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SYNTHESIS OF α-AMYLASE AND α-GLUCOSIDASE BY MEMBRANE BOUND RIBOSOMES FROM BACILLUS LICHENIFORMIS

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α-Glucosidase was membrane bound during exponential growth of Bacillus licheniformis but was released into the medium during stationary phase. It could be partially removed from exponential phase cells by washing with NaCl (0.5 M). α-Amylase was exclusively extracellular and could not be detected in cells. Polysomes were prepared from exponential phase cells and separated into membrane bound and soluble fractions. In vitro chain completion and immunoprecipitation showed that α-glucosidase and α-amylase were synthesized by membrane bound and not by soluble ribosomes.

Protein secretion in prokaryotes largely conforms to the signal hypothesis (1). Precursor forms of exported proteins possess an NH_2 terminal extension of 18-33 amino acid residues that interacts with the cytoplasmic membrane as it emerges from the ribosome. This associates the polysome with the membrane and, as the mRNA is translated so the nascent polypeptide chain traverses the membrane. On the outside of the membrane the signal peptide is proteolytically removed allowing the enzyme to adopt its mature configuration (1). This cotranslational secretion is common, but in some cases protein accumulates in the cytoplasm and is secreted post-translationally. Toxin secretion by Vibrio cholerae (2) is probably post-translational, as may be α-glucosidase secretion by two thermophilic Bacillus strains (3,4).

Bacillus licheniformis secretes an α-amylase that hydrolyzes starch to predominantly maltopentaose (5) which is hydrolyzed by α-glucosidase to glucose. In this paper we report that the α-amylase is an extracellular enzyme that cannot be detected in the cytoplasm or cell surface but the α-glucosidase is primarily a membrane bound enzyme. Despite

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their different locations, both proteins were synthesized exclusively by membrane bound polysomes and not by soluble polysomes providing strong evidence for cotranslational secretion or insertion of these proteins across, or into the membrane.

MATERIALS AND METHODS

Organism and growth conditions. Bacillus licheniformis NClB 6346 was grown in minimal medium (6) containing $MgCl_2$, $ZnSO_4$, $FeCl_3$ and $CaCl_2$ at 10^{-6} M and 2 mg/ml maltose (Sigma grade 1) in 500 ml indented flasks at 37°C in an orbital incubator. Flasks were inoculated with washed cells from a 16 h culture grown under the same condition but with xylose (2 mg/ml) as carbon source. Growth was followed as absorbance at 570 nm (A_{570}). Protoplasts were prepared by incubating cells at 37°C for 10 min in phosphate buffer (0.1 M, pH 6.5) containing 100 µg/ml lysozyme (Sigma grade 1) and 0.5 M sucrose.

Enzyme assays. The reaction mixture for the α-glucosidase assay contained 2 ml phosphate buffer (0.1 M, pH 6.5), 250 µg p-nitrophenyl-α-D-glucoside and enzyme solution (0.1 ml). The mixture was incubated at 37°C for 20 min, the reaction was stopped by the addition of 1 ml Na_2CO_3 (0.5 M) and the absorbance determined at 420 nm. One unit was defined as the increase of one absorbance reading per ml of enzyme solution under these conditions. α-Amylase was assayed as described previously (7).

In vitro translation. Exponential phase B. licheniformis (A_{570} = 1.0) was poured into frozen buffer (10 mM Tris-HCl, 10 mM magnesium acetate and 60 mM NH_4Cl, pH 7.6). Cells were harvested by centrifugation at 4°C and lysed with lysozyme (200 µg/ml) containing DN'ase (5 µg/ml) at 0°C in the same buffer. Total cell lysis (as determined by phase contrast microscopy) took 4 h but this was reduced considerably by first freezing and thawing the cells in liquid nitrogen. Cell lysate (1 ml) was loaded onto a Sepharose 2B column (1 x 50 cm) equilibrated in the same buffer. Elution was carried out with this buffer, 1 ml fractions were collected.

A 'high speed supernatant' was prepared from Escherichia coli NCTC 8164 grown in the minimal medium supplemented with yeast extract (0.25%). Mid-exponential phase cells (A_{570} = 0.75) were harvested by centrifugation, washed twice with physiological saline and frozen at -20°C. Cells (10 g) were ground in a pre-chilled mortar with 20 g alumina (70-250 mesh) to a semi-fluid paste. The mortar was rinsed twice with 5 ml of the Tris HCl buffer containing 6 mM 2-mercaptoethanol, DN'ase (50 µg/ml) was added and after 20 min at 0°C the debris was removed by centrifugation (3000 g, 5 min, 4°C). The supernatant was centrifuged at 235,000 g for 3 h, the supernatant removed, and dialysed against the same buffer. It was stored in liquid nitrogen.

