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LOCALIZATION OF SOME PHOSPHATASES IN YEAST

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SUMMARY

1. The localization of some phosphatases has been studied in yeast cells that were either fragmented by shaking intact cells with glass beads or by hypotonic or isotonic disruption of protoplasts prepared from intact cells.

2. The non-specific acid phosphatase with optimum activity at pH between 3 and 4 was shown to occur in the cell wall of commercial baker's yeast. As some substrates of the enzyme can only enter that part of the cell volume corresponding to the cell wall and the activity is not increased by freezing and thawing the yeast, it was concluded that all the enzyme is located here.

3. The highly specific α-glycerophosphatase is entirely present in the unstructured cytoplasm of a 17-h culture of *Saccharomyces carlsbergensis* (No. 74).

4. *S. carlsbergensis* (No. 74) contains only one non-specific alkaline phosphatase. The distribution depends upon the age of the culture: In a culture aged 17 h two-thirds of the enzyme is bound to particles sedimenting from 3000 × *g* to 100 000 × *g*, and one-third is soluble. In a culture aged 24 h 70–85% of the enzyme is found in the soluble fraction, and only a small amount is particle bound.

INTRODUCTION

Many phosphatases have been reported to occur in yeast, enzymes of low as well as of high substrate specificity. But it is not at all clear from the literature whether each enzyme will always be found in every type of yeast and under all circumstances of culture and age.

Very little is also known about the location of phosphatases within the yeast cell. Such knowledge would seem to be of prime importance for a better understanding of the role the different phosphatases play in the metabolism of the yeast cell. Therefore we have attempted to localize some of the better described phosphatases by means of fractionation and analysis of yeast cell components.

It is still very difficult to achieve a satisfactory fractionation of the components of the yeast cell by applying the procedure which has worked so well for animal

Abbreviations: α-GP, α-glycerophosphate; β-GP, β-glycerophosphate; pNPheP, *p*-nitrophenyl phosphate; PheP, phenyl phosphate; TPP, thiamine pyrophosphate.

tissues, namely desintegration of the cells, followed by differential centrifugation. To begin with, rough treatment is needed to disrupt the yeast cell with its tough wall, risking damage to some particles. Recently this problem has been successfully circumvented by EDDY AND WILLIAMSON[1], who made use of a discovery by GIAJA[2] in 1913 that the gut juice of the snail *Helix pomatia* contains enzymes capable of dissolving the yeast cell wall. Under suitable osmotic conditions, and using susceptible yeast strains in the logarithmic phase of growth, nearly all the cells can thus be converted into intact protoplasts, which desintegrate very easily. Starting from such protoplasts, attempts have been made to separate various particles of the yeast cell, but here our very incomplete knowledge of the properties of these particles has been a hindrance, making it difficult to ascertain their integrity and the purity of the fractions obtained.

Within the limitations imposed by these circumstances we have examined the location in the yeast cell of two phosphatases of low substrate specificity: the acid phosphatase with optimum activity at a pH between 3 and 4 and the alkaline phosphatase (optimum activity at a pH between 9 and 10), and one highly specific phosphatase: the α-glycerophosphatase.

MATERIALS AND METHODS

The yeasts

For the experiments concerning the acid phosphatase a commercial baker's yeast (*Saccharomyces cerevisiae*) rich in this enzyme was used ("Koningsgist", Delft). The other experiments were performed with *Saccharomyces carlsbergensis*, No. 74 from the British National Collection of Yeast Cultures. This yeast was grown in Wickerham's malt extract medium[3], in 1-l bottles each containing 500 ml medium, inoculated with 0.2 ml of a mature culture and shaken overnight during 17 h at 26 to 26.5°. The cells were collected by centrifugation and washed three times with distilled water.

Yeast preparations

Protoplasts: Protoplasts were prepared from a 17-h culture of *S. carlsbergensis* according to EDDY AND WILLIAMSON[1], but using 10% mannitol instead of 0.55 M rhamnose, and 10 mg/ml of the snail enzyme preparation. The latter was obtained by centrifuging, dialysing, and freeze-drying the gut juice of *Helix pomatia*. After about 1 h in a water-bath of 30° all the cells are converted into protoplasts. These are centrifuged (5 min at 2000 × *g*) and washed three times with ice-cold 0.01 M acetate buffer (pH 6.0) containing 10% mannitol.

The protoplasts can be kept in this medium at 0–4° for 48 h without microscopically visible changes or changes in phosphatase content and distribution. The number of protoplasts in a suspension was either determined turbidimetrically, using a calibration curve previously obtained by measuring the turbidity of suspensions in which the number of cells had been determined by counting them in a counting chamber, or, when greater accuracy was desired, by direct counting in a counting chamber.

Frozen and thawed yeast: A suspension of intact cells, in a thin layer in an

aluminium beaker (10–20 ml spread out over a bottom 9 cm in diameter) was immersed in a mixture of dry ice and acetone (temperature approx. −80°) for 45 sec, and thawed by running tap water along the wall of the beaker. This treatment was repeated 4 to 5 times.

