physical properties, amino acids composition, mode of action and product specificity [1]. α-Amylase will only hydrolize 1,4-α-glucosidic linkages in appropriate substrates and does not attack 1,6-α-bonds of branched α-glucans. The enzyme is thought to function in the degradation of α-glucans making these macromolecules available to the cell as a metabolizable carbon source.

In the course of an investigation of α-glucan metabolism in bacteria [2,3,4], we found it worthwhile to re-examine, in *B. amyloliquefaciens*, the enzymes possibly involved in the utilization of branched α-glucans. This report communicates the identification of a hydrolase which will selectively cleave 1,6-α-glucosidic bonds in branched α-glucans. According to its substrate specificity, this enzyme may be named isoamylase or glycogen 6-glucanohydrolase (EC 3.2.1.68).

2. Materials and methods

2.1. *Chemicals and accessory enzymes*

Carbohydrates used as a carbon source in growth experiments and as a substrate in enzyme digests were obtained from the following suppliers: Amylose No. 4561, maltose No. 5910 and 5912, D-glucose No. 8346, soluble starch No. 1253 from E. Merck, Darmstadt, GFR; pullulan from Serva, Heidelberg, GFR; amylopectin from Koch-Light, Colnbrook, U.K.; oyster glycogen, type II, from Sigma, St. Louis, USA. Phytoglycogen was isolated from sweet corn [5], commercial amylopectin was freed from small amounts of amylose [6], a maltodextrin mixture (average chain length 7 glucosyl units) was a gift of Corn Products International, USA. D-Glucose oxidase (grade III) and horse radish peroxidase (grade II) were purchased from Boehringer Mannheim, GFR, glucoamylase was a partly purified [7] commercial preparation ("Diazyme" Miles Labs., Elkhart, USA). In the standard purification of β-amylase from sweet potatoes [8], a procedure removing traces of α-glucosidase was included [9]. All other chemicals were of the highest grade commercially available.

2.2. *Culture conditions of* B. amyloliquefaciens

B. amyloliquefaciens ATCC 23350 was grown aerobically at 32°C in liquid media of the following composition per litre: Maltose or another carbon source as indicated in Results and discussion, 10 g; NH_4Cl, 7 g; $Na_2SO_4 \cdot 10\,H_2O$, 1 g; KH_2PO_4, 3.25 g; K_2HPO_4, 12.50 g; aspartic acid, 1 g; sodium glutamate, 1 g; stock solution of trace elements according to Chen and Segel [10], 2 ml. Cells harvested in their mid-exponential phase of growth on maltose were used as the source of isoamylase. Roughly the same amount of activity of isoamylase, in addition to an α-amylase, was also present in the culture fluid. A cell sonicate was purified by removal of nucleic acids with streptomycin sulphate and by fractional precipitation with

ammonium sulphate followed by column chromatography on DEAE-Sephadex. Fractions with isoamylase activity were pooled, dialyzed and stirred with an amylose suspension. After centrifugation, the dialyzed supernatant still contained α-amylase, which was found to separate from isoamylase on a molecular sieve (Acrylex P 100). A partially purified enzyme preparation from this step was used for the identification of isoamylase as described in the Results and discussion section. The optimal conditions for isoamylase formation and details of the purification procedure will be published elsewhere. One unit of enzyme activity is the amount of protein which releases 1 mole of reduction end-groups per second from glycogen at pH 5.0 and 37°C.

2.3. *Analytical methods*

D-Glucose was measured with the specific glucose oxidase reagent [11], α-glucan was determined with a combined glucoamylase/glucose oxidase reagent calibrated against soluble starch (G. Wöber and W. J. Whelan, unpublished work). Reducing power was measured according to a modification of Nelson's procedure [12] with maltose hydrate as a standard. Colorimetric determinations were performed on a Corning–EEL 222 colorimeter. The instrument was zeroed with appropriate blank solutions. Multiple ascending paper chromatography was performed on Schleicher u. Schüll, No. 2043 b, Dassel, GFR, with *n*-butanol/pyridine/H_2O, 6/4/3 (v/v/v) as the solvent. Before developing the chromatograms with $AgNO_3$ – NaOH – $Na_2S_2O_3$ [13], the paper was sprayed with a solution of glucoamylase in 20 mM sodium acetate buffer, pH 5.

