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Induction of Glucoamylasen in the Yeast *Candida tsukubaensis*

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Abstract: The induction of glucoamylase biosynthesis from the yeast *Candida tsukubaensis* by different carbon sources was investigated by using either an enzyme activity assay or immunoblot analysis. The induction by *C. tsukubaensis* appears to be independent of the carbon sources, although the level of enzyme activity was lower in slowly utilizable carbon sources such as galactose. This glucoamylase is a constitutive enzyme and its biosynthesis is resistant to carbon catabolite repression. Glucose was more effective for the enzyme induction than starch, maltose or glycerol. In addition, this enzyme is regulated by both induction and repression.

Key words: *Candida tsukubaensis*, catabolite repression, glucoamylase, induction.

Glucoamylase catalyzes the liberation of glucose from the non-reducing endsof starch or dextrins. The latter two and/or maltose induce glucoamylase biosynthesis whereas glucose or other easily utilizable monosaccharides repress its formation. Such a carbon catabolite repression, or glucose repression, is an important and global regulatory system in both prokaryotic and eukaryotic cells. Glucoamylase induction in different microorganisms varies greatly. Soluble starch and/or maltose induces glucoamylase synthesis in different microorganisms such as *Aspergillus niger* (Barton *et al.*, 1972), *Aspergillus terrus* (Ventura *et al.*, 1995), Schwanniomyces occidentalis (Dowhanick *et al.*, 1988), and *Clostridium thermohydrosulfuricum* (Hyun and Zeikus, 1985). However, the former disaccharide has been reported to repress glucoamylase formation in *Rhizopus chinensis* (Wang, 1988). On the other hand, Enseley *et al.* (1975) have reported that *Clostridium acetobutylicum* glucoamylase was induced by glucose but not by starch or maltose.

Good starch degradation and amylase secretion have been obtained with *Candida tsukubaeansis* (DeMot *et al.*, 1984a). The purification and characterization of glucoamylase from this yeast has been reported by DeMot *et al.* (1985) and Kim *et al.*(1992). However, information concerning induction of the enzyme is limited. We have examined the induction of glucoamylase biosynthesis in *C. tsukubaensis* with different carbon sources.

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Materials and Methods

Microorganism and enzyme induction

The yeast used was *Candida tsukubaensis* CBS6389 obtained from the Centraal Bureau voor Schimmelcultres (Yeast Division, Delft, The Netherlands). The basal medium for enzyme induction consisted of 0.67% yeast nitrogen base (Difco, Detroit, USA) in 75 mM sodium diphosphate-tartaric acid buffer (pH 5.5; DeMot *et al.*, 1985). The yeast was grown in the basal medium containing 0.5% glucose or 1% glycerol at 30℃ for 48 h on a gyratory shaker (125 rpm). The glucose or glycerol-grown cells were washed twice with sterile water. The washed cells were inoculated into 2-l Erhlenmyer flasks containing 200 ml of the above medium supplemented with 0.5 to 2% of different carbon sources and grown up to 2 or 3 days. The final cell concentration of inoculum showed an absorbance at 650 nm (A_{650}) of 0.25. For the initial formation of enzyme, yeast cells were grown in 100 ml basal medium supplemented with 1.0% glycerol up to 40 h under the culture conditions already described. After 40 h incubation, cells were harvested and washed three times to remove any remaining glycerol. The resultant cells were resuspended in 50 ml of fresh medium supplied with glucose, maltose or starch at a concentration of 1.0% to give an A_{650} reading of about 5.3, and grown up to 30 h. In all cases, the time course of growth and enzyme induction was determined by periodical sampling of the growth medium. The culture samples (10 ml) were centrifuged at 8000 rpm for 5 min. For immunoblot anal-