The reaction mixture (RM) for the in vitro translation was that of Randall and Hardy (8) and contained 0.05µM [^{35}S] methionine (1455 Ci/mmol, Radiochemical Centre, Amersham, UK). Polysome fractions were diluted with buffer to A_{254} = 1.0, 0.5 ml was added to 25 µl high speed supernatant with 0.5 ml RM and incubated at 37°C. At the time intervals, samples (0.1 ml) were precipitated with 5 ml TCA (10% w/v), heated at 80°C for 20 min and filtered on Whatman GF/C glass fibre filters. The filters were washed twice with 10 ml TCA, dried and counted in toluene containing PPO, 3 g and POPOP, 0.3 g per litre. Alternatively samples were immunoprecipitated in 5 ml of 2% Triton X-100, 10 mM Tris HCl pH 7.3, 5 mM EDTA, 0.1 mM phenylmethylsulphonyl fluoride and 50 mM

o-phenanthroline containing 0.025 ml antiserum. Non-radioactive carrier protein, α-amylase or α-glucosidase (5 μg/ml) was added and the mixture incubated at 4°C for 4 h. The precipitate was filtered, washed in the buffer and counted as above.

Preparation of antiserum. α-Amylase and α-glucosidase were purified to homogeneity (9) and contained about 1 mg/ml protein. They were sterilized by filtration, mixed with an equal volume of Freund's adjuvant (Difco) and injected (1 ml) intramuscularly into rabbits. After 14 d a second 1 ml was injected and after a further 14 d the rabbits were bled. Specificity of the antisera was determined by immunodiffusion, there was no cross-reactivity (9).

RESULTS

Localisation of α-amylase and α-glucosidase in Bacillus licheniformis. During exponential growth, B. licheniformis secretes α-amylase into the environment (7) but the enzyme could not be detected in the cytoplasm nor the cell surface of the organism (data not shown). α-Glucosidase, on the other hand, occurred as a tightly membrane bound (or cytoplasmic enzyme) in young cells but, as the culture aged a greater proportion of the enzyme could be released by protoplasting. Finally, as the culture entered stationary phase, α-glucosidase was detected in the culture fluid (Fig. 1). There was some loss of enzyme activity during these experiments, probably due to proteolysis since the rapid decline in total α-glucosidase as stationary phase begins suggests that the enzyme is susceptible to proteinases.

To investigate the association of the α-glucosidase with the cell surface, exponential phase cells (A_{570} = 1.0) were harvested by centrifugation and resuspended in the following aqueous solutions (2.5 mg/ml); Triton X-100, Tween-80, sodium taurocholate, sodium dodecyl sulphate, EDTA, cetyltrimethyl ammonium bromide, NaCl (0.5 M) and distilled water. The suspensions were shaken for 2 min on a vortex mixer, centrifuged, and the supernatant assayed for α-glucosidase. The activity released was related to the total activity from lysed cells assayed under identical conditions. Triton released 7% of the total activity, distilled water 4.5% and NaCl 33%. All other reagents released <1% of the total activity.

In vitro synthesis of α-glucosidase and α-amylase. Sepharose 2B chromatography effected a clean fractionation of cytoplasmic and

