EVIDENCE FOR THE PERIPLASMIC LOCATION OF GLYCOGEN IN SACCHAROMYCES

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SUMMARY: Treatment of yeast cells with hot alkali fails to solubilize a significant amount of glycogen. The insoluble glycogen is readily hydrolyzed by insolubilized α-amylase indicating that the apparent insolubility of this glycogen fraction does not result from its physical entrapment within an insoluble glucan membrane. The alkali-insoluble glycogen fraction of glutaraldehyde treated-cells is rapidly degraded by a mixture of snail gut enzymes during the formation of spheroplasts but the alkali-soluble glycogen fraction is unaffected. These results indicate that a major fraction of yeast glycogen is located outside the cellular membrane.

A water-soluble glycogen fraction is readily extracted from Saccharomyces cells under alkaline conditons, but extraction of the major glycogen fraction from the insoluble cellular debris requires repeated treatments under acidic conditions at high temperatures (1). Ling et al (2) and Trevelyan et al (3) suggested that yeast cells contain two glycogen components, one of which is bound to insoluble cell wall material. Northcote (4, 5) claimed that the apparent insolubility of the major glycogen fraction results from its physical entrapment within an insoluble glucan membrane and that all cellular glycogen is released as a single component by acidic or mechanical disruption of this membrane. In recent studies in our laboratory, however, the yields of glycogen obtained in the soluble and insoluble fractions after mechanical disruption of yeast cells were identical to those obtained after alkali treatment (6). The report of a glycogen fraction associated with the cell wall of Dictyostelium discoideum (7) and the several reports of amylaceous polysaccharide in the cell walls of fungi (see ref. 8) made it of interest to undertake the studies described in this report on the location of the insoluble glycogen fraction in Saccharomyces.

MATERIALS AND METHODS

Rabbit liver and shellfish glycogen, glutaraldehyde (25% in water)

and Bacillus subtilis α-amylase (EC.3.2.1.3) were purchased from Sigma Chemical Company. Aspergillus niger glucoamylase (EC.3.2.1.3) was prepared by the method of Fleming and Stone (9), the second enzyme peak from the DEAE-cellulose fractionation being used. Glusulase was purchased from Endo Laboratories Inc.

Glycogen contents of solutions or suspensions were determined by incubating them with a mixture of glucoamylase and α-amylase and measuring the total conversion into glucose using the glucose oxidase method (EC.1.1.3.4) (10). Reducing sugars were determined as by Robyt and Whelan (11).

Saccharomyces cerevisiae was one of several yeast strains kindly provided by Dr. F.J. Roth of the Department of Microbiology, University of Miami, School of Medicine. The cells were grown with shaking at 22° for 18 hr in liquid media containing 1% glucose, 0.5% Bacto peptone, 0.3% Difco yeast extract and 0.3% Difco malt extract. Cells were collected by centrifugation at 5,000 X g and washed three times with water (4°). Cells were fixed by suspending them in 1.5% glutaraldehyde solution at 22°. After treatment for 5 min, excess glutaraldehyde was removed by washing the cells with water. Spheroplasts were formed from glutaraldehyde-treated and untreated cells by the method of Duell et al. (12) in which the cells were preincubated with 140 mM 2-mercaptoethanol and 40 mM EDTA before treatment with a mixture of snail gut enzymes (Glusulase) in the presence of 0.72M glucitol.

For determination of glycogen content, cells were heated at 100° for 60 min in 20% KOH solution (about 0.05-0.40 g wet cells/ml); the cooled suspension was adjusted to pH 7 with 5N HCl and two volumes of 95% ethanol were added. After centrifugation the sedimented pellet was washed three times with 66% ethanol, extracted three times with warm water (1 ml) and resuspended in water (2 ml). The water extract and the pellet suspension were used to determine glycogen in the soluble and insoluble fractions respectively.

RESULTS AND DISCUSSION

The pellet obtained from yeast cells following treatment with hot 20% KOH

Table 1. Action of Insolubilized α-Amylase on Insoluble Yeast Glycogen and Rabbit Liver Glycogen.

Time (min)	Insoluble glycogen (yeast) Iodine stain (visual)	Rabbit liver glycogen Iodine stain (490 nm)
0	+++++	1.80
10	++	0.64
20	+	0.33
35	-	0.16
60	-	0.05

Immediately before use the insoluble enzyme was washed in 1% glycogen solution to absorb unbound α-amylase, centrifuged at 5,000 x g and residual glycogen removed by exhaustive washing with 0.2M sodium acetate buffer pH 5.0. Insoluble enzyme (2 IU) was incubated at 37° with the insoluble yeast fraction or with soluble rabbit liver glycogen (5 mg) in 50 mM sodium acetate buffer pH 5.0. Hydrolysis of glycogen was followed by staining portions at the indicated times with a 0.2% I_2-2% KI solution and reading the absorbance at 490 nm (rabbit liver glycogen) or assessing the stain visually (insoluble yeast glycogen).

