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Synthesis and secretion of bacterial α-amylase by the yeast *Saccharomyces cerevisiae*

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α-Amylase from *Bacillus amyloliquefaciens*, synthesized in yeast *Saccharomyces cerevisiae* without substitution of the signal sequence, is efficiently secreted from yeast cells: 60–70% of the overall amount of the enzyme is found in the culture fluid. In contrast to many yeast secretory proteins, which accumulate in the periplasmic space and in the cell wall, intracellular α-amylase is localized mainly in the cytoplasm. Obviously, transfer across the cell wall is not a rate-limiting step in α-amylase export from the cell. The glycosylated forms of proteins are predominantly found both inside the cell and in the culture medium.

Amylase, α-; Yeast; Secretion; Glycosylation; Bacteria

1. INTRODUCTION

Recently attempts were made to obtain yeast strains with a starch-degrading ability. Genes of α-amylases from different sources (wheat [1], murine [2], *Bacillus amyloliquefaciens* [3,4] and human [5]) have been expressed in *Saccharomyces cerevisiae*.

It is known that foreign protein secretion from yeast is restricted for at least two reasons: (i) incomplete correspondence between the natural signal peptides and the yeast transport system [6]; (ii) low permeability of the yeast cell wall [7]. To overcome these restrictions, some secretory signal peptides of yeast origin are usually substituted for natural signal sequences of foreign proteins [8,9].

The peculiarity of α-amylase genes expression in yeast consists in the effective secretion of enzymatically active proteins into the culture medium without substitution of secretion signals. As much as 60–70% of the overall synthesized enzyme can be secreted [2,5]. To explain this phenomenon, several questions ought to be answered such as: what is the pathway of the foreign secretory protein inside the yeast cell? Is the level of secretion dependent on intracellular modifications of the transferred protein? And so forth.

Analysis of glycosylated forms of *B. amyloliquefaciens* α-amylase in yeast and the results of experiments with secretion-defective mutants of *S. cerevisiae* [4] showed that the transfer of the bacterial protein proceeds by a pathway usual for yeast protein secretion [10].

This work deals with the rate-limiting step in secretion of *B. amyloliquefaciens* α-amylase by yeast and with glycosylation of the bacterial enzyme in yeast cells.

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2. MATERIALS AND METHODS

The strain of yeast *S. cerevisiae* (AH-22-pYT3/203), containing the gene of *B. amyloliquefaciens* α-amylase with its own signal sequence in the vector plasmid, and construction of the plasmid were described in detail in [11].

Yeast cells were grown at 30°C in a minimal medium [3], containing 40 mg/l histidine. Amylolytic activity was assayed by the chromogenic method using amylopectin azure as described in [12]. Cell extracts were prepared by vortexing yeast cells with glass beads and subsequent removal of the debris [3]. Yeast

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spheroplasts were prepared by treating the cells with Zymolyase as performed in [13]. Spheroplasts were lysed in the presence of 0.2% Triton X-100. For preparation of anti-α-amylase serum, rabbits were immunized with commercial *B. amyloliquefaciens* α-amylase (Sigma A-6380). Immunoblotting was performed as described in [14], using antibodies purified by immuno-affinity chromatography. The sorbent was obtained by immobilization of commercial α-amylase on cyanogen bromide-activated Sepharose 4B. The conjugate of anti-rabbit antibodies with peroxidase (Sigma P-8250) was prepared according to [15]. Chromatography on concanavalin A-agarose was performed as in [16]. Samples of the culture fluid were treated with endoglycosidase H (Sigma E0636) as described in [17]. SDS-PAGE was performed as in [18] and PAGE under nondenaturating conditions, as in [19]. α-Amylase activity in PAGE was assayed according to the procedure described in [20].

3. RESULTS AND DISCUSSION

It was reported elsewhere that the yeast *S. cerevisiae*, transformed with a plasmid containing the gene of *B. amyloliquefaciens* α-amylase with its natural secretion signal, produced bacterial enzyme and effectively secreted it into the culture fluid. After growth on a starch-containing solid medium and subsequent staining with I_2-KI solution, areas of clearing could be detected around the yeast colonies. Protein, active both in enzymatic and immunological respects, was found in the culture medium [3].

