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α-GLUCOSIDASE, A MEMBRANE-BOUND ENZYME OF α-GLUCAN METABOLISM IN *BACILLUS AMYLOLIQUEFACIENS*

PURIFICATION AND PARTIAL CHARACTERIZATION

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Summary

The organism *Bacillus amyloliquefaciens* is capable of producing α-amylase (1,4-α-D-glucan glucanohydrolase, EC 3.2.1.1) and isoamylase (glycogen 6-glucanohydrolase, EC 3.2.1.68) extracellularly and a membrane-bound, intracellular α-glucosidase (α-D-glucoside glucohydrolase, EC 3.2.1.20).

The amounts of α-glucosidase in cells of *B. amyloliquefaciens* grown on amylaceous polysaccharides were significantly higher than in cells grown on non-carbohydrate carbon sources. α-Glucosidase was exclusively found associated with membranes from ruptured spheroplasts by subcellular fractionation and solubilization studies. Salt solutions and chelating agents alone did not dislodge α-glucosidase from membranes, but in combination with detergents were most effective in solubilizing active enzyme (0.1% sodium cholate (pH 8.0)/0.4 M sodium chloride).

Purified α-glucosidase very rapidly hydrolized *p*-nitrophenyl α-D-glucopyranoside and sucrose. Maltose, maltotriose, isomaltose and isomaltotriose were hydrolized at slower rates, whereas β-glucosides and polymeric α-glucans were not attacked. Other properties of the purified enzyme were as follows: Temperature optimum for catalysis = 39 ± 1°C; pH optimum = 6.8; molecular weight = 27 000 ± 1000.

α-Glucosidase is proposed to function in the endogenous metabolism of α-glucans provided extracellularly as carbon sources for growth of *B. amyloliquefaciens*.

Introduction

Various bacteria exhibit excellent growth patterns when grown on a broad spectrum of amylaceous polysaccharides, and the enzymes thought to be

involved have been investigated [1—4]. The α-glucan utilization system previously proposed as operative in these organisms comprises the enzymes α-amylase (1,4-α-D-glucan glucanohydrolase, EC 3.2.1.1), pullulanase (pullulan 6-glucanohydrolase, EC 3.2.1.41), amylomaltase (1,4-α-D-glucan:1,4-α-D-glucan 4-α-glycosyltransferase, EC 2.4.1.25) maltodextrin phosphorylase (1,4-α-D-glucan:orthophosphate α-glucosyltransferase, EC 2.4.1.1) and maltodextrin permease(s) [3,5].

Bacillus amyloliquefaciens can grow on various linear and branched α-glucans, but the set of enzymes that function in the degradation of α-glucans was significantly different to that proposed by Palmer et al. [3]. Neither 4-α-glucanotransferase (1,4-α-D-glucan:1,4-α-D-glucan 4-α-glycosyltransferase, EC 2.4.1.25) nor polyglucose phosphorylase (EC 2.4.1.1) was detected intracellularly during the exponential phase of growth. Together with a well-known α-amylase which is secreted into the culture fluid [6], *B. amyloliquefaciens* produced extracellular isoamylase (EC 3.2.1.41) and cell-bound α-glucosidase (α-D-glucoside glucohydrolase, EC 3.2.1.20) and α-amylase [7]. The results of a detailed investigation of α-glucosidase will be reported here. (The results on subcellular localization of α-glucan-degrading enzymes were presented as a poster at the 558th Meeting of the Biochemical Society [7].)

Materials and Methods

Carbohydrates

Amylose, soluble starch, maltose, glucose, galactose, sucrose, *p*-nitrophenyl α-D-glucopyranoside and *p*-nitrophenyl β-D-glucopyranoside from E. Merck (Darmstadt, G.F.R.); Pullulan from Serva (Heidelberg, G.F.R.); methyl α-D-glucopyranoside, isomaltose and isomaltotriose from Sigma (St. Louis, U.S.A.). A maltodextrin mixture (average chain length, 7 glucosyl units) was a gift of Corn Products International (U.S.A.). Phytoglycogen was isolated from sweet corn according to Schoch [8]. Maltotriose was prepared from an incubation mixture of pullulan and a partially-purified pullulanase from *Klebsiella pneumoniae* [9]. [U-^{14}C]Glucose was prepared from radioactive *Anacystis nidulans* glycogen [10]. Enzymes: D-Glucose oxidase (β-D-glucose:oxygen 1-oxidorextase, EC 1.1.3.4) (grade III) and peroxidase (donor:hydrogen-peroxide oxidoreductase, EC 1.11.1.7) (grade II) were products of Boehringer Mannheim (G.F.R). Exo-1,4-α-glucosidase (1,4-α-D-glucan glucohydrolase, EC 3.2.1.3) was a partly-purified [11] commercial preparation (Diazyme, Miles Laboratories, Elkhart, U.S.A.). Lysozyme (mucopeptide *N*-acetylmuramoylhydrolase, EC 3.2.1.17) was purchased from Serva (Heidelberg, G.F.R.). EDTA, cetylpyridinium chloride, Triton X-100 and sodium cholate were purchased from Merck (Darmstadt, G.F.R.). Tween 20 and Tween 85 are products of Koch-Light Laboratories Ltd. (Colnbrook, U.K.). The zwitterionic detergents, Empigen BB and Empigen BT, were kindly supplied by Marchon France S.A. (France). Sodium dodecyl sulphate was obtained from Serva (Heidelberg, G.F.R.). All other chemicals were of the highest grade commercially available.

