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BIOSYNTHESIS OF ACID PHOSPHATASE OF BAKER'S YEAST. FACTORS INFLUENCING ITS PRODUCTION BY PROTOPLASTS AND CHARACTERIZATION OF THE SECRETED ENZYME

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SUMMARY

1. The secretion of acid phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.2) by protoplasts prepared from baker's yeast has been studied. Secretion into the incubation medium begins after a lag period, is linear for 2 to 3 h and stops after about 5 h. During the linear phase the secretion rate is about 50 molecules of enzyme/s per protoplast. Renewed vigorous secretion can be induced by refreshing the medium or replenishing it with glucose.

2. Secretion of acid phosphatase only occurs when (1) the protoplasts are prepared from log-phase yeast cells containing less than 3 μmoles P_i per 10^8 cells; (2) the protoplasts are incubated in a medium containing less than 10 μM P_i, *i.e.* less than 10^{-2} μmole P_i per 10^8 protoplasts; (3) the medium contains at least 56 μmoles glucose per 10^8 protoplasts.

3. The secreted acid phosphatase appears to be identical with the enzyme located in the cell wall of the intact yeast (a mannan–protein complex). It has the same pH optimum, the same K_m towards the substrates *p*-nitrophenyl phosphate and β-glycerophosphate, the same atypical transphosphorylation behaviour, is inhibited in the same way by molybdate ions, and its synthesis requires a low-phosphate medium and an unimpeded production of mannan.

4. Throughout the secretion of acid phosphatase, and also after its termination, a small amount of enzyme remains firmly bound to the protoplasts, even after lysis. After a transient decrease in the beginning this membrane-bound fraction remains constant.

INTRODUCTION

Previous work in this laboratory on the acid phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.2) of baker's yeast has been concerned with its purification, characterization and mechanism of action[1–3]. Present studies are directed towards its biosynthesis and its role in phosphate metabolism in the yeast cell.

The acid phosphatase is known to be mainly located in the cell wall[4-7], although some acid phosphatase activity has also been demonstrated in the cytoplasm[6-8]. A comparison with the yeast β-fructofuranosidase (β-D-fructofuranoside fructohydrolase, EC 3.2.1.26; formerly known as invertase) presents itself. This enzyme has clearly been proven to exist in two forms: an external, mannan-containing form and an internal, almost carbohydrate-free form[9]. Although the internal form is not simply a direct precurser of the external enzyme, a generally accepted point of view is that the presence of the enzyme in the cell wall is the result of a series of processes, such as: biosynthesis of the protein moiety in the cytoplasm, biosynthesis and attachment of mannan to the enzyme protein, transport from the cytoplasm and fixation in the cell wall matrix.

Considering the great similarity between the external forms of β-fructofuranosidase and acid phosphatase (both contain about 50% mannan and 4% glucosamine, their amino acid composition is fairly comparable, their molecular weights are in the same order of magnitude [β-fructofuranosidase $2.7 \cdot 10^5$, acid phosphatase $2.9 \cdot 10^5$ (refs 9, 10)], and the fact that both activities are also found in the cytoplasm, these enzymes could well depend upon the same factors for the attachment of mannan to the protein moiety and transport to the cell wall. On the other hand, bearing in mind their different physiological roles in the yeast cell, factors acting upon the biosynthesis of the internal forms might be expected to differ.

A study of the biosynthesis of the acid phosphatase and the relationship between internal and external forms along the lines previously followed for β-fructofuranosidase seems valuable, and may give more insight in the nature of common pathways for secretion.

For studying the internal form of the acid phosphatase protoplasts have some important advantages: they are devoid of most of the total acid phosphatase activity present in the yeast cell, so changes in the amount of the internal fraction are easier to detect, while this fraction is more accessible to substrates. Furthermore, protoplasts no longer divide, so their concentration remains fairly constant. Under favourable conditions protoplasts are able to secrete newly-formed cell wall components, such as mannan, β-fructofuranosidase and acid phosphatase[7,11,12].