ysis, the resultant supernatants were dialyzed exhaustively against 75 mM disodium tartaric acid (pH 5.5) and concentrated by ultrafiltration and/or freezed drying 20-fold for the overall formation of enzyme and 50-fold for its initial synthesis. The remaining cell pellets were disolved in a buffer consisting of 50 mM Tris (pH 7.5), 5 mM EDTA (pH 8.0), 150 mM NaCl, 0.05% Nonidet P-40 and 20 mM phenylmethylsulfonyl fluoride (DoWhanick *et al.*, 1988). The cellular proteins were extracted from the pellets, which had been immediately frozen in liquid nitrogen, by vortexing an equal volume of cell pellets and glass beads (0.2 diameter) at high speed for 10 min, and then centrifuged at 15000 rpm for 5 min. Extra- and cellular supernatants prepared were stored at 4℃ until enzyme assay or immunoblotting.

Enzyme purification and Western immunoblot

The *C. tsukubaensis* glucoamylase was purified by using ultrafiltration, Sephacryl gel filtration and SP-Sephadex cation ion exchange chromatography (Parmacia; Upspsala, Sweden). The molecular weight of the purified enzyme was approximately 50 kDa, estimated by sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE) which was the same size as that purified by Kim *et al.* (1992) but lower than the 55kDa obtained by DeMot *et al.* (1985). A polyclonal antibody to 50 kDa enzyme was prepared as described elsewhere (Jonston and Thorpe, 1987). Each 40 μl amount of extra and cellular samples was boiled for 1 min with a half volume of 3 x Laemmli buffer and then subject to SDS-PAGE and immunoblotting as described by Kubicek *et al.* (1987) using peroxidase conjugated rabbit secondary antibody.

Analytical methods

Growth was monitored by measuring the absorbance of the cultures at 650 nm on a Spectronic 20 spectrophotometer. Residual soluble starch in the culture medium was determined by an iodine coloration method (DeMot *et al.*, 1984b). Glucoamylase was assayed by determining the amount of the glucose liberated from soluble starch. The substrate solution consisted of 0.5% soluble starch in 50 mM McIlvaine buffer (pH 5.4). The substrate (990 μl) and enzyme solutions (10 μl) were mixed and incubated at 55℃ for 30 min. The reaction was stopped by boiling for 5 min, and the glucose concentration in either reaction mixture or all culture filtrates was assayed by the peroxidase-glucose oxidase method with a commercial kit (Sigma; St. Louis, USA; Technical Bulletin, NO. 510). One glucoamylase unit is defined as the amount of enzyme producing 1 mol of glucose/min. Protein concentration was estimated according to the method of Lowry *et al.* (1951) with bovine serum albumin as a standard. All assays were repeated at least three times with results not varying by greater than 10%.

Results and Discussion

We have made an attempt to induce glucoamylase biosynthesis by adding different carbon sources to the basal medium. When compared with both enzyme activity and growth on different carbon sources, the activity of extracellular glucoamylase was maximum with glucose, followed by maltose and soluble starch, and was also detectable in slowly utilizable substrates such as glycerol, raffinose, sucrose, cellobiose, or lactose (Table 1). The induction of *C. tsukubaensis* glucoamylase measured by enzyme activity appears to be independent of the carbon source. This result was similar to that of α-glucosidase obtained from *Saccharomyces cerevisiae* harboring the *C. tsukubaensis* α-glucosidase gene (Kinsella and Cantwell, 1991). An increased level of glucoamylase activity in *C. tsukubaensis* was, however, observed when glucose rather than other carbohydrates was the sole carbon source in the growth medium.