Desintegrated cells: Intact cells were desintegrated by shaking a yeast suspension with glass beads, either in the apparatus of NOSSAL[4] or that of MERKENSCHLAGER[5]. In the first case about 9 g of yeast (wet, packed weight) were suspended in water to 15 ml, 17 g of Ballotini beads No. 12 added, the mixture cooled to 0° and shaken in a steel capsule for 20 sec a number of times, with intermittent cooling, because the temperature rose to 13° in 20 sec. In the second case the yeast harvested from 1 l of growth medium was suspended to 10 ml in the desired medium in a 50-ml glass bottle, 25 g of Ballotini beads No. 9 were added, the mixture cooled to 1° and shaken for 2 min. In this time the temperature rose 5° and 60% of the cells were disrupted. Intact cells were removed by centrifuging for 30 min at 1000 × *g*.

Desintegrated protoplasts: Protoplasts were either desintegrated hypotonically, by suspending them in a hypotonic buffer, in which they swell and burst, or isotonically, by homogenizing a suspension, in a medium containing 10% mannitol, in a Potter–Elvehjem tissue homogenizer with close-fitting perspex pestle. Three movements up and down with the tube along the rotating pestle (500 rev./min, each taking about 2 min) sufficed to disrupt practically all the protoplast membranes. Any intact protoplasts remaining could be removed by centrifuging at 250 × *g* for 10 min. Also after hypotonic desintegration the suspension was homogenized in the Potter–Elvehjem apparatus, moving the tube up and down once, to suspend the cell particles which had clumped together. The tube of the apparatus was always surrounded by a jacket with crushed ice during homogenization.

Determinations of enzyme activity

For each phosphatase the optimum conditions were determined under which the amount of substrate hydrolysed was a measure for the amount of enzyme present. All determinations were performed in 12 ml centrifuge tubes that were shaken in a water bath of 30°.

Acid phosphatase: Reaction mixture of 0.05 M β-GP in 0.1 M acetate (pH 3.5), 0.5 ml; 0.1 M acetate (pH 3.5), 1.5 ml; yeast preparation, 0.5 ml. Incubation for 30 min; reaction stopped by adding 2.5 ml 10% trichloroacetic acid. After centrifuging, P_i was determined according to SUMNER[6] in an aliquot of the supernatant. The value obtained was corrected by subtracting the P_i found in blank determinations, lacking either substrate or yeast preparation.

α-Glycerophosphatase: Reaction mixture of 0.2 M α-GP in M/35 veronal–acetate buffer (pH 6.5), 1.0 ml; 0.5 M $MgSO_4$ in the same buffer, 0.4 ml; a mixture of 0.1% Triton X-100 and 0.01 M EDTA in the same buffer, 0.1 ml; yeast preparation, 0.5 ml[7]. After incubating for 20 min the reaction was stopped by adding 2 ml 10% trichloroacetic acid. P_i was determined in the supernatant after centrifuging, and corrected for blank values as above.

Alkaline phosphatase: Three different substrates were used: β-glycerophosphate, *p*-nitrophenyl phosphate and phenyl phosphate.

With β-glycerophosphate the reaction mixture was as follows: 0.08 M β-GP in

0.1 M glycine–NaOH (pH 9.2), 0.5 ml; glycine–NaOH (pH 9.2), 1.0 ml; yeast preparation, 0.5 ml. After incubating for 20 min the reaction was stopped by adding 2 ml 10% trichloroacetic acid. P_i was determined in the supernatant after centrifuging and corrected as mentioned above.

With *p*-nitrophenyl phosphate the reaction mixture contained 0.1 M pNPheP in 0.05 M Tris–H_2SO_4 (pH 10.0), 0.1 ml; 0.05 M Tris–H_2SO_4 (pH 10.0), 0.3 ml; yeast preparation, 0.1 ml. Incubation lasted 15 min; then 8 ml 2N NaOH were added to stop the reaction. After centrifuging, free *p*-nitrophenol was measured in the clear yellow supernatant in a Beckman DU spectrophotometer at 400 mμ (see ref. 8), and corrected for blank values as mentioned above*.

With phenyl phosphate the reaction mixture contained 0.12 M PheP in 0.025 M ethanolamine–veronal buffer (pH 9.4), 0.5 ml; 0.025 M ethanolamine–veronal (pH 9.4), 1.2 ml; yeast preparation, 0.3 ml. Incubation during 15 min; reaction stopped by adding 2 ml of the reagent of FOLIN AND CIOCALTEU[9] in a three-fold dilution. After centrifuging, free phenol was determined according to KING AND ARMSTRONG[10] by adding 3 ml 5% Na_2CO_3 to 2 ml of the supernatant, heating in a water bath of 60° for 10 min and measuring the absorbancy of the blue color in the Beckman DU spectrophotometer at 760 mμ.