This paper reports a study of some factors acting upon the secretion of acid phosphatase by yeast protoplasts and a characterization of the secreted enzyme.

MATERIALS AND METHODS

Materials

All chemicals used were analytical grade.

The yeast used in the experiments was *Saccharomyces cerevisiae* (Koningsgist), which was inoculated monthly in a Wickerham medium and kept at 4 °C. Starting material for preparing protoplasts was obtained by transferring a yeast inoculum ($0.4 \cdot 10^7$ cells per ml) from the Wickerham medium to a very rich medium as described by Markham *et al.*[13], but with 1 mM additional P_i. During cultivation the yeast was shaken in a conical flask at 30 °C. The yeast was harvested when the cell density was more than $3.2 \cdot 10^7$ cells per ml. The cell density was determined by measuring the absorbance at 420 nm with an EEL-Spectra photometer. By counting in a Bürker chamber we determined that $A_{420\ nm} = 0.250$ was equivalent to $1.0 \cdot 10^7$ cells per ml.

For the conversion of yeast cells into protoplasts we used a preparation of snail gut enzymes obtained from L'Industrie Biologique Française (Genevilliers, France).

The incubation medium for protoplasts was a Markham medium containing 12% (w/v) mannitol, henceforth called Markham–mannitol medium.

Procedures

After determining the amount of cells, the yeast was harvested and washed once with distilled water, followed by two washes with 12% (w/v) mannitol in water. Cells were then suspended in 0.1 M succinate buffer (pH 5.8) by adding 1.5 ml buffer per $5 \cdot 10^8$ cells. Thereupon the same volume of a solution containing 24% (w/v) mannitol, 50 mM 2-mercaptoethanol, 30 mM EDTA and 30 mM $MgSO_4$ was added. Finally 0.1 ml of snail enzyme per $5 \cdot 10^8$ cells was added and the suspension was incubated at 30 °C, shaking gently. The conversion of the cells into protoplasts (usually complete within 90 min) was followed by observing small samples of the suspension with a phase contrast microscope after dilution with water. Subsequently the protoplasts were centrifuged (10 min, 1000 × *g*, 4 °C) and washed twice with 12% (w/v) mannitol. They were then ready for use in experiments. The protoplasts could be stored in 12% (w/v) mannitol at 4 °C for a few days without losing their capacity for synthesizing acid phosphatase.

We studied the secretion of acid phosphatase by protoplasts as follows. A suspension (10^8 protoplasts per ml in Markham–mannitol medium) was incubated at 30 °C. During the incubation a sample of 2 ml was taken every hour and treated according to the scheme shown in Fig. 1. The activity determined in Supernatant I was