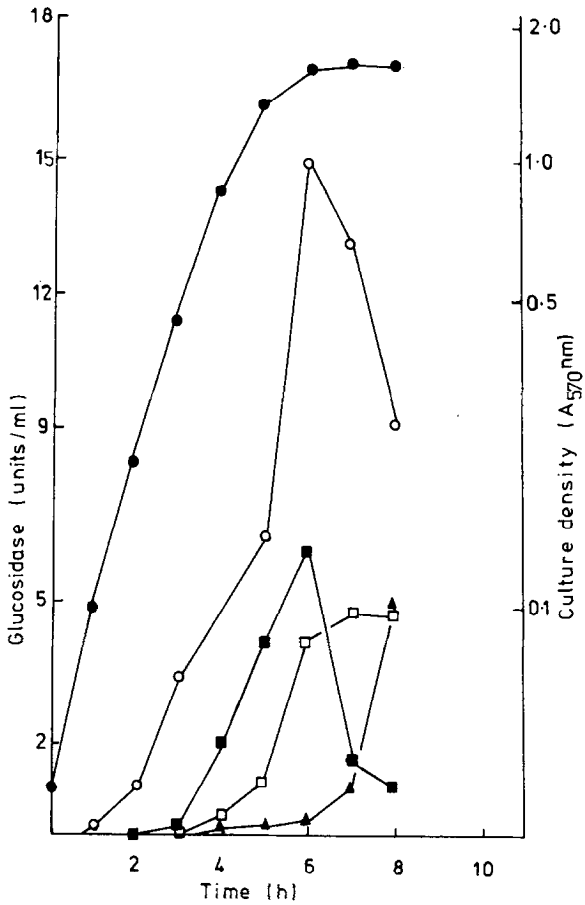

Figure 1. Distribution of α-glucosidase during growth (●) of B. licheniformis. Total enzyme activity (○) was determined after lysis of a culture sample with lysozyme (200 μg/ml) and DN'ase (5 μg/ml) at 37°C for 20 min. A second portion was centrifuged to remove the cells and the supernatant assayed (▲). The cells were protoplasted and centrifuged (12 000 g, 4°C, 10 min) and the supernatant assayed (□). The protoplasts were lysed in phosphate buffer (1 ml, 25 mM containing 5 μg/ml DN'ase) and assayed (■).

membrane bound polysomes (Fig. 2). The first peak from the column contained both [^{3}H] oleic acid and [^{14}C] uracil corresponding to membrane bound polysomes. The second peak was only labelled with [^{14}C] uracil indicating that this comprised the soluble polysomes.

The activity of the polysomes was assessed by estimating the incorporation of [^{35}S] methionine into acid insoluble peptides. There was a steady incorporation of amino acids with time in both systems (Fig. 3). Finally, the synthesis of α-amylase and α-glucosidase by these polysomes was determined by immunoprecipitation of the

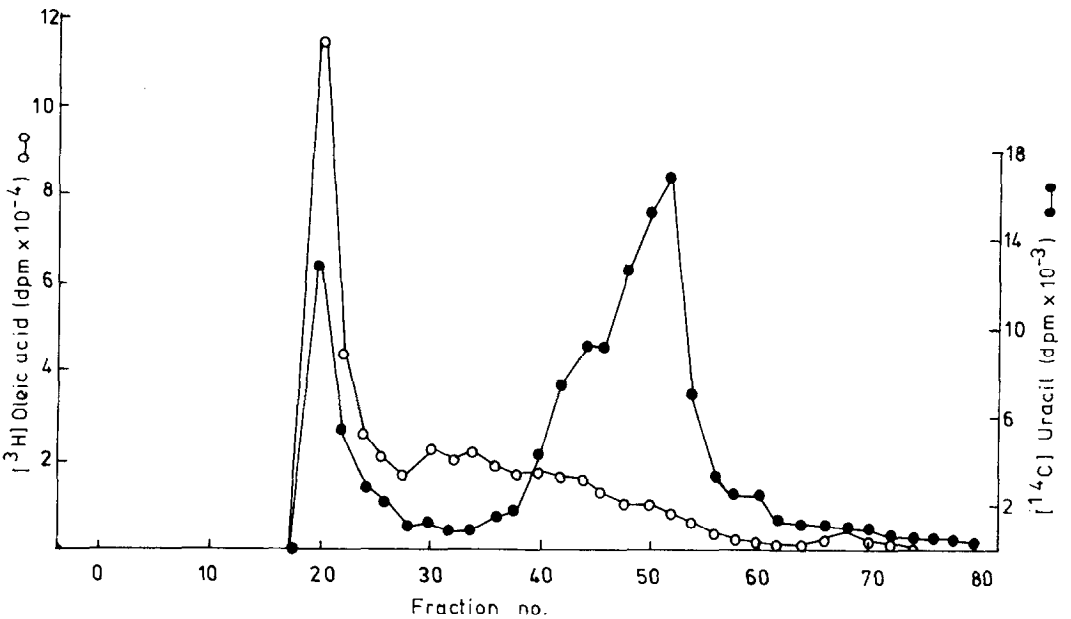

Figure 2. Separation of membrane-bound and cytoplasmic polysomes by Sepharose 2B chromatography. Cells were grown until A_{570} = 0.4 and [^{3}H] oleic acid (0.175 µM, 1.0 µCi/ml) and [^{14}C] uracil (1.5 µM, 0.087 µCi/ml) were added. At A_{570}= 1.0 cells were harvested and lysed as described in Materials and Methods. Fractions (1 ml) from the column were precipitated in 5 ml of 10% TCA for 30 min, filtered and counted.

products. Both α-amylase and α-glucosidase were synthesized exclusively by membrane-bound polysomes; there was negligible synthesis by the soluble polysomes.