With all three substrates the relationship between the rate of substrate hydrolysis and the amount of enzyme was not linear over the whole range of enzyme concentrations tested: at the lowest concentrations the increment of activity per unit of enzyme increased gradually, until at higher concentrations a linear relationship was reached (see Fig. 1). This proved to be due to a co-factor of the alkaline phos-

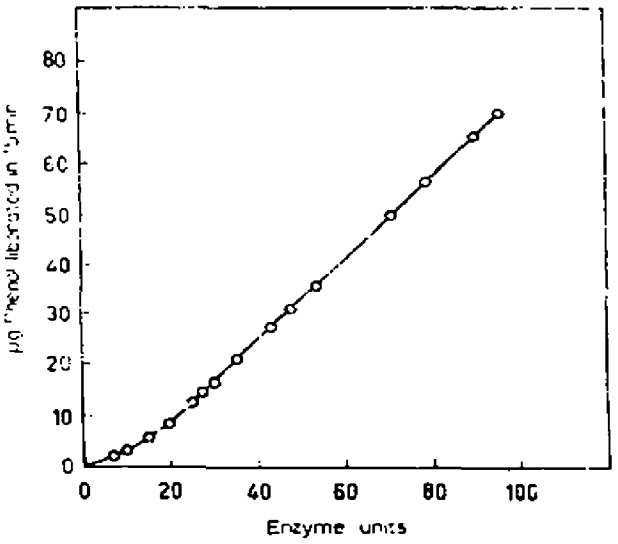

Fig. 1. Relationship between enzyme concentration and activity of alkaline phosphatase in *S. carlsbergensis*. Substrate: phenyl phosphate. Enzyme preparation: disintegrated intact cells or protoplasts. Activity determined as described above.

* This substrate was no longer used after we discovered that in its presence various anions, *e.g.* Cl^-, have an anomalous effect upon the activation of alkaline phosphatase by Mg^{2+}.

phatase, present in the yeast preparations, which becomes limiting in high dilutions. In dialysed preparations this co-factor can be replaced by Mg^{2+}, and the relationship is then linear over the whole range of enzyme concentrations used. When Mg^{2+} is added to undialysed preparations the relationship also becomes linear with low enzyme concentrations, but at higher concentrations Mg^{2+} has an inhibitory action. Undialysed preparations were always tested without added Mg^{2+}, as the relationship between enzyme concentration and activity proved to be extremely reproducible, and the measured activities were translated into (arbitrarily chosen) units of alkaline phosphatase with the aid of a carefully established calibration curve, as shown in Fig. 1.

Chemicals and other materials

All chemicals used were of analytical reagent grade. Disodium α-glycerophosphate was obtained from Light and Co. (Great Britain); disodium β-glycerophosphate (max. content of α-isomer 0.1%) from Eastman Kodak Co., New York; phenyl disodium orthophosphate from Merck, Darmstadt; p-nitrophenyl disodium orthophosphate from British Drug Houses, London; thiamine pyrophosphate from Hoffmann-La Roche, Basle; malt extract and yeast extract from Difco Lab., Detroit; peptone from Oxo Ltd., (Great Britain); Ballotini beads from English Glass Co., Leicester.

EXPERIMENTS AND RESULTS

Acid phosphatase

It is very likely that the non-specific acid phosphomonoesterase with optimum activity between pH 3 and 4 will be located in the cell wall, as intact yeast cells possess this activity[11-15], and all the radioactive phosphate liberated from [^{32}P]ATP by intact cells is recovered in the medium[16].

A comparison of the acid phosphatase activity of fresh baker's yeast and frozen and thawed yeast showed that both preparations hydrolyse β-GP at the same rate (34.6 μg P_i/10 mg yeast/30 min *versus* 33.2 μg). Therefore in intact cells the substrate has access to all the enzyme. This observation is in favour of the conception of an exclusive location of the enzyme in the cell wall, as phosphate esters can only penetrate into the interior of a yeast cell after it has been frozen and thawed.

This conception might be confirmed by transforming yeast cells into protoplasts according to EDDY AND WILLIAMSON[1]: as the cell wall dissolves the enzyme will pass into solution.

However, neither our commercially available baker's yeast, nor a 17-h culture of the same, both rich in this acid phosphatase, proved to be susceptible to the treatment with snail enzyme. The intact cells of a 17-h culture of *S. carlsbergensis* (No. 74), which can easily be converted into protoplasts, do not yet show any acid phosphatase activity. The enzyme is present in a 40-h culture of this yeast, but then the cell walls cannot be dissolved without affecting the integrity of the protoplasts. A 17-h culture of a hybrid of several species of *Saccharomyces* (No. 303-3 from the Carlsberg collection), from which the wall can be "peeled off", does possess acid phosphatase activity, but is less suitable because a pH-activity curve of the

intact cells shows a second optimum, close to the first, at pH 4.7. So we had to confine our experiments to mechanically desintegrated commercial baker's yeast.

When a suspension of baker's yeast was shaken in the NOSSAL apparatus, even for short periods of time (10–40 sec), in which many cells still remain intact, a considerable part of the enzyme was no longer sedimentable upon centrifuging for 1 h at 100 000 × *g*. Thus, even if all the acid phosphatase were originally located in the cell wall, the greater part would seem to be so loosely bound that it is easily dislocated when the cell breaks up.