When comparison of glucoamylase formation on glucose, glycerol, maltose and starch was made by either enzyme assay or immunoblot analysis (Fig. 1), the concentration required to obtain a maximal induction by these carbon sources was approximately 1% (wt/vol)

Table 1. Effect of different carbon sources on the induction of glucoamylase in *Candida tsukubaensis*

Carbon source	Concentration[a] (%[wt/vol])	Growth[b] (A_{650} nm)	Glucoamylase (U/ml)	Ratio[c] (U/ml to A_{650})
Glucose	1.0	7.0(36)	3.0	0.43
Fructose	2.0	6.6(42)	1.5	0.23
Galactose	0.5	3.0(72)	0.2	0.06
Maltose	1.0	7.2(36)	1.9	0.26
Lactose	2.0	9.4(60)	2.1	0.22
Cellobiose	0.5	3.5(72)	1.5	0.42
Sucrose	1.0	3.9(60)	1.2	0.31
Xylose	0.5	3.0(42)	0.7	0.23
Raffinose	4.0	6.2(66)	2.1	0.34
Soluble starch	1.0	6.2(42)	1.8	0.29
Glycerol	1.0	10 (72)	2.0	0.18

[a]Substrate concentration required to reach the highest activity of enzyme.
[b]The value in parenthesis is the incubation time (h) required.
[c]Calculated ratio between enzyme activity and cell concentration (A_{650}) in the culture fluids.

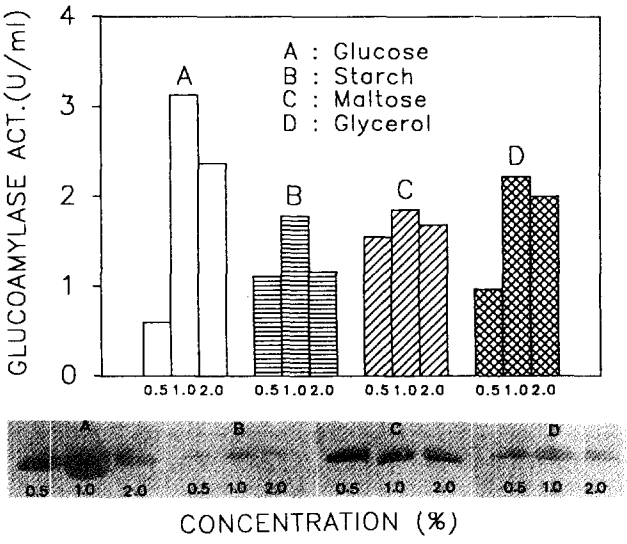

Fig. 1. Comparison of glucoamylase product during growth on glucose (A), starch (B), maltose (C) and glycerol (D) at different concentration (0.5, 1.0 and 2.0%). Enzyme product was subject to SDS-PAGE and proved with antiserum raised against to 50 kDa glucoamylase. The supernatant sampled from the highest enzyme activity at at each concentration of carbon source was 20-fold concentrated and resultant sample (40 μl) was applied to each lanes.