Microorganism and culture conditions

B. amyloliquefaciens ATCC 23350 was grown aerobically at 37°C in a liquid medium of the following composition per litre: Maltose (or another carbon

source as indicated in Results) 10 g/NH_4Cl, 7 (5) g/$Na_2SO_4 \cdot 10H_2O$, 1 g/KH_2PO_4, 3.24 g/$K_2HPO_4 \cdot 3H_2O$, 16.28 g/aspartic acid, 1(0.5) g/sodium glutamate 1(0.5) g/stock solution of trace elements according to Chen and Segel [12], 2 ml. The concentrations of medium components used in experiments on spheroplast formation are given in parentheses. With the exception of citric acid and malic acid, carbon sources and salt medium (pH 7.0) were sterilized separately and then mixed under aseptic conditions.

Analytical methods

The activities of α-amylase [13], isoamylase [4] and pullulanase [14] were determined with their respective substrates. Enzyme activity was measured as the release of reducing end-groups by a modification of Nelson's procedure [15] with glucose hydrate as a standard. α-Glucosidase was assayed with *p*-nitrophenyl α-glucoside. The standard enzyme digest contained 0.3 ml 2% (w/v) substrate in 0.1 M potassium phosphate buffer (pH 6.8), 50—200 µl enzyme solution plus the same buffer to give a final volume of 1 ml. After incubation for up to 1 h, 4 ml of 0.1 M Na_2CO_3 solution was added. Enzyme activity is expressed in mol sodium *p*-nitrophenolate formed per s. The release of glucose from various amylaceous polysaccharides was determined by the glucose oxidase method [16]. Amylomaltase and maltodextrin phosphorylase were measured by techniques described previously [1,17].

Protein content of cells was measured by the modified Folin method [18]. Iodine staining of amylose and soluble starch was carried out according to Krisman [19]. Growth of the organisms was followed by measuring the cell dry weight of culture aliquots [20]. Polyacrylamide gel electrophoresis with 7.5% cylindrical gels was routinely used to follow the progress of enzyme purification. Native proteins were run in an anionic system (7.5) according to Williams and Reisfeld [21]. Molecular weight estimations on sodium dodecyl sulphate (SDS) polyacrylamide gels were obtained with the system described by Davis [22].

Results

Physiology of α-glucosidase formation by B. amyloliquefaciens

Cells of *B. amyloliquefaciens* were grown either on amylaceous polysaccharides or with carbon sources having little or no inductive effect and the results are shown in Fig. 1a. Irrespective of the degree of polymerization or the presence or absence of 1,6-α-glucosidic linkages in the α-glucans provided as carbon sources, the rate of growth was essentially the same. Cell yields determined with amylaceous polysaccharides were significantly higher than those with sodium acetate. The oligo- and polysaccharides yielded increased amounts of α-glucosidase activity in comparison with glucose or acetate (Fig. 1b) and also sucrose, malate and citrate (unpublished).

The following conclusions can be drawn from these observations: (i) α-Glucosidase is subject to graded induction when grown on various carbon sources; (ii) the amounts of α-glucosidase determined in cells grown with non-carbohydrates as carbon sources correspond to a constitutive level of the enzyme: (iii) catabolite repression is observed when glucose is present in the culture fluid.

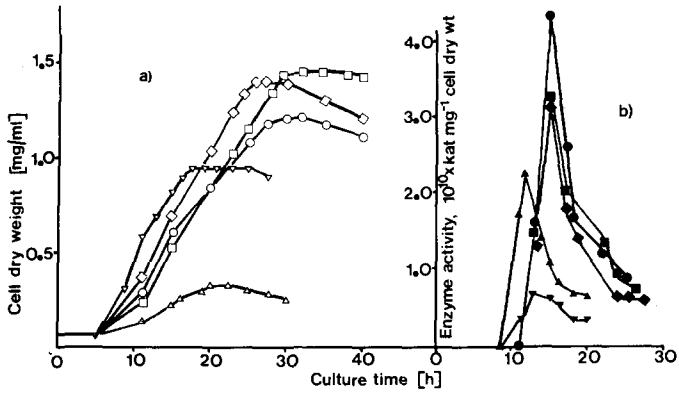

Fig. 1. Growth of *B. amyloliquefaciens* supported by various carbon sources (a) and time course of specific activity of α-glucosidase during growth of organism (b). For growth experiments, cells maintained on agar plates were inoculated into 125 ml of medium containing maltose as the sole carbon source. At 0.8 mg/ml cell dry weight, cells were harvested by centrifugation (14 000 × *g* for 20 min), washed twice with fresh salt medium without carbon source and resuspended to the same cell density with pure salt medium. 10 ml each of this suspension were inoculated into 500 ml of medium before solutions of the respective carbon sources were added aseptically. ◇, Soluble starch; ○, maltodextrins; □, maltose; ▽, glucose; △, acetate.