It was possible to demonstrate that the enzyme is indeed present in the cell wall by preparing a fairly pure cell wall fraction from the desintegrated yeast. To carry out an adequate fractionation we had to start with a yeast preparation consisting mostly of large cells. The smaller cells were removed from a sample of fresh baker's yeast by suspending the yeast in 20% glycerol and centrifuging for 3 min at 600 × *g*. The sediment, mainly comprising the larger cells, was treated twice more in the same manner. It was then desintegrated in the NOSSAL apparatus. The material sedimenting from the desintegrate after 10 min at 1000 × *g* was suspended in 10% glycerol and a fraction mainly composed of large cell wall fragments collected by alternate centrifuging at 250 × *g* (5 min) and 600 × *g* (3 min), seven times in all. Finally the glycerol was removed by washing the cell walls several times with water, the number of fragments and intact cells in the fraction were counted and its acid phosphatase activity determined. The results obtained with varying times of desintegration are shown in Table I.

TABLE I

ACID PHOSPHATASE ACTIVITY OF CELL WALLS ISOLATED FROM BAKER'S YEAST

Preparation of cell wall fractions described in text; activity determined with β-GP (see p. 455).

Desintegration time (sec)	*No. of intact cells/0.5 ml suspension*	*No. of cell walls/0.5 ml suspension*	*P_i liberated (μg/30 min)*	*P_i μg/30 min / 10^8 cells or cell walls*
0	$1.2 \cdot 10^8$	—	48.7	40.6
10	$2.2 \cdot 10^6$	$0.9 \cdot 10^8$	9.8	10.8
20	$2.3 \cdot 10^5$	$1.7 \cdot 10^8$	17.9	10.5
30	$5.1 \cdot 10^5$	$4.1 \cdot 10^8$	46.3	11.2
40	$6.2 \cdot 10^5$	$2.3 \cdot 10^8$	21.7	9.3

As can be seen, all cell wall fractions possess acid phosphatase activity. The contribution to this activity from intact cells still present can be calculated and is at most 0.2 μg P_i. Assuming that the size of the fragments corresponds to a more or less "complete" cell wall, the figures show that about 25% of the total enzyme activity remains bound in the cell wall upon desintegration of the yeast cell.

Additional evidence that not only a part, but all the acid phosphatase activity is present in the wall of the intact yeast cell was obtained in an entirely different manner, by determining the "apparent free space" of baker's yeast for two substrates of the enzyme, β-glycerophosphate and thiamine pyrophosphate, according

to CONWAY AND DOWNEY's method[17]. The "apparent free space" R is that part of the volume of the yeast cell into which a substance can readily penetrate.

We determined R for various pH values at 27°, using washed and centrifuged baker's yeast. When TPP was the substrate under investigation 10^{-3} M sodium molybdate was also added to inhibit the phosphatase[16,18]. With β-GP some of the added substrate was decomposed by phosphatase action during incubation, but as the reaction products were included in our determination of β-GP the error thus introduced proved to be very small. The results are given in Fig. 2.

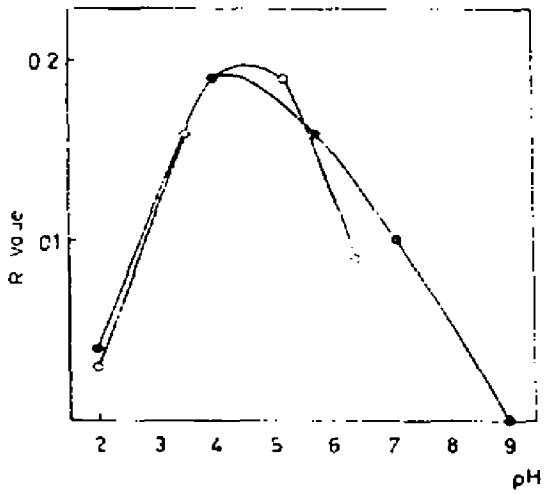

Fig. 2. Relationship between pH and R value in fresh baker's yeast. ●—●, β-GP. 5 g baker's yeast, washed and centrifuged, mixed with 5 ml 0.2 M β-GP, incubated 15 min; β-GP determined in dry weight before and after mixing with the yeast. ○—○, TPP. 5 g baker's yeast mixed with 5 ml 0.0047 M TPP, containing 10^{-3} M sodium molybdate, incubated 5 min; TPP determined manometrically before and after mixing[19].

At a pH of about 4 the value of R is 0.19 for both substrates. This means that they can rapidly occupy at most about 19% of the total cell volume. According to CONWAY AND DOWNEY[17] a number of substances readily enter a space corresponding to 10–20% of the cell volume but do not or only very slowly penetrate further into the cell. They have called this space the "outer region" of the yeast cell, and advanced reasons for the view that this region may be identified with the cell wall. As the substrates β-GP and TPP do not penetrate any further into the cell, and the acid phosphatase activity cannot be augmented by freezing and thawing the yeast, we may conclude that all acid phosphatase is located exclusively in the cell wall.

α-Glycerophosphatase

α-Glycerophosphatase is a highly specific phosphomonoesterase with a sharp optimum of activity at pH 6.5 (see ref. 20). It is present in a high concentration in *S. carlsbergensis* (No. 74) as compared to the non-specific phosphatases that are also capable of decomposing α-glycerophosphate (but with different pH optimum). The

rate of liberation of P_i from this substrate by this yeast at pH 6.5 can therefore be regarded as an exclusive measure of α-glycerophosphatase activity.