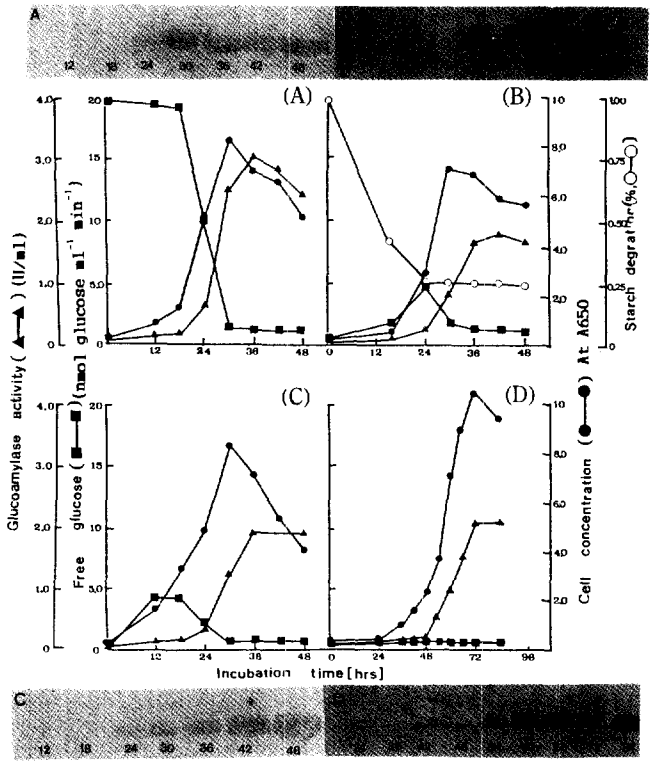

Fig. 2. Time course of growth, starch degradation and extracellular glucoamylase synthesis by *Candida tsukubaensis*, and glucose concentration in the culture. The immunoblot can be deduced from the superimposed photographs. Yeast was grown in 1% glucose (A), 1% starch (B), 1% maltose (C) and 1% glycerol (D). Ten ml of supernatant was 20-fold concentrated and resultant samples (40 μl) was applied to each lane.

but beyond these concentrations glucoamylase formation showed a lower level, suggesting that enzyme synthesis is regulated by both induction and repression as has been observed in the cellulase formation of *Trichoderma reseei* (Coughlan, 1985) and *Aspergillus terreus* (Ali and Sayed, 1992).

The experiment shown in Fig. 2 was carried out to examine the growth and extracellular enzyme induction of *C. tuskubaensis* on glucose, glycerol, maltose and starch in more detail. The rationale behind their use was that glucose is commonly believed to repress an amylolytic enzyme but starch and/or maltose to induce it. Glycerol is included because it is used as a respiratory substrate. Growth on glucose, maltose and soluble starch reached a maximum level at 36 h, and thereafter the cultures rapidly switched to autolysis. Growth on glycerol was very slow and required 72 h for the maximum level. Such a slow growth on glycerol could be due to slow transport of it into the cell (Gancedo *et al.*, 1968) or, in part, due to the prior induction of enzyme required for the utilization of glycerol (Ganced and Serrano, 1989) which could cause a long lag to induce glucoamylase. The overall extracellular enzyme synthesis increased concomittantly with cell densities and reached the maximum level at or after the end of exponential growth, as has been observed in *Schwanniomyces alluvius* (Wilson *et al.*, 1982). Regardless of carbon sources, except for glycerol, the rapid increase in either glucoamylase synthesis or growth was accompanied by the corresponding reduction of glucose in the culture medium, indicating the correlation of enzyme formation with the utilization of glucose or cell growth. *C. tsukubaensis* produced a trace amount of α-amylase (DeMot *et al.*, 1985). As shown in Fig. 2B, the approximately 25% dextrin remained constant from 24 to 48 h, reflecting a trace amount of α-amylase production by *C. tsukubaensis*. Growth on starch rather than on glucose resulted in lower enzyme activity. The low level of enzyme accompaning growth on starch may be, in part, due to the inherent low production of α-amylase by *C. tsukubaensis*, the action of which could release inducer molecules from starch hydrolysis (Forgaty and Kelly, 1980). Another possibility for the low enzyme production on starch could be the greater adsorption on the remaining dextrin (Ensley *et al.*, 1972).

Cellular glucoamylase, on the other hand, was also detectable, albeit at very low level, during growth on 1% glucose, maltose, and starch, and showed similarity to the pattern of enzyme activity obtained from the culture fluid (Fig. 3). When calculated as the percentage of the highest total activity, glucoamylase activities on

Table 2. The comparison of localization of glucoamylase activity in *C. tsukubaensis* with different carbon sources

Carbohydrate source	Total[a] activity	% of cell fraction activity[b]	
		Cellular	Extracellular
Glucose	3.8	20	80
Maltose	2.6	29	71
Starch	2.2	19	81
Glycerol	4.1	46	54

[a]Total activity equals the sum of cellular and extracellular activity (μ/ml). This was determined at the highest activity produced.
[b]Represents cell fraction activity ×100/total activity.