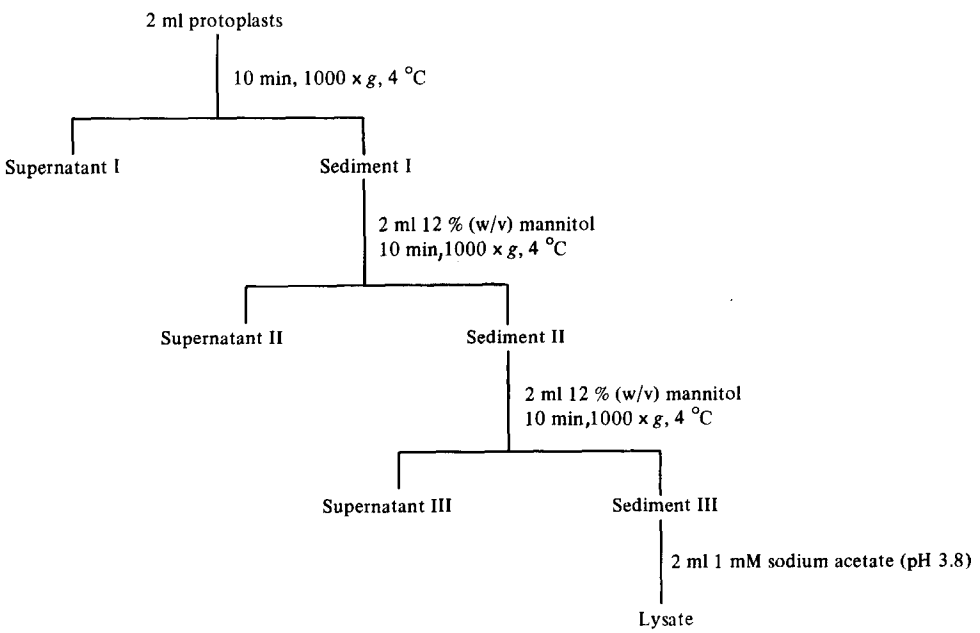


Fig. 1. Scheme for washing yeast protoplasts. Experimental conditions and procedures for determination of acid phosphatase activities in the supernatants as described in Materials and Methods.

a measure of secreted enzyme and the activity measured in the lysate we defined as membrane-bound activity.

To characterize the secreted acid phosphatase, Supernatant I had to be concentrated. This was done by dialysis against distilled water (24 h), followed by dialysis (24 h) against 0.1 M sodium acetate (pH 3.8) and placing the dialysis bags in solid sucrose at 0 °C. After concentration the preparation was stored at −20 °C.

Analytical methods

Acid phosphatase activities were routinely measured by determining the rate of liberation of *p*-nitrophenol from *p*-nitrophenyl phosphate. Assay mixtures contained 0.4 ml *p*-nitrophenyl phosphate (11 μmoles/ml) in 0.1 M sodium acetate (pH 3.8) and 0.1 ml enzyme solution. After incubation at 30 °C for 15 min the reaction was stopped by the addition of 8 ml 0.2 M NaOH and the absorbance of the solution was measured at 401 nm. One unit of activity was defined as the amount of enzyme which liberates 1 μmole *p*-nitrophenol per min under specified conditions (0.1 M sodium acetate (pH 3.8), 30 °C). Enzyme activity towards β-glycerophosphate was determined by measuring the liberation of P_i. Assay mixtures contained 0.5 ml 50 mM β-glycerophosphate in 0.1 M sodium acetate (pH 3.8), 1.5 ml of this buffer and 0.5 ml enzyme solution. The reaction was stopped by adding 2.5 ml 10% (w/v) trichloroacetic acid solution. P_i was determined by the method of Eibl and Lands[14].

Transphosphorylation experiments were performed as described by Boer and Steyn-Parvé[3], except that the amount of liberated P_i was estimated by the method of Eibl and Lands.

Carbohydrate was estimated according to Trevelyan and Harrison[15], using mannose as standard.

Glucose was determined with glucose oxidase by the so-called GOD-perid method with reagents from Boehringer, Mannheim Gmbh.

RESULTS

Secretion of acid phosphatase

Curve A of Fig. 2, represents a secretion curve. There is a lag period (1–1.5 h), followed by a practically linear increase of acid phosphatase activity in the medium during 2 to 3 h. Then secretion stops and the activity remains at a constant value. The secretion rate during the linear phase is about 0.4 unit/h per 10^8 protoplasts. The secretion curve always has a sigmoid shape, but slope and height of the plateau vary slightly from one experiment to another. We shall return to this point in the discussion.

Fig. 2 also shows the time-course of the membrane-bound acid phosphatase activity (Curve B). The freshly prepared protoplasts still have some activity (0.05 unit/10^8 protoplasts). This decreases during the first hour of incubation to about 0.04 unit/10^8 protoplasts, but then climbs to a constant level (about 0.06 unit/10^8 protoplasts). This level is reached at a time when the secretion of acid phosphatase is still fully linear. The activity in the membrane fraction seems to be caused by strongly bound acid phosphatase, for the acid phosphatase activity in Supernatant III (Fig. 1) was low (15%) compared to that of the lysate and after centrifugation (10 min, 1000 × *g*, 4 °C) of the lysate 94% of the activity of the latter was found in the sediment.