Subcellular localization of α-glucosidase

α-Glucosidase was exclusively determined in whole-cell homogenates, because no activity of the enzyme was detectable in the culture fluid. Exact information about the subcellular localization of enzymes of α-glucan metabolism is provided by preparation of spheroplasts (detailed in Fig. 2). The results of enzyme assays with subcellular components which served as enzyme

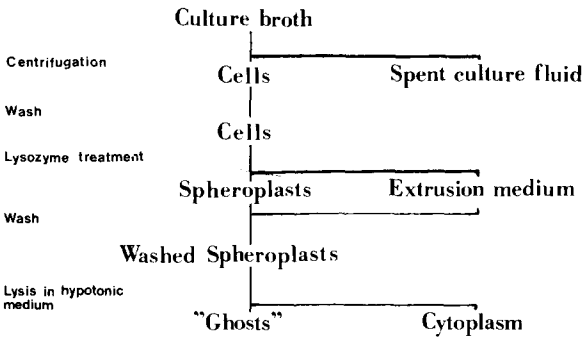

Fig. 2. Flow scheme for the preparation of spheroplasts form cells of *B. amyloliquefaciens*. Cells from 500 ml of culture in the mid-exponential phase of growth were harvested by centrifugation (28 000 × *g* for 40 min), washed twice with 20 mM potassium phosphate buffer (pH 6.0), centrifuged and resuspended in 16 ml of 0.4 M sodium maleate buffer (pH 6.0). Lysozyme was added to give a final concentration of 50 μg/ml. After 2 h of incubation at 37°C, conversion into spheroplasts was complete, as monitored with a phase contrast microscope. Spheroplasts were collected from the extrusion medium by centrifugation (7000 × *g* for 20 min), resuspended in 0.4 M sodium maleate buffer (pH 6.0) and centrifuged again. The supernatant of the latter centrifugation was combined with the extrusion medium. Spheroplasts were then ruptured by suspending them in 10 mM sodium maleate buffer (pH 6.0). Lysed spheroplasts were centrifuged (28 000 × *g* for 40 min) to separate cytoplasm and ghosts. Pellet (ghosts), supernatant (cytoplasm) and extrusion medium were used for enzyme assays.

TABLE I

SUBCELLULAR DISTRIBUTION OF ENZYMES POSSIBLY INVOLVED IN α-GLUCAN CATABOLISM IN *B. AMYLOLIQUEFACIENS*

+, Enzymes detected; —, enzymes not detectable.

	α-Amylase	Isoamylase	α-Glucosidase
Culture fluid	+	+	—
Extrusion medium	+	+	—
'Ghosts'	—	—	+
Cytoplasm	+	—	—

sources are summarized in Table I. Neither amylomaltase nor maltodextrin phosphorylase were detected in the organism, but α-glucosidase attached to membrane fragments, and an intracellular α-amylase, were found.

In order to confirm the membrane-bound character of α-glucosidase, the relative enzyme activity found in sediments and supernatants after differential centrifugation of whole-cell homogenates was determined. The results of this fractionation procedure (cf. Table II) indicated that α-glucosidase was a particulate protein.

Solubilization of membrane-bound α-glucosidase

The question of whether α-glucosidase was a peripheral or a integral membrane protein was examined by subjecting membranes of *B. amyloliquefaciens* to a treatment with various detergents. The results of detergent treatments are compiled in Table III. The highest degree of solubilization of α-glucosidase activity was observed with sodium cholate in 50 mM sodium phosphate buffer (pH 8.0) in the presence of 0.4 M sodium chloride. When detergent was omitted in a control experiment, it became apparent that high ionic strength by itself did not solubilize the enzyme significantly. Higher concentrations of

TABLE II

DIFFERENTIAL CENTRIFUGATION OF *B. AMYLOLIQUEFACIENS* CELL HOMOGENATE FOR SUBCELLULAR LOCALIZATION OF α-GLUCOSIDASE

Cells of 100 ml of culture were harvested in the mid-exponential phase of growth by centrifugation. The cells were washed twice with 0.1 M potassium phosphate buffer (pH 6.8), centrifuged again and resuspended in 10 ml of the same buffer. After sonication for 1.5 min, the homogenate was centrifuged at 28 000 × *g* for 20 min. The supernatant was centrifuged at 104 000 × *g* for 60 min and the supernatant of the latter centrifugation centrifuged again at 104 000 × *g* for 240 min. Whole-cell homogenates, pellets and supernatants were assayed for enzyme activity. (Activity of α-glucosidase in whole-cell homogenates was taken as 100%).