The α-glycerophosphatase activity of intact cells is less than 1% of that of the same amount of yeast after freezing and thawing (Table II). Neither can intact protoplasts decompose α-glycerophosphate, in contrast to frozen and thawed protoplasts (Table III). Thus, removal of the cell wall does not make the cell accessible to the substrate; it cannot pass the protoplast membrane. Evidently the enzyme must be located inside the protoplast membrane.

TABLE II

α-GLYCEROPHOSPHATASE ACTIVITY OF INTACT CELLS AND FROZEN AND THAWED *S. Carlsbergensis*

17-h yeast culture, suspended in M/35 veronal-acetate (pH 6.5), frozen and thawed 4 times. Activity determinations described on p. 455. Values refer to 10 mg yeast (dry wt.).

State of yeast cells	*P_i liberated (μg/20 min)*
Intact	6
Frozen and thawed	813

TABLE III

α-GLYCEROPHOSPHATASE ACTIVITY OF INTACT AND FROZEN AND THAWED PROTOPLASTS OF *S. carlsbergensis*

Reaction mixture: 0.2 M α-GP in M/35 veronal acetate (pH 6.5), 1 ml; 0.07 M $MgSO_4$ in same buffer, also containing 0.0035 M EDTA, 0.3 ml; 0.45 M mannitol, 0.7 ml; suspension of protoplasts in 0.01 M acetate (pH 6.0), also containing 0.55 M mannitol, 0.5 ml.

State of protoplasts	*P_i liberated (μg/5 min/10^9 protoplasts)*
Intact	0
Frozen and thawed	117

To further localize the enzyme, protoplasts of *S. carlsbergensis* were desintegrated, either hypotonically or isotonically, in media varying in composition and pH, and the distribution of α-glycerophosphatase ascertained between supernatant and sediment after centrifuging at 100 000 × *g* for 45 min. The results of these experiments are assembled in Table IV.

The figures show that at pH 6 and 7 practically all the enzyme is recovered in the supernatant, irrespective of the mode of desintegration or composition of the medium. The small amount of enzyme found in the sediments may largely be attributed to the supernatant with which they were still saturated, as the sediments were not washed prior to the determination of enzyme activity. Only at pH 5

TABLE IV

DISTRIBUTION OF α-GLYCEROPHOSPHATASE BETWEEN 100 000 × *g* SEDIMENT AND SUPERNATANT, OBTAINED FROM DESINTEGRATED PROTOPLASTS OF *S. carlsbergensis*

Buffers: 0.01 M acetate (pH 5.0 and 6.0); 0.025 M maleate (pH 7.0). Isotonic media always contained 10% mannitol. Concentrations of additions Mg^{2+} 0.0005–0.008 M; Ca^{2+} 0.005 M; EDTA 0.0003 M. Centrifuged for 45 min at 100 000 × *g*. Activity determinations described on p. 455. – between figures: more than 2 experiments. ; between figures: 2 experiments.

Tonicity and pH of medium	Additions to medium	Activity, per cent of total desintegrate		
		Sediment	Supernatant	Sediment + Supernatant
Hypotonic 5.0	Mg^{2+}	12–41	11–70	43–82
	Mg^{2+} + Ca^{2+}	14–27	47–64	74–78
	Mg^{2+} + EDTA	17	55	72
Isotonic 5.0	Mg^{2+}	19–30	20–46	63–70
	Mg^{2+} + Ca^{2+}	14	75	89
Hypotonic 6.0		4	89	93
	Mg^{2+}	3	89	92
	Mg^{2+} + Ca^{2+}	3 ; 1	83 , 91	86 , 92
	Mg^{2+} + EDTA	4	96	100
Isotonic 6.0	–	3	95	98
	Mg^{2+} + EDTA	9	91	100
Hypotonic 7.0		6	88	94
	Mg^{2+}	4	93	97
Isotonic 7.0	Mg^{2+}	0	93	93

appreciable but varying amounts of enzyme are found in the sediment. Also at this pH the recovery of total activity is poor, but α-glycerophosphatase is known to be fairly labile at pH 5.

If the distribution found at pH 6 and 7 would reflect the true situation, the different distribution found at pH 5 might be caused by iso-electric precipitation of the enzyme and/or adsorption of soluble enzyme onto sedimentable particles. Further experiments showed that this is indeed the case. When a suspension of desintegrated protoplasts prepared at pH 6.0 is brought to pH 5.0 the sedimentable α-glycerophosphatase increases and attains the same values as when desintegration takes place at pH 5.0 (20–40% of total activity). Also, when a supernatant obtained by desintegrating protoplasts and centrifuging at pH 7.0 is adjusted to pH 5.0, some protein precipitates, carrying α-glycerophosphatase activity, which can be separated from the supernatant by a second centrifugation. So both causes mentioned above can contribute to the partial sedimentation of the enzyme observed at pH 5.0, which is therefore an artifact.

Thus we may conclude that all the α-glycerophosphatase is located in the soluble fraction of the yeast cell.