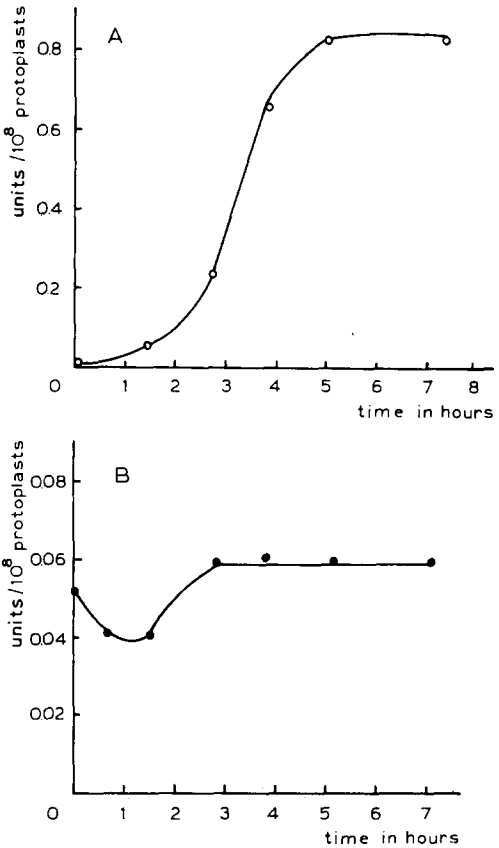


Fig. 2. (A) Time-course of secretion of acid phosphatase by yeast protoplasts (10^8 protoplasts per ml) in Markham–mannitol medium. (B) Time-course of membrane-bound phosphatase activity of the same secreting protoplasts. Experimental conditions and procedures as described in Materials and Methods.

The secretion of the enzyme is dependent on the concentration of P_i and glucose in the medium. Fig. 3 shows that when protoplasts are incubated in a Markham–mannitol medium containing 10 μM P_i there is no effect upon the secretion, while at 100 μM P_i secretion decreases, and becomes almost negligible at 1 mM P_i. Fig. 4 shows the dependency of the secretion on the glucose concentration present at the beginning of the incubation. There is a maximal secretion at 5% (w/v) glucose, while at higher and lower glucose concentrations a decrease of secretion was observed.

We can envisage three possible causes for the stopping of the secretion after about 5 h, *viz.* aging of the protoplasts, intoxication of the protoplasts by products secreted in the medium and exhaustion of one or more components of the medium.

Aging of the protoplasts could be ruled out as a cause by demonstrating that enzyme secretion is resumed when the medium is refreshed. Table I shows the result of an experiment in which after 4 h incubation under standard conditions the protoplasts were collected by centrifuging when the concentration of secreted phosphatase was 0.91 unit/10^8 protoplasts. After resuspension to the same protoplast concentration