Centrifugation	Relative activity in %	
	Pellet	Supernatant
20 000 × *g* for 20 min	73.0	27.0
104 000 × *g* for 60 min	8.6	18.4
104 000 × *g* for 240 min	14.2	4.2
Recovery:	95.8	4.2

TABLE III

DIFFERENTIAL SOLUBILIZATION OF α-GLUCOSIDASE FROM MEMBRANES OF *B. AMYLOLIQUEFACIENS* WITH VARIOUS DETERGENTS

Cells from 0.75 l of culture in the mid-exponential phase of growth (0.9 mg/ml cell dry weight) were harvested, washed twice with 0.2 M sodium phosphate buffer (pH 6.8) and resuspended with the same buffer to a final volume of 70 ml. After sonication (1.5 min at 30 kHz and 4°C), cell debris was removed by centrifugation (48 000 × *g* for 1 h). The pellet was resuspended in 70 ml of 50 mM sodium phosphate buffer (pH 6.8), homogenized with a Braun potter and divided into 4-ml fractions. To each of the aliquots, 0.1 ml of the respective detergent in buffered solution was added. After 2 h of incubation at 37°C with gentle shaking, non-solubilized material was removed by centrifugation (28 000 × *g*, 1 h). The supernatant was used for enzyme assays. 100% activity corresponds to 48.58 kat × 10^{-10}/ml cell homogenate with a specific activity of 9.72 kat × 10^{-10}/mg protein.

Detergent	Concn. (%)	kat × 10^{-10}	Enzyme activity solubilized (%)	Protein solubilized (mg/ml)	Spec. act. kat × 10^{-10}/mg protein
0.4 M NaCl in 50 mM sodium phosphate buffer pH 6.8 (control)	—	2.42	4.92	0.363	6.67
EDTA	0.1	2.73	6.03	0.355	8.25
EDTA	0.2	2.61	5.37	0.336	7.74
Anionic					
Na-cholate, pH 6.8	0.1	3.08	6.34	0.271	11.37
Na-cholate, pH 6.8	0.2	3.38	6.95	0.392	8.62
Na-cholate, pH 8.0/0.4 M NaCl	0.1	19.85	40.85	0.319	62.23
SDS	0.01	2.40	4.94	0.262	9.16
SDS	0.02	3.20	6.58	0.311	14.91
Zwitterionic					
Empigen BT	0.1	7.55	15.53	0.422	17.89
Empigen BT	0.2	4.62	9.51	0.506	9.13
Empigen BB	0.1	9.09	18.70	0.472	19.23
Empigen BB	0.2	2.93	6.03	0.554	8.26
Non-ionic					
Tween 20	0.1	2.40	4.94	0.365	6.58
Tween 20	0.2	4.00	8.23	0.301	13.29
Tween 85	0.1	11.26	23.17	0.417	27.00
Tween 85	0.2	7.75	15.95	0.505	15.35
Cationic					
Cetylpyridinium chloride	0.05	2.37	4.88	0.291	12.41
Cetylpyridinium chloride	0.1	1.93	4.03	0.307	6.38

detergent than those shown in Table III resulted in an increased amount of protein solubilized from membranes, but drastically decreased the enzyme activity (by 80% at 1 mg/ml). An aliquot of the protein solution solubilized with sodium cholate was dialyzed exhaustively against 50 mM sodium phosphate buffer (pH 6.8); this procedure inactivated the enzyme and the activity was not restored after the addition of sodium cholate and readjustment of the pH to 8.0.

Attempts were made to replace sodium cholate immediately after solubilization by non-ionic detergents with the objective of increasing the stability of α-glucosidase in detergent solution. Aliquots of protein (final concentration, 0.46 mg/ml) in a solution of 2 mg/ml sodium cholate in 50 mM sodium phosphate buffer (pH 8.0)/0.4 M sodium chloride were dialyzed against 50 mM

sodium phosphate buffer (pH 6.8) containing varying concentrations of Tween 20, Triton X-100 and Tween 85. With decreasing hydrophile-lipophile balance number of the detergent, recovery of enzyme activity increased.

The following optimal values for residual α-glucosidase activity were obtained under otherwise identical conditions: Tween 20 (2 mg/ml), 20%; Triton X-100 (5 mg/ml), 33% Tween 85 (2 mg/ml), 37%; sodium cholate (1 mg/ml), 20%.