Alkaline phosphatase

S. carlsbergensis (No. 74) contains at least one non-specific phosphomonoesterase with optimum activity in the alkaline region at pH 9 to 10, judged from the pH-activity curves established with various substrates and using either frozen and thawed yeast or lysed yeast protoplasts (Fig. 3).

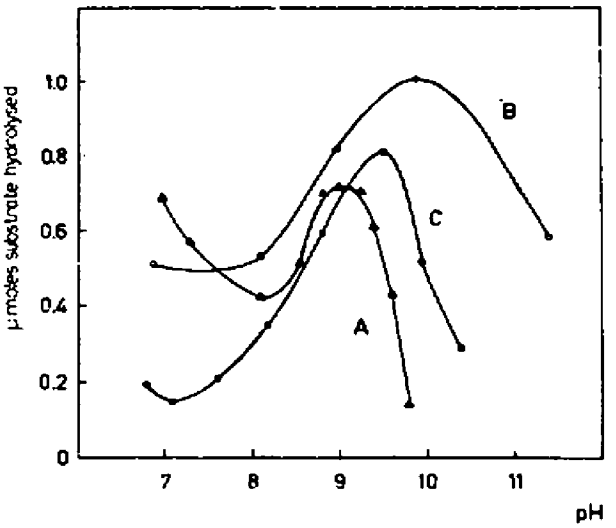

Fig. 3. Dependence upon pH of the activity of alkaline phosphatase in *S. carlsbergensis*. A, substrate β-GP, frozen, thawed and washed yeast; B, substrate pNPheP, frozen and thawed yeast; C, substrate PheP, hypotonically desintegrated protoplasts. Activity determined as described on p. 4. The activities towards the different substrates are not comparable as different amounts of yeast were used.

It is not likely that there will be more than one alkaline phosphatase, as with each of three different substrates (β-glycerophosphate, *p*-nitrophenyl phosphate and phenyl phosphate) optimum activity is reached at the same substrate concentration (approx. 0.03 M), and an identical non-linear relationship is observed between enzyme concentration and activity (Fig. 1). Also, with two substrates (pNPheP and PheP) low concentrations of cyanide and EDTA inhibit the enzyme markedly, while inorganic phosphate has no effect (but see ref. 21).

The alkaline phosphatase is located entirely within the protoplast membrane, as neither intact yeast cells nor intact protoplasts exhibit any activity towards phenyl phosphate at alkaline pH, in contrast to frozen and thawed cells or protoplasts (Table V). Moreover, frozen and thawed cells treated with the snail enzyme

TABLE V

DEPENDENCE OF ALKALINE PHOSPHATASE ACTIVITY UPON THE STATE OF YEAST CELLS AND PROTOPLASTS

Yeast cells (17-h culture of *S. carlsbergensis*) suspended in 0.025 M maleate (pH 7.0); part of suspension frozen and thawed several times. Protoplasts suspended in same buffer, but containing 10% mannitol; part of suspension frozen and thawed once. Activity determined with phenyl phosphate as a substrate (see p. 456).

Yeast preparation	*Amount*	*Phenol liberated (µg/20 min)*
Intact cells	2.1 mg dry wt.	0
Frozen and thawed cells	2.1 mg dry wt.	14.2
Intact protoplasts	$5.5 \cdot 10^7$	0
Frozen and thawed protoplasts	$5.5 \cdot 10^7$	40.0

preparation appeared to have the same activity as frozen and thawed protoplasts (60.8 and 63.3 μg phenol liberated/20 min/10^8 cells, respectively), so there is indeed no alkaline phosphatase in the cell wall.

Protoplasts of *S. carlsbergensis* were desintegrated either hypotonically or isotonically at varying pH and the distribution of the enzyme ascertained between supernatant and sediment after centrifuging for 45 to 60 min at 100 000 × g. As Table VI shows, 60–75% of the enzyme is always found in the sediment, irrespective of the manner of desintegration*.

TABLE VI

DISTRIBUTION OF ALKALINE PHOSPHATASE BETWEEN 100 000 × g SEDIMENT AND SUPERNATANT, OBTAINED FROM DESINTEGRATED PROTOPLASTS OR CELLS OF *S. carlsbergensis*

Buffers: 0.01 M acetate (pH 5.0 and 6.0); 0.025 M maleate (pH 7.0 and 7.5). Isotonic media always contained 10% mannitol. Protoplasts desintegrated in Potter Elvehjem tissue homogenizer; cells desintegrated in MERKENSCHLAGER apparatus. Centrifuged for 45–60 min at 100 000 × g. Activity determined with phenyl phosphate as substrate (see p. 456). – between figures: more than 2 experiments; ; between figures: 2 experiments.