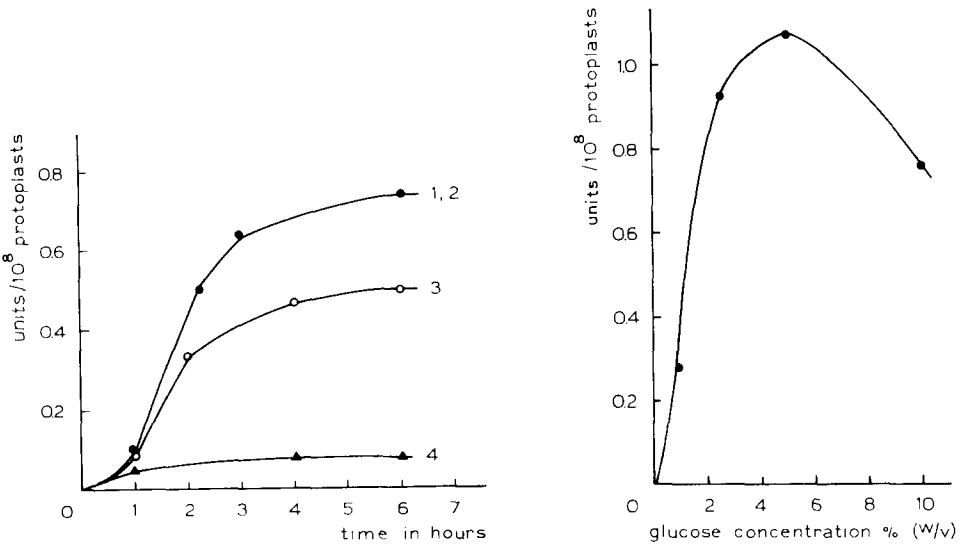


Fig. 3. Effect of P_i in the incubation medium on the secretion of acid phosphatase. 1, no P_i added (blank experiment); 2, 10 μM P_i added at time zero; 3, 100 μM P_i added at time zero; 4, 1 mM P_i added at time zero. Experimental conditions and procedures otherwise the same as in Fig. 2.

Fig. 4. Secretion of acid phosphatase by yeast protoplasts as a function of glucose concentration. Activity accumulated in the medium was determined after incubating 4.5 h in Markham–mannitol medium containing varying amounts of glucose.

in fresh medium secretion started again, at an even greater rate. After 2 h the protoplasts had already produced 0.94 unit/10^8 protoplasts, bringing the total in the two secretion periods (total 6 h) to 1.85 units/10^8 protoplasts. Repeated refreshing again provoked a considerable secretion. A microscopical check of the protoplasts after 6 h incubation did not reveal any morphological changes.

Thus, deterioration of the medium seems a more likely reason for the termination of enzyme secretion. To explore this matter further, we first checked the pH.

During the first 2 h of incubation the pH declines from 5.2 to 4.3 and thereafter remains constant. However, this cannot be deleterious, because after the pH has attained this constant value the secretion still continues at the same rate for another

TABLE I

EFFECT OF REFRESHING THE INCUBATION MEDIUM UPON THE SECRETION OF ACID PHOSPHATASE BY YEAST PROTOPLASTS

10^8 protoplasts/ml; details in the text.

Incubation time (h)	*Acid phosphatase secreted (units/10^8 protoplasts)*	
	First medium	*After refreshing*
0	0.07	0.040
1.25	–	0.393
1.75	0.192	–
2	–	0.943
3	0.631	
4	0.910	