Purification of α-glucosidase

Cells from 3 l of culture were harvested by centrifugation (10 000 × *g* for 1 h). The pellet was washed twice with 0.2 M potassium phosphate buffer (pH 6.8), centrifuged again and suspended in 20 ml of the same buffer precooled to 4°C and sonicated for 1.5 min. The homogenate was centrifuged at 20 000 × *g* for 1 h. After precipitation of nucleic acids with 2% (w/v) streptomycin sulphate [23], the supernatant was centrifuged again and dialyzed against 0.1 M potassium phosphate buffer (pH 6.8) in an Amicon ultrafiltration cell (XM 50 membrane). The crude extract containing particulate α-glucosidase was applied to a molecular sieve, Ultrogel AcA 44, and eluted with 0.1 M potassium phosphate buffer (pH 6.8) (Fig. 3). Fractions containing α-glucosidase activity were pooled. α-Glucosidase was subsequently eluted from a DEAE-cellulose column with a continuous salt gradient of increasing ionic strength (Fig. 4). Samples of vesicular α-glucosidase, after dialysis, lost only 12% of activity after 6 days at 4°C. Repeated freezing and thawing resulted in practically no loss of activity of the enzyme.

To find optimal solubilization conditions, aliquots of vesicular enzyme

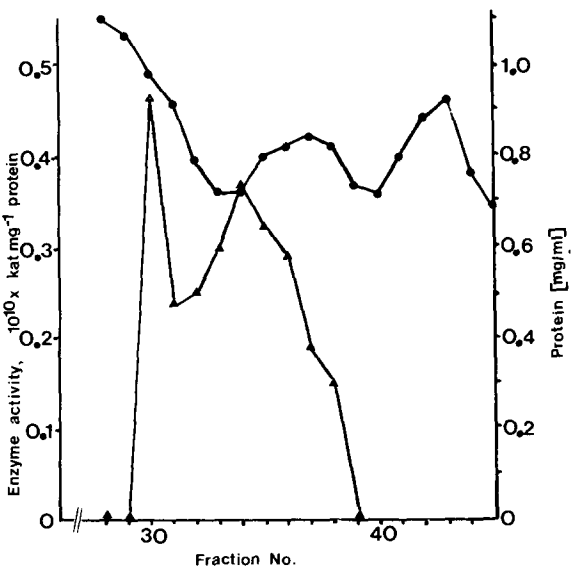

Fig. 3. Elution pattern of α-glucosidase from a molecular sieve, Ultrogel AcA 44, (column 120.0 × 2.0 cm) previously equilibrated with 0.1 M potassium phosphate buffer (pH 6.8). Fractions of 4 ml were collected. ●, Protein: ▲, specific activity of α-glucosidase.

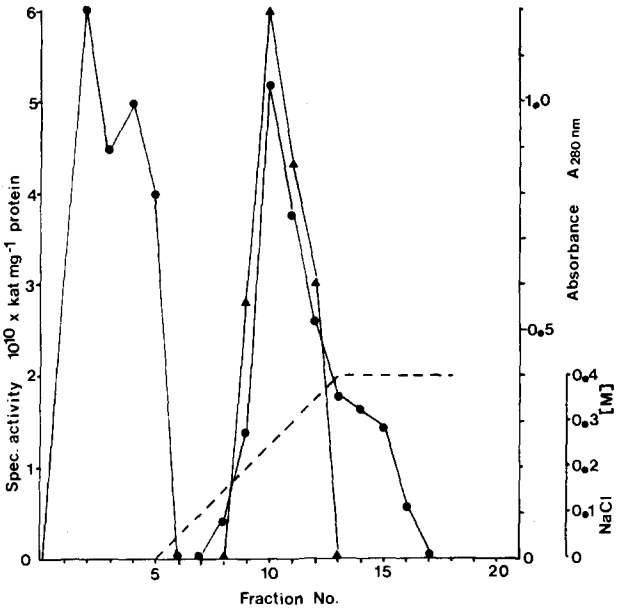

Fig. 4. Ion-exchange chromatography of α-glucosidase on DEAE-cellulose (column 10.0 X 1.2 cm) pre-equilibrated with 0.05 M potassium phosphate buffer (pH 6.8). Protein bound to the resin was eluted with a linear salt gradient (0—0.4 M sodium chloride in 0.05 M potassium phosphate buffer (pH 6.8)). Fractions of 8 ml were collected. ●, Protein: ▲, specific activity of α-glucosidase.