In contrast to the majority of yeast secretory proteins, bacterial α-amylase is efficiently transferred across the cell wall. The data on the distribution of the active enzyme between the culture medium and yeast cells are shown in table 1. More than 60% of the overall amount of the active enzyme was found in the culture medium. Only insignificant α-amylase activity was found in the cell wall (the data for whole cells are given in table 1) and in the periplasm (table 2). In contrast, spheroplasts lysed by 0.2% Triton X-100 displayed a high activity (table 2). Such localization of the α-amylase suggests that the rate-limiting step in enzyme secretion precedes its transfer across the cell wall.

Table 1

Distribution of bacterial α-amylase between the culture medium and the yeast cells

Sample	α-Amylase activity[a] (units/absorbance of cells at 600 nm)	% of total activity
Whole cells	0.095	5
Cell extracts	0.570	30
Culture fluid	1.240	65

[a] Enzyme units as described in [12]

Table 2

α-Amylase content in the periplasmic space and yeast spheroplasts

Sample	α-Amylase activity[a] (units/absorbance of cells at 600 nm)
Periplasmic space[b]	0.048
Spheroplasts	0.370

[a] Enzyme units, as described in [12]

[b] Yeast cells were treated with Zymolyase and centrifuged at 1000 × *g* for 10 min. α-Amylase activity was assayed in the post-spheroplastal supernatant

Spheroplasts were lysed in 50 mM phosphate-citrate buffer (pH 7.0) with 0.2% Triton X-100

Further information about secretion of bacterial α-amylase in yeast was obtained after analysis of glycosylated forms of the enzyme inside and outside the cell. Four forms of the enzyme with different gel mobilities were observed after SDS-PAGE and subsequent immunoblotting of the culture medium. One of them (55 kDa) was identical in size with the commercial *B. amyloliquefaciens* α-amylase. Molecular masses of other forms were 59, 62 and 64 kDa, the most abundant was the heaviest form (fig.1).

Bacterial α-amylase is not glycosylated originally, but has four potential sites for glycosylation [4]. Proceeding from the estimated molecular masses of different enzyme forms and assuming the molecular mass of one core oligosaccharide unit to be equal to 2–3 kDa, one can suggest that the heavier forms result from core glycosylation of original proteins of two, three and four sites.

For identification of glycosylated forms, samples of the culture medium were analyzed by affinity chromatography on concanavalin A-agarose. Some portion of the active enzyme (60% of activity) appeared to be bound by the sorbent and was then eluted with α-methyl-D-mannoside, while some portion remained unbound. Immunoblotting of a fraction obtained after α-methyl-D-mannoside elution showed three bands with gel mobilities slower than wild-type α-

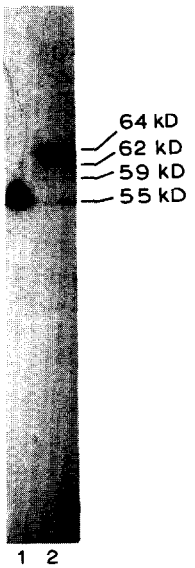

Fig.1. Immunoblotting of molecular forms of secreted bacterial α-amylase in the yeast culture medium. Lanes: 1, commercial α-amylase from *B. amyloliquefaciens*; 2, α-amylase secreted by yeast.

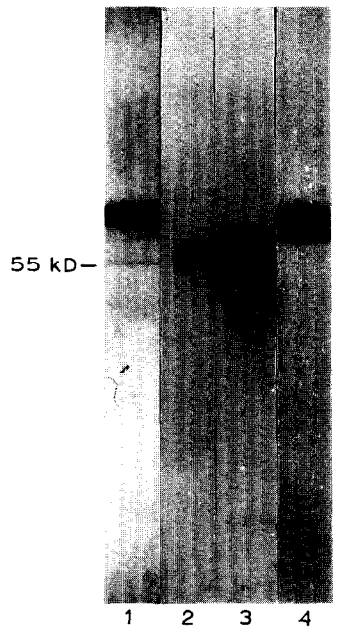

Fig.2. Separation of glycosylated forms of α-amylase. After chromatography on concanavalin A-agarose, fractions were concentrated by lyophilization, subjected to SDS-PAGE and immunoblotted. Lanes: 1, nontreated culture medium; 2, commercial α-amylase; 3, fraction not bound to concanavalin A-agarose; 4, fraction eluted with α-methyl-D-mannoside.

amylase. Only one band was observed in the unbound fraction, and this band corresponded to the nonglycosylated bacterial enzyme (fig.2). Thus, the heavier forms of α-amylase with affinity for concanavalin A can be considered as glycoproteins.