3. Results and discussion

3.1. *In search of a debranching enzyme: rationale*

We were led to consider the operation, in *B. amyloliquefaciens*, of a branched α-glucan-hydrolizing enzyme, different from α-amylase, on the basis of the following observation: The rate of cell multiplication and carbohydrate consumption was essentially the same irrespective of the presence or absence of 1,6-α-glucosidic linkages in the α-glucan provided as a carbon source. Among several growth substrates tested, the results of experiments employing maltose,

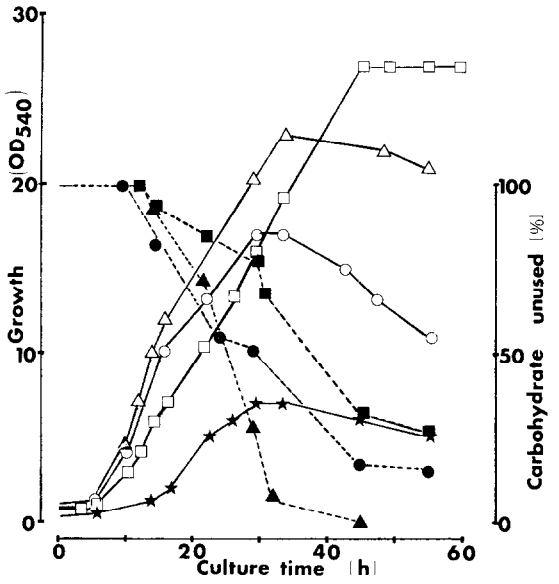

Fig.1. Different carbohydrates support growth of and are consumed by *Bacillus amyloliquefaciens*. Open symbols: growth, full symbols: carbohydrate utilization, squares: maltose, circles: glycogen, triangles: glucose, stars: control (no carbohydrate added). At regular intervals, an aliquot of the culture fluid was removed aseptically for the measurement of both a growth parameter, i.e. OD_{540}, and residual carbohydrate, after appropriate dilution. Carbohydrate was determined enzymatically (for details see Materials and methods).

oyster glycogen and, as a reference, glucose are illustrated in fig.1. An analogous pattern was obtained with maltodextrins, amylopectin, or different brands of soluble starch (data not shown). All attempts, however, to detect, either in the culture fluid or in a cell-bound form, an enzyme presumed to be capable of hydrolizing the 1,6-α-interchain linkages of branched substrates were unsuccessful due to the presence of an α-amylase interfering with the assay. We therefore set out to purify the enzyme premised to be a 6-α-glucanohydrolase with particular emphasis on removing the accompanying α-amylase.

3.2. *Criteria of freedom from α-amylase*

Care was taken to ensure the absence of any detectable trace of α-amylase in the enzyme preparation to be characterized. To this end, the change in iodine-staining power of a digest with amylose was

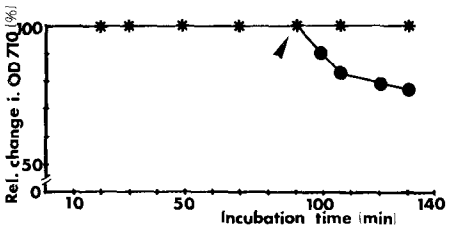

Fig.2. Criterion for the absence of α-amylase in purified *Bacillus amyloliquefaciens* isoamylase. The incubation contained (final concentration per ml): amylose, 5 mg; enzyme solution, 17 μg; 50 mM sodium acetate buffer, pH 5.0. After various incubation periods, an aliquot was removed and treated with iodine solution according to Krisman [17]. OD_{710} is expressed relative to a blank incubation ommitting enzyme. The arrow indicates addition of α-amylase (1.2×10^{-9} kat/mg protein) to a parallel incubation.

recorded (fig.2). Over the period observed, no amylose-degrading enzyme could be detected. At the time indicated by an arrow, α-amylase separated in the last step of the enzyme purification was added to an aliquot of the incubation mixture resulting in a rapid decrease of the absorbance of the amylose iodine complex.