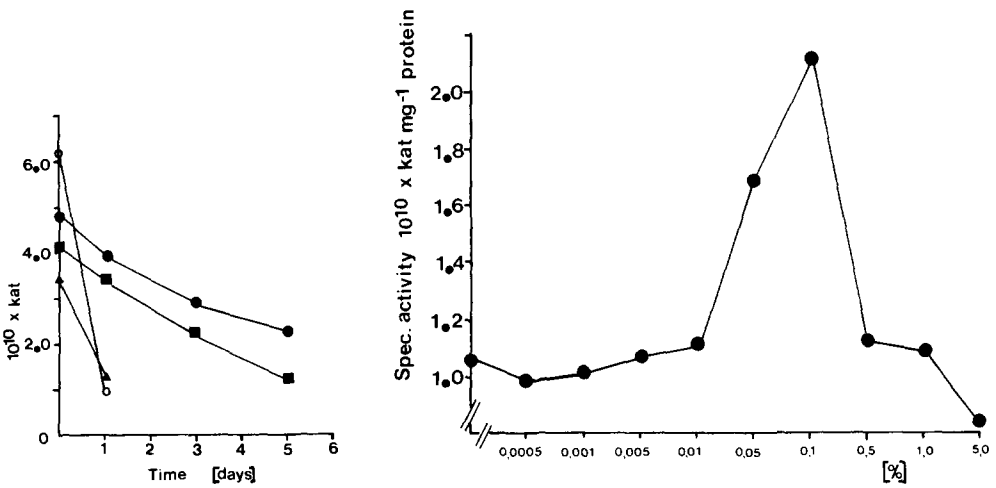

Fig. 5. Stability of α-glucosidase solubilized with various detergents. 0.330 mg/ml protein (final concentration) were incubated with Tween 20 (0.2%, w/v, final concentration) (▲), Tween 85 (0.1%, w/v) (●) and Triton X-100 (0.5%, w/v (■). 0.64 mg/ml protein were incubated with sodium cholate (0.1%, w/v, plus 0.4 M sodium chloride) (○). The incubation mixture was kept at 8°C for 5 days. In 24 h intervals, α-glucosidase activity was assayed.

Fig. 6. Solubilization by Tween 85 of partly purified vesicular α-glucosidase. Aliquots of 0.17 mg/ml of protein (final concentration) were incubated each with varying concentrations of Tween 85 (0.005—50 mg/ml detergent, final concentration in the digest) and assayed for activity.

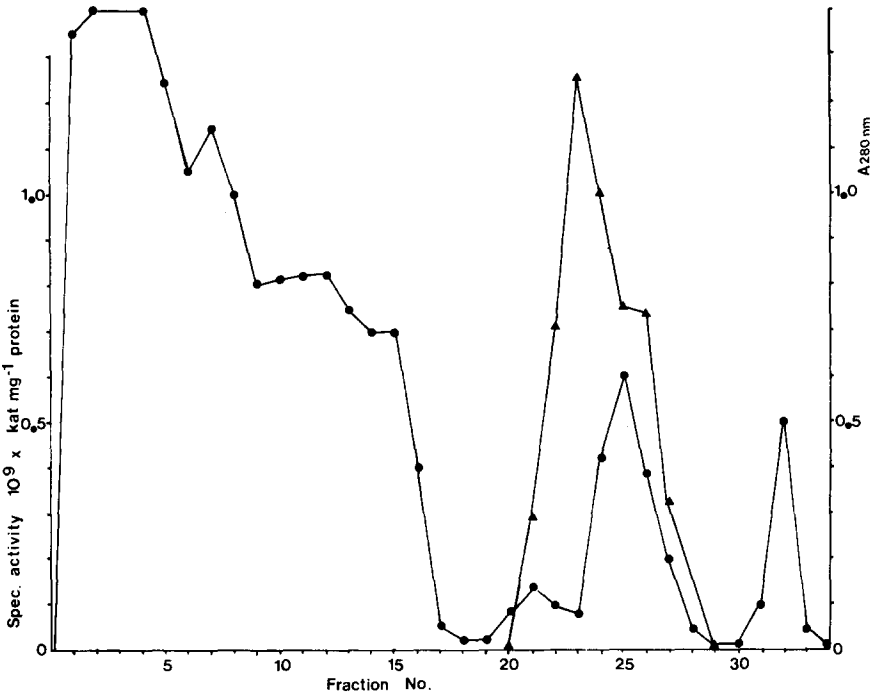

Fig. 7. Purification of solubilized α-glucosidase on a molecular sieve, Biogel P-100 (column 60.0 × 2.25 cm). The column material had been equilibrated with 50 mM sodium phosphate buffer (pH 6.8) containing 0.05% (w/v) Tween 85. The sample containing detergent (50 mg/ml final concentration) was placed on top of the column and eluted with 50 mM sodium phosphate buffer (pH 6.8, 0.05% Tween 85). Fractions of 1.5 ml were collected. ●, Protein: ▲, specific activity.

preparation were incubated with Tween 20, Tween 85, Triton X-100 and sodium cholate then stored at 8°C for 5 days. After removal of 0.1-ml aliquots in 24 h intervals, the incubation mixture was assayed for residual α-glucosidase activity. As shown in Fig. 5, least degradation was obtained with Tween 85.