Tonicity and pH of medium	Initial preparation	Activity, per cent of total desintegrate		
		Sediment	Supernatant	Sediment + Supernatant
Hypotonic 5.0	Protoplasts	75	0	75
Isotonic 5.0	Protoplasts	72	10	82
Hypotonic 6.0	Protoplasts	64 ; 73	34 ; 35	98 ; 108
Isotonic 6.0	Protoplasts	59–70	20–40	91–110
Isotonic 6.0	Cells	68–75	21–32	91–107
Hypotonic 7.0–7.5	Protoplasts	60–71	35–43	95–106
Isotonic 7.5	Protoplasts	61	34	95
Isotonic 7.0–7.5	Cells	60–70	14–21	84–88

It has not been possible so far to characterize the particles containing alkaline phosphatase by their sedimentatory behaviour. The enzyme already starts sedimenting at forces between 1000 and 5000 × g, and sedimentation is not complete before centrifuging 45 min at 100 000 × g (Table VII). When a desintegrate of protoplasts is centrifuged in a discontinuous sucrose gradient (10–60%), the activity is spread diffusely over the length of the tube. Experiments performed in a medium suitable for the preparation of ribosomes[22] gave the usual sedimentation pattern. A preparation rich in protoplast membranes[23] contained hardly any alkaline phosphatase. When protoplasts were desintegrated in a medium suitable for the isolation of intact nuclei[23] (0.01 M acetate (pH 5.0) containing 10^{-3} M Mg^{2+} and 10^{-4} M Ca^{2+}), 60%

* The poor recovery at pH 5 will probably be due to instability of the soluble alkaline phosphatase at this pH; the reasons for the poor recovery upon desintegrating whole cells at pH 7.0–7.5 are not clear.

TABLE VII

SEDIMENTATION OF ALKALINE PHOSPHATASE OF *S. carlsbergensis* WITH INCREASING CENTRIFUGAL FORCE

Protoplasts obtained from 17-h culture desintegrated hypotonically in 0.01 M acetate (pH 6.0). Centrifugation time 45 min at each force indicated. Activity determined with phenyl phosphate as substrate (see p. 456).

Centrifugal force (× g)	*Activity, per cent of total desintegrate*	
	Sediment	*Supernatant*
1 000	—	97
5 000	27	63
25 000	57	42
100 000	68	29

of the enzyme already sedimented at 3000 × *g*. But this did not mean that most of the enzyme is located in the nuclei, as subsequent experiments showed that Mg^{2+} and Ca^{2+} activate the particle-bound enzyme when added after preparing the desintegrate, and reduction of the pH from 6.0 to 5.0 increases the amount of enzyme sedimenting (cf. α-glycerophosphatase). Finally, it was not possible to correlate the sedimentation pattern of alkaline phosphatase with that of the exclusively mitochondrial enzyme succinic dehydrogenase, as our culture of *S. carlsbergensis* did not yet contain measurable amounts of the latter enzyme after 17 h, although it did appear in older cultures (see also ref. 24).

A number of experiments were performed to ascertain whether the sedimentable

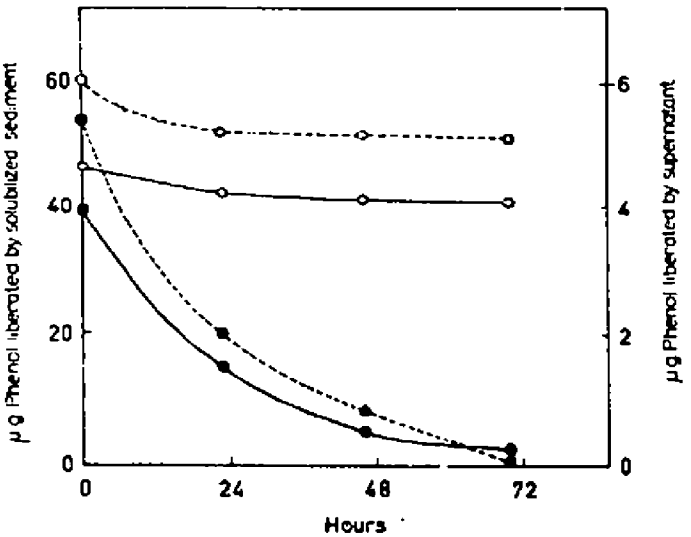

Fig. 4. Influence of dialysis upon particle-bound and soluble alkaline phosphatase after deoxycholate treatment. Cell fractions of *S. carlsbergensis* containing alkaline phosphatase prepared as described in Table VIII. Substrate: PheP. Activity determined as described on p. 456. ○—○, 0.01 M Mg^{2+} added before determination; ●—●, no Mg^{2+} added; ———, sediment solubilized with deoxycholate; - - - -, supernatant with added deoxycholate.

and soluble alkaline phosphatase are identical. To this purpose the following properties were compared of the enzyme in the 100 000 × *g* sediment and the supernatant: pH-activity curve, behaviour during dialysis, activity towards different substrates, and thermostability. To achieve the fairest comparison possible the enzyme in the sediment was solubilized with sodium deoxycholate. It could then no longer be sedimented by centrifugation at 100 000 × *g*. The enzyme thus solubilized appeared to be much more strongly activated by Mg^{2+} than that in the supernatant (50–100% *versus* 20% increase in activity). To compensate for this difference the 100 000 × *g* sediment obtained from the original desintegrate of protoplasts was resuspended to the original volume in supernatant inactivated by heating before applying the deoxycholate treatment. The supernatant was also treated with deoxycholate.