DISCUSSION

The location of α-glucosidase in bacilli varies according to the strain. In *B. cereus* it is intracellular (10), in *B. subtilis* (11) and *B. brevis* (12) it is largely extracellular, and in *B.amyloliquefaciens* it is cell bound and can be released by washing cells with detergents (13). In some thermophilic bacilli it is probably cytoplasmic during exponential growth but subsequently secreted (3,4). Assignment of an enzyme to a particular location is notoriously difficult but our data suggest that in young cells of *B. licheniformis* NCIB 6346 α-glucosidase is either tightly cell bound or possibly cytoplasmic. Since NaCl removed much of this enzyme it is presumably attached to the membrane and the NaCl acted either by ionic interaction or

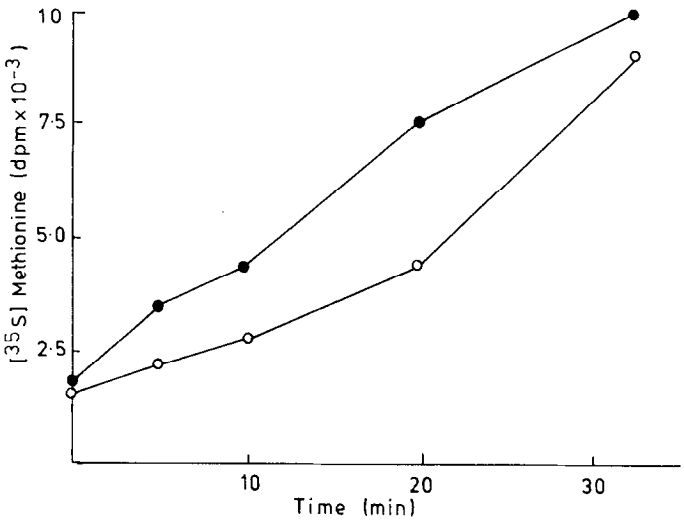

Figure 3. Incorporation of [^{35}S] methionine into acid-insoluble peptides by membrane-bound (●) and soluble (○) polysomes from Bacillus licheniformis.

by activation of a specific proteinase. In older cells, however, α-glucosidase was less tightly bound or the proteinase more effective since removal of the cell wall released much of the enzyme. This system is reminiscent of the penicillinase of B. licheniformis which exists as membrane bound and extracellular proteins (14). Here, the

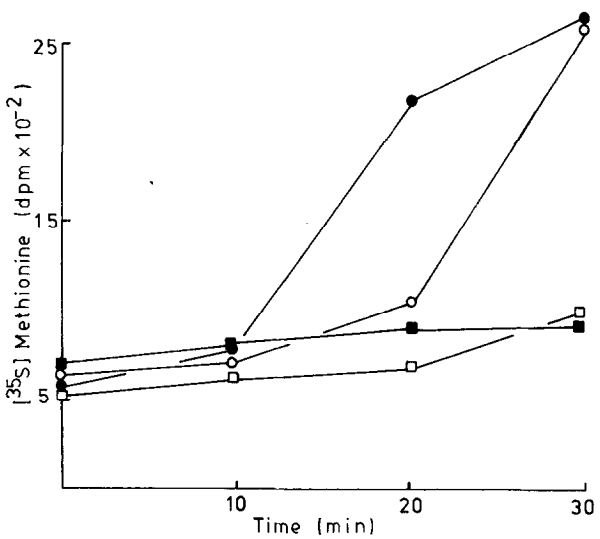

Figure 4. Incorporation of [^{35}S] methionine into α-glucosidase (solid symbols) and α-amylase (open symbols) by membrane bound (circles) and soluble (squares) polysomes.

membrane bound form contains covalently linked glyceride and can be removed by protoplasting, washing cells with sodium deoxycholate or by a specific protease (14,15). We have no evidence for covalently linked lipid in the membrane bound form of α-glucosidase (9).

Despite the different locations of α-amylase and α-glucosidase in B. licheniformis both enzymes were synthesized exclusively by membrane bound polysomes. This is strong evidence for cotranslational secretion of these proteins and indicates that α-glucosidase does not accumulate in the cytoplasm, as it does in some thermophilic bacilli (3,4) but is cotranslationally inserted into the membrane and subsequently released. The signal peptides for α-amylase and α-glucosidase must therefore differ, the former promoting efficient and rapid secretion and the latter containing a 'stop' sequence. This therefore provides an ideal system for analysis of the molecular details of protein localization in a Gram positive bacterium.

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