Vesicular α-glucosidase was incubated with varying concentrations of the detergent and assayed for activity. Under standard assay conditions, we found highest activity at a 1 : 0.34 ratio (detergent to protein) using 0.1% (w/v) Tween 85 in the incubation mixture (Fig. 6). Fractions containing α-glucosidase (from DEAE-cellulose chromatography) were pooled, dialyzed and concentrated to 1 ml with an Amicon cell (XM 50 membrane). The concentrated suspension was brought to a final concentration of 50 mg/ml detergent and immediately placed on top of a molecular sieve. Solubilized α-glucosidase was found to separate from the bulk of protein (Fig. 7). Fractions containing α-glucosidase were pooled and applied to a second DEAE-cellulose column. α-Glucosidase was eluted from the column at an initial concentration of 0.4 M NaCl in sodium phosphate buffer (pH 6.8) containing 0.05% Tween 85 as shown in Fig. 8. The results of this purification schedule are summarized in Table IV.

Criteria of purity of α-glucosidase

No α-amylase or isoamylase activity was observed by an iodine-staining tech-

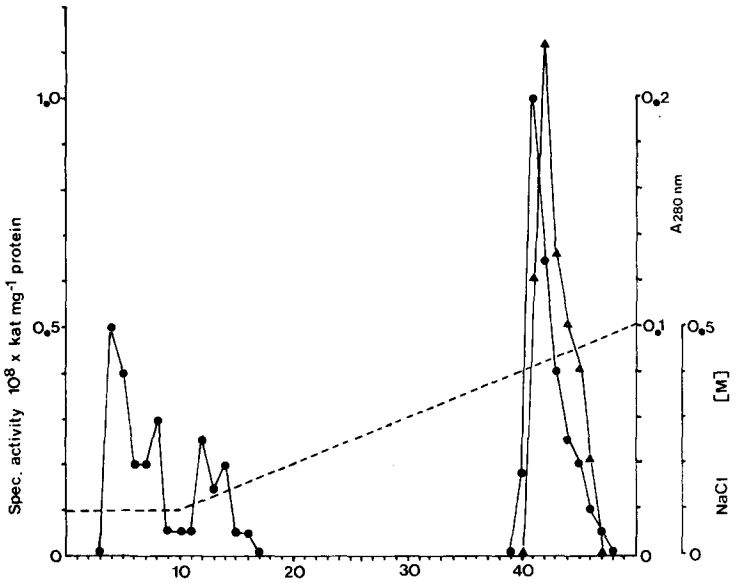

Fig. 8. Binding to, and elution from, DEAE-cellulose (column 2.0 X 1.2 cm) of solubilized α-glucosidase. The enzyme was eluted from the column pre-equilibrated with 0.5 M sodium phosphate buffer (pH 6.8, 0.05%, w/v, Tween 85). Protein was eluted from the column with a linear salt gradient (0.1—0.5 M NaCl in the same buffer plus 0.05% Tween 85. Fractions of 1.2 ml were collected. ●, Protein; ▲, specific activity of α-glucosidase.

nique. Furthermore, polyacrylamide gel electrophoresis with native protein showed only two bands. The major protein band coincided with an activity stain [23,24]. Polyacrylamide gel electrophoresis with dodecyl sulphate showed a single band, and the molecular weight of the protein was calculated as 27 000 ± 1000.

TABLE IV

PURIFICATION SCHEME OF α-GLUCOSIDASE

Preparation	Specific activity X 10^{10} kat/mg protein	Protein (mg/ml)	Total activity X 10^{10} kat	Volume (ml)	Fold-purification	Yield (%)
Particulate enzyme *	0.34	49.80	338.50	20	—	100
Ultrogel AcA 44 (120 X 2.0 cm)	1.34	10.80	144.70	10	3.94	42.75
1. DEAE-cellulose (10 X 1.2 cm)	4.61	3.39	117.10	7.5	13.54	34.60
Biogel P-100 (60 X 2.25 cm)	75.00	0.29	110.35	5.0	220.61	32.60
2. DEAE-cellulose (2.0 X 1.2 cm)	118.00	0.12	71.00	7.5	347.10	20.97

* 20 000 X *g* supernatant after centrifugation of sonicated cells.

Properties of purified α-glucosidase

A distinct peak of enzyme activity was found at pH 6.8, whereas vesicular α-glucosidase showed a braod optimum between pH 6.5 and 7.0. After incubation for 30 min at pH 6.8 at different temperatures, the optimum for enzyme activity was found to be about 38°C. Above 45°C, the activity of the enzyme decreased rapidly to a residual activity of 24% at 50°C (compared to 100% at 38°C). To determine the stability of the enzyme, aliquots of the preparation were held at 11°C for 24 h at various pH values, readjusted to optimal pH and assayed for residual enzyme activity. α-Glucosidase exhibited a broad maximum between pH 6.2 and 7.0.

The enzymic reaction with *p*-nitrophenyl α-D-glucoside was found to proceed linearly up to 60 min and 6.0 μg/ml protein. Consequently, the initial rate of reaction with various substrates was measured in this range (Table V).