This conclusion is confirmed by endoglycosidase H treatment of the culture medium aliquots. Subsequent immunoblotting showed that the treatment resulted in a decrease of intensity of the band with the slowest mobility (heavily glycosylated form) and in an increase in the intensities of other bands (fig.3).

The presence of a form with a molecular mass identical to that of the wild-type α-amylase is obviously a result of signal sequence cleavage in or near the proper site.

The same pattern of α-amylase molecular forms was observed in cell homogenates after immunoprecipitation with anti-α-amylase serum and SDS-PAGE. Experiments with concanavalin A-agarose showed that the major portion of α-amylase inside the cell is glycosylated. After electrophoresis under nondenaturing conditions and subsequent identification of active bands mainly glycosylated forms were observed which had similar mobilities with those in the culture medium (fig.4). These data suggest that glycosylated bacterial α-amylase retains its enzymatic activity. Both after immunoblotting and identification of active bands in the gels the nonglycosylated form was observed not in all the samples, obviously as a result of its low content.

Usually yeast secretory proteins are subjected to further glycosylation in Golgi, resulting in a significant increase in their molecular mass [22]. Such fully glycosylated form was not observed in yeast; obviously α-amylase is subjected only to core glycosylation in the endoplasmic reticulum. So, according to the data in the literature, α-amylases of bacterial [3,4], plant [1] and human [5] origin are synthesized in yeast and efficiently secreted into the culture fluid owing to their native secretory signals. It was shown that signal peptides are correctly cleaved [4,5]. Human [5] and bacterial [4] amylases are subjected to core glycosylation. These observations are in agreement with our results here. Figs 1 and 3 show that the nonglycosylated form is also secreted, although in

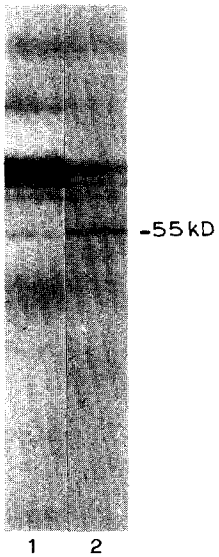

Fig.3. Immunoblotting of α-amylase in the yeast culture medium treated with endoglycosidase H. Lanes: 1, control; 2, treated with endoglycosidase H. SDS and β-mercaptoethanol were added to 40 μl samples of the concentrated culture fluid to final concentrations of 1% and 0.2 M, respectively, followed by 5 min boiling. Then the samples were diluted to 400 μl and their treatment by endoglycosidase H (5 mE) was carried out at 37°C for 24 h in the presence of 0.3 M sodium citrate (pH 5.8) and 50 mM PMSF.

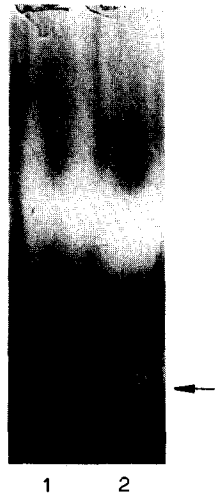

Fig.4. Identification of bands displaying amylolytic activity in PAGE after electrophoresis under nondenaturing conditions. Lanes: 1, culture medium; 2, cell extract. The arrow indicates the position of commercial α-amylase.

[4] secretion of the nonglycosylated form was observed only in the presence of tunicamycin.

Surprisingly, transfer across the cell wall is not a rate-limiting step in α-amylase secretion, which contradicts the data on the majority of yeast [7] and foreign [8] secretory proteins, usually accumulating in the periplasm and in the cell wall.

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