and ethanol precipitation (Materials and Methods) was exhaustively extracted with warm water to remove soluble glycogen. The residue stained dark brown with iodine solution but failed to stain after the bound glycogen component was destroyed by treatment with glucoamylase or soluble α-amylase. A similar loss of iodine staining power occurred when the residue was mixed with a suspension of α-amylase covalently attached to a cellulose support. The rate of reaction of the insoluble enzyme on the bound glycogen fraction appeared to be about equal to its action on soluble rabbit liver glycogen (Table 1). Determination of reducing sugars (as glucose equivalents) indicated that after 24 hr incubation, the insoluble enzyme had hydrolyzed each glycogen to about 20%. Filtered portions (glass wool) of the supernatant solutions (5,000 X g), taken from the insoluble α-amylase digests after about 30 min incubation, failed to decrease the iodine stain of fresh glycogen solutions over 24 hr. This confirmed that no soluble α-amylase activity was associated with the cellulose-bound activity. The susceptibility of the insoluble glycogen fraction to the action of the immobilized α-amylase therefore argues against the proposed

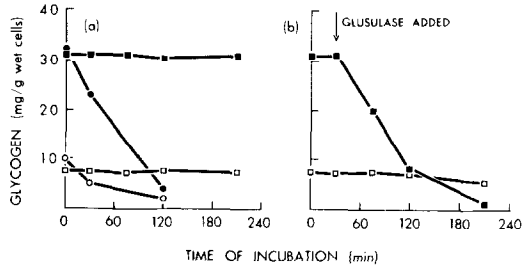

Fig.1. Effects of Glutaraldehyde and Glusulase-Treatment on The Soluble and Insoluble Glycogen Fractions in Yeast Cells. (a) Yeast cells (0.2g) were suspended at 30° in 140 mM mercaptoethanol and 40 mM EDTA (0.5 ml) before (circles) and after (squares) glutaraldehyde treatment. Soluble (open symbols) and insoluble (solid symbols) glycogen was estimated at the intervals indicated as described in the text. (b) Glutaraldehyde treated-cells were pre-incubated under the conditions described in (a). After 30 min, Glusulase stock solution (0.05 ml containing $9x10^4$ units glucuronidase and $2.5x10^3$ units sulfatase) was added and soluble (□) and insoluble (■) glycogen was determined as in (a).

encapsulation of the polysaccharide within an insoluble glucan membrane as proposed by Northcote (5).

The possible location of the insoluble glycogen fractions in the cell wall of Saccharomyces cerevisiae was studied by examining the effect of protoplast formation on the amount of insoluble glycogen recovered from the cells after hot KOH treatment. Under the incubation conditions chosen for protoplast formation, the soluble and insoluble glycogen of the yeast cell suspensions rapidly disappeared (Fig 1a). A controlled fixation of the cells with glutaraldehyde, however, prevented this endogenous catabolism of the glycogen reserves (Fig 1a) without altering the capacity of the cells to form protoplasts (Table 2). Using the fixed cells it was observed that the formation of protoplasts by the action of Glusulase was accompanied by a progressive decrease in the amount of glycogen in the insoluble fraction. In contrast, the level of soluble glycogen in the cells remained essentially constant during protoplast formation (Fig 1b). This is consistent with the insoluble glycogen fraction being located outside the cytoplasmic membrane of the yeast cell. Destruction of the outer regions of the cell wall then would expose the insoluble glycogen fraction to rapid hydrolysis by a high amylolytic activity of the Glusulase enzyme mixture. However, so long as the protoplasmic membrane

Table 2. Formation of Spheroplasts by Glusulase Action on Untreated and Glutaraldehyde-fixed Yeast Cells.

Time of Glusulase Treatment (min)	Untreated Cell count glucitol 720 mM	9 mM	spheroplasts (%)	Glutaraldehyde-fixed Cell count glucitol 720 mM	9 mM	spheroplasts (%)
0	510	505	0	450	435	0
30	412	214	51	433	230	46
120	-	-	-	406	127	70

As indicated in the text cells were incubated with Glusulase under the conditions described by Duell et al (12). The formation of spheroplasts was followed by observing microscopically the number of cells lysed when the glucitol concentration was decreased from 720 mM to 9 mM by adding portions of the Glusulase treated-cell suspensions to 80 volumes of water.

remains intact, intracellular soluble glycogen would not be degraded.

It is proposed therefore that most of the glycogen of yeast cells is located in the periplasmic space and is probably linked to an insoluble component of the cell wall. Studies on the metabolism of this glycogen fraction and on the nature of the linkage(s) by which it is attached to the cell wall are in progress.

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