Discussion

α-Glucosidase, a membrane-bound enzyme

Evaluation of experiments with spheroplasts indicated that α-glucosidase is associated with the membrane of *B. amyloliquefaciens*. To learn more about the membrane-bound character of the enzyme, we found it necessary to remove α-glucosidase from membranes of the organism. Plenty of data are available about solubilization of membrane proteins with detergents and the different interactions of detergents with membrane proteins [25—28]. Solubilized α-glucosidase which was intensively dialyzed against buffer in the absence of detergent completely lost its biological activity. This activity was not restored by the addition of amphiphiles. The small effect of increased ionic strength of the extraction medium and the negligible amounts of α-glucosidase liberated from the membranes in comparison with the results obtained with sodium cholate or Tween 85, lead us to suggest that α-glucosidase is a tightly-bound integral membrane protein.

The denaturing effect of ionic detergents upon α-glucosidase activity is similar to those observed with other integral membrane-bound enzymes [28]. Membrane-bound enzymic activities may be activated, inactivated or even modified by detergents [25,29].

Since solubilized α-glucosidase appeared rather unstable, the high stability of vesicular α-glucosidase was exploited by removing contaminating protein before solubilizing the enzyme. It proved more convenient to purify α-glucosidase-containing vesicles rather than to deal with the difficulties of carrying a solubilized protein through several steps of purification.

Comparison of B. amyloliquefaciens α-glucosidase with similar enzymes of various origin

Even though α-glucosidases from various sources have been investigated in depth, little is known about membrane-bound α-glucosidases. Extracellular maltases were reported to be secreted by *Bacillus subtilis* P-11 [30] and a thermophilic *Bacillus* KP 1035 [31]. An extracellular α-glucosidase was found in *Bacillus* KP 1006 [32]. On the contrary, the glucosidases of *Pseudomonas fluorescens* [33], *Myxobacter* Al-1 [34], *Bacillus cereus* [35] and *Strepto-*

TABLE V

INITIAL RATE OF REACTION OF *B. AMYLOLIQUEFACIENS* α-GLUCOSIDASE WITH VARIOUS SUBSTRATES

Standard digest: 6.0 μg/ml protein, substrate (final concentration buffer, pH 6.8, which contained 50 μg/ml Tween 85. Incubation time, 15 min (100% corresponds to 5.9 kat × 10^{-10}.)

Substrate	Relative enzyme activity (%)
Maltose	40
Maltotriose	32
Isomaltose	30
Isomaltotriose	18
Soluble starch	—
Amylose	—
Phytoglycogen	—
Sucrose	140
p-Nitrophenyl-α-D-glucoside	100
p-Nitrophenyl-‖ β-D-glucoside	—
Methyl-α-D-glucoside	Trace

coccus pyogenes [36], including *Munor javanicus* [37] and *Saccharomyces cerevisiae* [38], were characterized as cytoplasmic enzymes. Large differences were found among maltases and α-glucosidases with respect to substrate specificity, pH optimum, temperature optimum and molecular weight. Despite the distinctions between these enzymes all were soluble. For the first time, this report describes a membrane-bound α-glucosidase of bacterial origin.

According to definitions given by Gottschalk [39], *B. amyloliquefaciens* α-glucosidase is a true α-glucosidase requiring α-configuration at carbon atom 1 of the glycon moiety of its substrates. Accordingly, *p*-nitrophenyl β-D-glucoside was not attacked by the enzyme. In its property of hydrolyzing *p*-nitrophenyl α-D-glucoside and sucrose faster than maltose, α-glucosidase of *B. amyloliquefaciens* seems to resemble α-glucosidases from yeasts [39,40] more than α-glucosidases from other bacteria [30—35,41]. No other α-glucosidases of the *Bacillus* genus have been reported to be capable of hydrolyzing sucrose, but some of them have a specificity towards methyl glucosides which the enzyme of *B. amyloliquefaciens* has not.

Physiological function of α-glucosidase in α-glucan catabolism in B. amyloliquefaciens

As established by growth experiments with various carbon sources, absolute activities of α-glucosidase in cells grown on carbohydrates were significantly higher than in those not grown on carbohydrates. This seems to be due to a graded induction of the enzyme. The low level of α-glucosidase activity in cells grown on glucose and the rapid decrease of specific activity in the mid-exponential phase of growth when cells were grown on α-glucans can possibly be related to catabolite repression of the enzyme. The extracellular enzymes, α-amylase and isoamylase, are secreted into the culture fluid, before active α-glucosidase is detectable in the membrane of the organism. By the concomitant action of both α-amylase and isoamylase, branched and linear α-glucans

can be degraded to maltodextrins, maltose and glucose. Maltodextrins and maltose may then be attacked by membrane-bound α-glucosidase, the products being transported by a yet uncharacterized permease into the cytoplasm of the cell. Because of the lack of maltodextrin phosphorylase and amylomaltase, α-glucosidase has to be regarded as a constituent of endogenuous α-glucan metabolism as previously proposed [7].

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