In order to confirm the absence of α-amylase by a different method, the enzyme preparation was tested with oxidized amylose [14]. No *endo*-1,4-α-glucanase was detectable.

3.3. *Characterization of* B. amyloliquefaciens *isoamylase*

Preliminary experiments had established the enzymic reaction (liberation of reducing end-groups from glycogen) to proceed linearly up to 60 min and 3.8 μg/ml of protein. Consequently, the initial rate of reaction with various substrates was measured in this range (table 1). The lack of action upon pullulan, maltodextrins and maltose indicated the absence of a pullulanase, α-glucosidase and 4-α-glucanotransferase, respectively. The products of hydrolysis of oyster glycogen and phytoglycogen were inspected by paper chromatography (fig.3). Maltodextrins with a chain length of 6–8 together with higher members of the series (not resolved in the chromatogram) are visible.

This evidence pointed towards the performance of an isoamylase. The complete debranching of glycogen with the concurrent liberation of maltodextrin chains

Table 1
Initial rate of reaction of *Bacillus amyloliquefaciens* isoamylase with various substrates

Substrate	Relative enzyme activity [%]
glycogen	100
soluble starch	75
amylopectin	75
pullulan	0
amylose	0
maltodextrin mixture, cf 7	0
maltose	0

100% corresponds to 6.25×10^{-9} kat/mg protein.

with exclusively 1,4-α-bonded glucosyl residues is a feature of other bacterial isoamylases [15,16]. We, then, incubated glycogen consecutively with an excess of *B. amyloliquefaciens* isoamylase and β-amylase. Glycogen was converted quantitatively (98.5 ± 0.5%) into maltose indicative of complete debranching of glycogen.

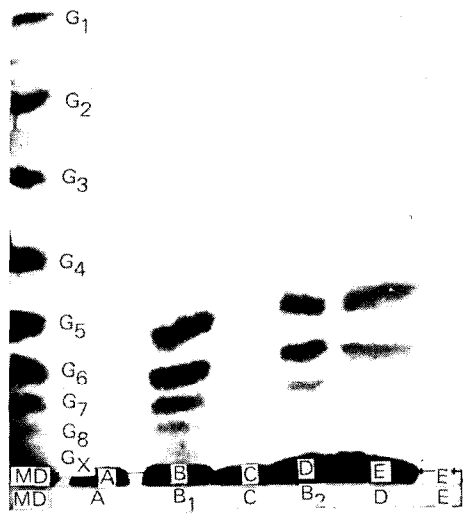

Fig.3. The products of action of *Bacillus amyloliquefaciens* isoamylase upon glycogen. MD: maltodextrin standard mixture where G_1, G_2, G_3, etc. stands for glucose, maltose and maltotriose, respectively; A, C: blank incubations of phytoglycogen and oyster glycogen without enzyme; B_1 and B_2: phytoglycogen incubated with isoamylase for 6 hr and 1 hr, respectively; C: oyster glycogen incubated with isoamylase for 1 hr; E: enzyme blank. Final concentration of protein in all digests: 9.5 μg/ml. For details of the chromatographic procedure see Materials and methods.

In summation, we believe to have presented sufficient evidence to support the statement that *B. amyloliquefaciens* will produce an isomylase under appropriate nutritional conditions. In terms of physiological function, isoamylase is assumed to assist α-amylase in the primary attack on branched α-glucans in the environment of the bacteria.

Acknowledgement

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