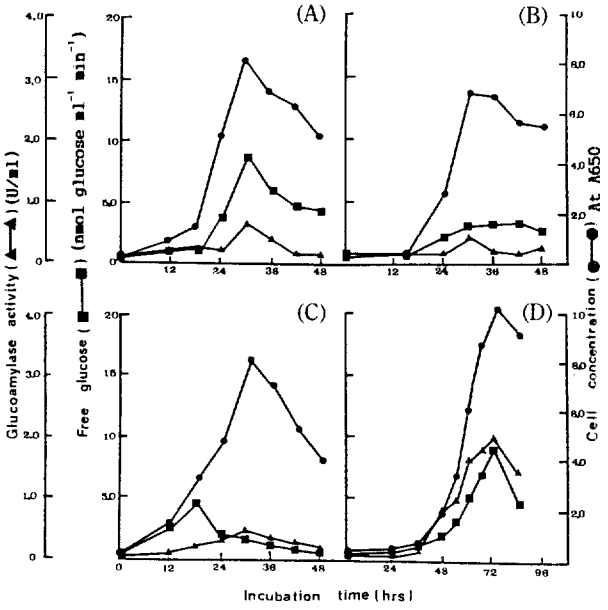

Fig. 3. Time course of growth, cellular glucoamylase activity and free glucose by *C. tsukubaensis*. This experiment was carried out as described in Fig. 1.

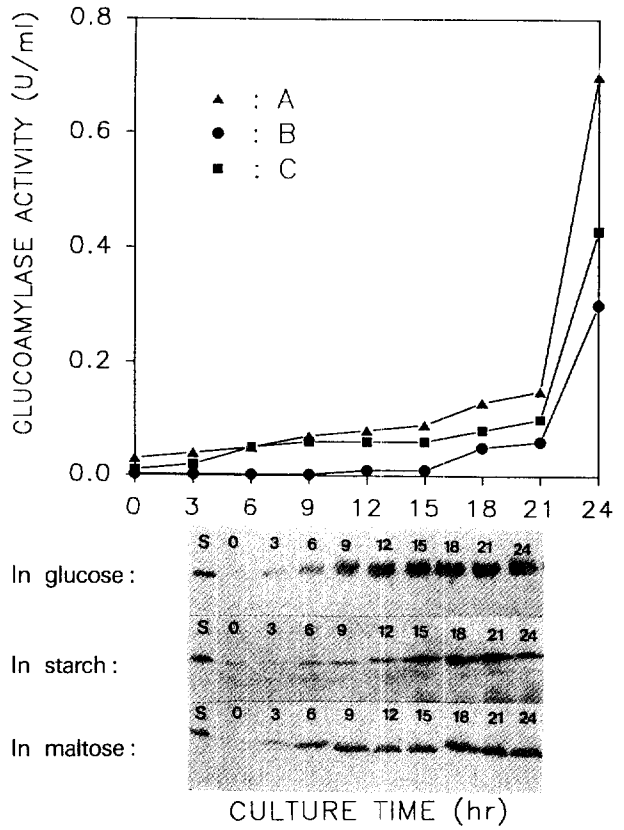

Fig. 4. Initial production of glucoamylase during growth on glucose (A) and starch (B) and maltose (C). *C. tsukubaensis* was grown on 1% glycerol upto 40 h and used as starter culture for enzyme production. Enzyme protein was subject to by SDS-PAGE and proved with antiserum raised against 50 kDa glucoamylase. S indicate the immunoblots of purified glucoamylase (1 μg). See Materials and Methods for details.

glucose, maltose, starch and glycerol were 80, 71, 81 and 53% extracellular, respectively (Table 2). The result indicates that *C. tsukubaensis* glucoamylase is primarily extracellular. However, the cellular enzyme activity from glycerol-grown cell maintained almost the same level as the extracellular throughout 96 h incubation (Fig. 3D). Gosh *et al.* (1990) reported that *T. reesei* grown in the presence of glycerol alone or with avicel did not increase the formation of endoplasmic reticulum capable of playing an important role in the synthesis of secretory proteins. This fungus grown in the presence of lactose or cellulose, on the other hand, has been known to relieve the secretion of cellobiohydrolase, probably by the derepression of glycosylation (Messner and Kubicek, 1991). The limited secretion of *C. tsukubaensis* glucoamylase by glycerol could be, in part, due to either parameters described above or other unknown factors such as protein malfolding (Romans *et al.*, 1992). However, the actual mechanism which limits the secretion of enzyme into the culture fluid by glycerol-grown cells remains to be solved.