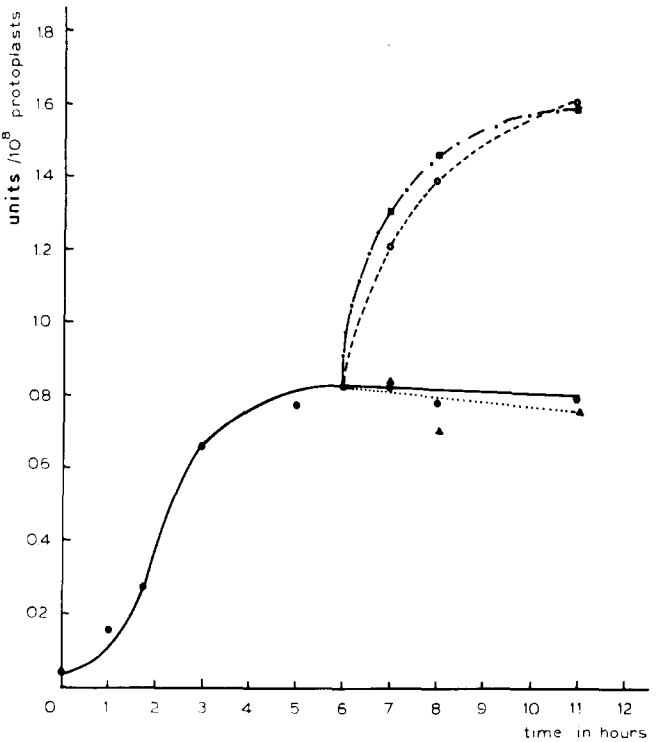


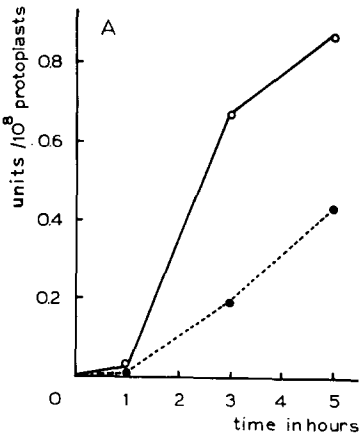
Fig. 5. Secretion of acid phosphatase by yeast protoplasts (10^8 protoplasts per ml) with extra addition of glucose and $(NH_4)_2SO_4$ after 6 h. ●——●, no additions (blank experiment); ▲ . . . . ▲, $(NH_4)_2SO_4$ added to the original concentration (0.38%); ■— . —■, glucose added to the original concentration of 5% (w/v); ○ – – – ○, glucose + $(NH_4)_2SO_4$ added to the original concentrations. Experimental conditions and procedures otherwise the same as in Fig. 2.

2.5 h. It is indeed quite possible that lowering of the pH is necessary for secretion, considering the lag period.

Two of the most likely candidates in the medium, $(NH_4)_2SO_4$ and glucose, were then checked for exhaustion. We observed that, starting with 5% (w/v), the glucose concentration in the medium falls to less than 1% (w/v) in about 4 h. Addition of glucose or of glucose *plus* $(NH_4)_2SO_4$ after enzyme production has stopped gives rise to a renewed secretion. $(NH_4)_2SO_4$ added alone has no effect, and only slightly enhances the secretion caused by the addition of glucose (Fig. 5).

From these experiments we conclude that exhaustion of glucose accounts for the stopping of secretion. It is therefore likely that the renewed secretion seen after refreshing the medium is also in a large measure due to replenishment with glucose.

To determine whether unimpeded formation of mannan is a necessary condition for the secretion of acid phosphatase we added 2-deoxyglucose (2 mg/ml) to a suspension of protoplasts in Markham–mannitol medium. This compound is known to inhibit synthesis of mannan[16], but not that of protein[12]. After 4 h incubation both the amount of acid phosphatase secreted and the concentration of non-diffusible carbohydrate in the medium (determined after 24 h dialysis against distilled water followed by 24 h dialysis against 0.1 M sodium acetate, pH 3.8) were reduced by 50%



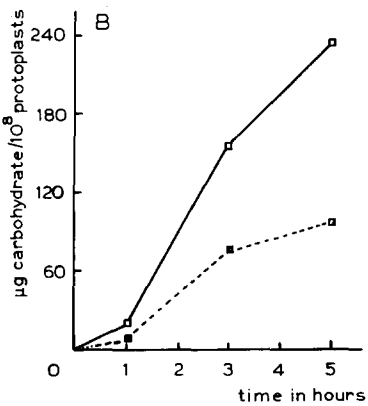


Fig. 6. Influence of 2-deoxyglucose upon the secretion of acid phosphatase and non-diffusible carbohydrate by yeast protoplasts (10^8/ml). (A) Acid phosphatase. ○——○, no 2-deoxyglucose added; ●...●, 2 mg/ml 2-deoxyglucose added at zero time. (B) Non-diffusible carbohydrate. □——□, no 2-deoxyglucose added; ■ – – – ■, 2 mg/ml 2-deoxyglucose added at zero time.