To determine the initial production of enzyme, yeast cells pre-grown in 1.0% glycerol medium for 40 h were inoculated into a fresh medium containing different carbon sources with a A_{650} reading of 5.0 and grown up to 24 h. As shown in Fig. 4, the immunoblot band of the extracellular glucoamylase, initially found after at least 3 h of growth in glucose or maltose medium, reached the maximum level at 9 h and thereafter maintained a similar level. In contrast, the corresponding bands from starch medium were first detected at about 9 h, reached the maximum at 18 h and after 24 h slightly decreased. This result indicated that enzyme induction occurs much faster and at a higher level in glucose than in starch, ruling out the possibility that the low level of glucoamylase formation obtained from the early culture (Fig. 2) is due to glucose repression. On the other hand, Reddy and Abouzied (1986) have

reported that in *Aspergillus* spp. glucose did not repress amylase synthesis but inhibited its activity. We have examined the possibility of the inhibition of enzyme activity by glucose (ca. 0.9%) present in the early culture (12 and 18 h in Fig. 2) of glucose grown cells. No discernable difference in enzyme activity between the dialyzable and nondializable supernatants in early cultures was found (data not shown), indicating the absence of feedback inhibition of enzyme by glucose.

Comparison of glucoamylase formation on four carbon sources revealed that *C. tsukubaensis* may produce even more enzyme by glucose than by other carbon sources. This result is in contrast to previous reports that glucose exerts a strong catabolite repression of glucoamylase synthesis in yeasts, fungi and bacteria such as *Schwanniomyces occidentalis* (Dowhanaick *et al.*, 1988; 1990; Horn *et al.*, 1988), *Lipomyces* species (Horn *et al.*, 1988), *Rhizopus chinensis* (Wang, 1988), *Aspergillus oryzae* (Wang, 1988), *Aspergillus terrus* (Ventura *et al.*, 1995), and *Clostridium thermohydrosulfuricum* (Hyun and Zeikus, 1985). On the other hand, Ensely *et al.* (1975) have reported that *Clostridium acetobutylicum* glucoamylase was induced by glucose but not by starch. However, the concentration of glucose they used for glucoamylase induction was below 0.4%. Such a low concentration of glucose has been reported to stimulate glucoamylase formation in *Aspergillus niger* (Reddy and Abouzied, 1986). In addition, the enzyme formation during growth on glucose and glycerol as a sole carbon source suggests a constitutive nature of *C. tsukubaensis* glucoamylase as has been observed in α-amylases obtained from fungus (Cruger and Cruger, 1989) and *Bacillus thuringensis* (Tobey *et al.*, 1977).

The combined results indicated that *C. tsukubaensis* glucoamylase is a constitutive enzyme and resistant to carbon catabolite repression. This enzyme synthesis is also regulated by both induction and repression. However, it is not clear whether the regulation take place at the level of the gene (Flick and Johnston, 1990; Rygus and Hillon, 1992; Kinsella and Cantwell, 1991) or transcription-translation (Innis *et al.*, 1985; Dowhanaick *et al.*, 1990; Lombardo *et al.*, 1992). Further studies at the molecular level are needed to gain a better understanding of the insensitivity of *C. tsukubaensis* glucoamyalse to glucose repression. Experiments are underway to isolate the glucoamylase gene from genome DNA of this yeast.

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