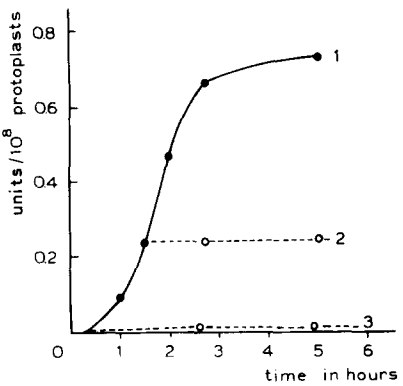


Fig. 7. Effect of cycloheximide on synthesis and secretion of the acid phosphatase by yeast protoplasts. 1, without cycloheximide (blank experiment); 2, cycloheximide (2 µg/ml) added after 1.5 h incubation; 3, cycloheximide (2 µg/ml) added at zero time. Experimental conditions and procedures otherwise the same as in Fig. 2.

as compared to a control without 2-deoxyglucose (Fig. 6). Thus, suppression of mannan synthesis suppresses acid phosphatase secretion.

Experiments with cycloheximide provided further proof that the secretion of acid phosphatase is indeed a result of *de novo* synthesis. Cycloheximide inhibits *de novo* protein synthesis on cytoplasmatic ribosomes in eukaryotic organisms, including yeast[17]. Added to a protoplast suspension in a final concentration of 2 µg/ml, it caused an instantaneous suppression of the secretion of acid phosphatase (Fig. 7). In this concentration cycloheximide does not affect the enzyme assay.

Characterization of the secreted acid phosphatase

To characterize the secreted acid phosphatase we compared some specific

TABLE II

COMPARISON OF PROPERTIES OF ACID PHOSPHATASE SECRETED BY PROTOPLASTS AND PURIFIED ACID PHOSPHATASE FROM THE CELL WALL OF BAKER'S YEAST

Property	*Secreted acid phosphatase*	*Purified acid phosphatase*
pH optimum (*p*-nitrophenyl phosphate)	4.0–4.5	4.0–4.5
pH optimum (*β*-glycerophosphate)	4.0–4.3	4.0–4.5
K_m (*p*-nitrophenyl phosphate)	1.0 mM	1.3 mM
K_m (*β*-glycerophosphate)	2.8 mM	3.1 mM

properties of the enzyme with those of a purified enzyme, prepared from the yeast cell wall[1,10]. Some results are presented in Table II. It can be seen that both enzyme preparations have the same pH optimum and K_m towards *p*-nitrophenyl phosphate and *β*-glycerophosphate as substrates. Furthermore the secreted enzyme was competitively inhibited by molybdate ions, using *β*-glycerophosphate as a substrate, in the same way as the purified enzyme.

One of the most remarkable properties of the purified cell wall enzyme is its typical kind of transphosphorylation[3]. Contrary to all other phosphatases investigated transphosphorylation is low and the rate of hydrolysis decreases with increasing amounts of acceptor alcohol. The secreted enzyme exhibits exactly the same kind of transphosphorylation behaviour.

All these similarities make it extremely likely that the enzyme secreted by protoplasts is identical with that located in the yeast cell wall.

DISCUSSION

The secretion of acid phosphatase by our yeast protoplasts resembles that observed by McLellan and Lampen[7], although there are some differences. We were not able to obtain any secretion in the medium used by these authors (0.6 M KCl, 2 mM glucose and 40 mM sucrose). Therefore we incubated our protoplasts in the richer Markham–mannitol medium, which contains, besides vitamins and minerals, 5% (0.28 M) glucose and 0.38% (29 mM) $(NH_4)_2SO_4$. On the other hand, in this medium our protoplasts produce about ten times as much acid phosphatase as those of McLellan and Lampen[7]. Armed with a knowledge of the molecular composition and the specific activity of the pure enzyme[10], we can further calculate that in the linear phase each protoplast secretes about 50 molecules of acid phosphatase per s.

To obtain actively secreting protoplasts it is not only necessary to keep down the concentration of inorganic phosphate in the medium ($\leqslant 10\ \mu$M, Fig. 3), but it is also essential to prepare the protoplasts from yeast cells grown in a low phosphate medium.

Experiments with yeast in batchwise culture and in continuous culture have shown us that synthesis of acid phosphatase in such cultures only takes place if two conditions are fulfilled: (1) the yeast cells must be dividing actively and (2) the total amount of phosphate in the culture must be less than 3 μmoles P_i per 10^8 cells (P. Boer, unpublished experiments). Protoplasts prepared from such cells exhibit a good

secretion, whereas protoplasts prepared from cells containing more phosphate than this critical value secrete poorly, and this only after a very long lag period. It is possible that the variations in the slope and final level of the secretion curve mentioned earlier (see *Secretion of acid phosphatase*) are due to small variations in the state of the yeast cells from which the protoplasts are prepared.

The decrease of membrane-bound acid phosphatase during the first hour of incubation probably reflects a release of acid phosphatase from the freshly-prepared protoplasts at a time when the synthesis of new enzyme molecules has not yet reached its maximal rate. It is remarkable that the membrane-bound phosphatase already reaches a constant level some hours before the protoplasts stop secreting enzyme. The membrane-bound phosphatase is probably in a steady-state situation. Islam and Lampen[11] found an analogous phenomenon in experiments concerning the secretion of β-fructofuranosidase by protoplasts. We cannot yet say whether or not the membrane-bound fraction of the acid phosphatase exclusively represents the internal fraction.

Glucose plays an important and indispensable role in the secretion of acid phosphatase. During the first 4 h of secretion glucose disappears in almost linear fashion from the medium as the concentration falls from 5% to less than 1%.

From the molecular composition of the pure enzyme[10] we can calculate that synthesis of the carbohydrate moiety of the enzyme in this period requires only 0.2% of the glucose consumed, while the non-diffusible carbohydrate secreted in the same time corresponds to 0.5%. So most of the glucose appears to serve primarily as a source of energy. This is in agreement with other data reported in the literature[18], which state that similar amounts of glucose are required as energy source for cultures of yeast cells. Viewed in this light, the renewed, vigorous secretion of acid phosphatase observed upon replenishment of the medium with glucose after secretion has stopped must be primarily due to a replenishment of the source of energy.

The secreted enzyme appears to be identical with the acid phosphatase ordinarily located in the yeast cell wall. This is not only apparent from the close resemblance of some kinetic properties (see *Characterization of the secreted acid phosphatase* and Table II), but also from the same requirement of a low phosphate medium for synthesis, and from the requirement of mannan synthesis for secretion of phosphatase (Fig. 6).

Considering the question of the biosynthesis of acid phosphatase, our attention was drawn by the hypothesis of Beteta and Gascón for the biosynthesis of yeast β-fructofuranosidase[19]. These authors envisage a role for yeast vacuoles in the secretion of invertase. However, we have not been able to detect any enrichment of acid phosphatase in a yeast vacuole fraction prepared according to the same method of Matile and Wiemken[20] as used by Beteta and Gascón.

On the other hand, from recent developments in the field of glycoprotein research there seems to emerge a general principle for the biosynthesis and secretion of these molecules, valid for both animal and plant cells[21]. The polypeptides of the glycoproteins are synthesized on the polysomes of the rough endoplasmatic reticulum, while the carbohydrate components are attached to the protein moiety in a stepwise fashion within the membranous entity of endoplasmatic reticulum and Golgi apparatus, followed by secretion of the completed glycoprotein molecule to the outside of the cellmembrane. The question, to what extent this picture for higher eukaryotes

would also hold for a primitive eukaryote like the yeast cell, encourages us to focus further studies on the membrane-bound fraction of the acid phosphatase.

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