With both solutions pH-activity curves were obtained with identical shape between pH 8.5 and 10.5, and a maximum at pH 9.4–9.5, using phenyl phosphate as a substrate. Both preparations lost their activity at exactly the same rate during dialysis against 0.025 M maleate (pH 7.0) at 5° and could be reactivated to the same extent by adding 0.01 M Mg^{2+} (Fig. 4). The ratio of the rates in which each preparation hydrolysed two different substrates, PheP and β-GP, was the same in both cases (Table VIII).

TABLE VIII

RELATIVE ACTIVITIES TOWARDS TWO DIFFERENT SUBSTRATES OF PARTICLE-BOUND AND SOLUBLE ALKALINE PHOSPHATASE AFTER DEOXYCHOLATE TREATMENT

Protoplasts from a 17-h culture of *S. carlsbergensis* desintegrated hypotonically in 0.025 M maleate (pH 7.0) and separated into sediment and supernatant by centrifuging 1 h at 100 000 × *g*. Sediment resuspended to original volume in supernatant inactivated by heating 20 min at 90°. Both fractions mixed with equal volumes of 0.5% sodium deoxycholate in 0.025 M maleate (pH 7.0), incubated 15 min at 30°, cooled, centrifuged 1 h at 100 000 × *g*. Both supernatants dialysed 50 h against 0.025 M maleate (pH 7.0); tested after adding Mg^{2+} (0.01 M). For activity determinations see p. 456.

Soluble enzyme obtained from	I PheP hydrolysed (μmoles/15 min)	II β-GP hydrolysed (μmoles/15 min)	Ratio I : II
Sediment	0.74	0.27	2.7
Supernatant	0.10	0.04	2.5

The experiments on thermostability were performed with a suspension of desintegrated whole cells (MERKENSCHLAGER apparatus, 0.01 M acetate (pH 6.0)) and the sediment and supernatant obtained therefrom by centrifuging for 1 h at 100 000 × *g*, without applying the deoxycholate treatment. All three preparations retained their alkaline phosphatase activity up to and including heating for 10 min at 50°; they all lost a little activity after 10 min at 60°. Thermo-inactivation followed the same course in all three cases at higher temperatures, resulting in a complete inactivation after 10 min at 80°.

From all these experiments we may conclude that *S. carlsbergensis* (No. 74) contains only one alkaline phosphatase, in part soluble, in part bound to cell particles.

The age of the yeast culture has a profound influence upon the location of the alkaline phosphatase. This became apparent upon a comparison of the distribution

of the enzyme between 100 000 × *g* sediment and supernatant obtained from desintegrates of 10, 17 and 24-h cultures of *S. carlsbergensis*. The experiments were performed with both hypotonically desintegrated protoplasts and cells disrupted by shaking with glass beads. To obtain protoplasts from the 24-h old yeast the cells had to be incubated for 3 instead of 1 h with the snail enzyme preparation. About half the number of cells are then completely lysed, the other half are in the protoplast stage. These protoplasts were washed repeatedly with ice-cold isotonic maleate buffer to free them from the contents of the lysed cells before disrupting

TABLE IX

DISTRIBUTION OF ALKALINE PHOSPHATASE OVER 100 000 × *g* SEDIMENT AND SUPERNATANT AT VARIOUS AGES OF A CULTURE OF *S. carlsbergensis*

Protoplasts and cells desintegrated in 0.025 M maleate (pH 7.0); centrifuged for 1 h at 100 000 × *g*. Protoplasts desintegrated in Potter–Elvehjem tissue homogenizer, cells in MERKENSCHLAGER apparatus. Enzyme activity determined with phenyl phosphate as a substrate (see p. 456). – between figures: more than 2 experiments. ; between figures: 2 experiments.

Age of culture (h)	*Initial preparation*	*Activity, per cent of total desintegrate*	
		Sediment	*Supernatant*
10	Protoplasts	65	32
17*	Protoplasts	60–71	35–43
	Cells	60–70	14–21
24	Protoplasts	15	85
	Cells	10 ; 23	84 ; 67

* Taken from Table VI.

them hypotonically. From the results, assembled in Table IX, it can be seen that the distribution of alkaline phosphatase was the same for the 10-and 17-h cultures (two-thirds in sediment, one-third in supernatant), but that at 24 h there was a complete change: 70–85% of the enzyme was now found in the supernatant and the remainder in the 100 000 × *g* sediment.

DISCUSSION

The experiments reported above indicate that the acid phosphatase with optimum activity between pH 3 and 4 is entirely located in the wall of the yeast cell. SUOMALAINEN *et al.*[15] reached a different conclusion, namely that 30% of the enzyme would be located within the protoplast membrane, as the acid phosphatase activity of their baker's yeast increased 30% after freezing and thawing. Perhaps not all baker's yeasts are alike in this respect, depending upon the strain and circumstances of culture.

In our baker's yeast 20–30% of the enzyme is firmly bound to the cell wall, and the remainder more loosely. Perhaps the enzyme is situated in two different areas of the cell wall; considering the fact that the cell wall largely consists of two polysaccharide layers: an outer glucan layer and an inner mannan layer[48], its

structure offers possibilities in this respect. This hypothesis might be tested with a yeast containing acid phosphatase and convertible into protoplasts, by comparing the rates of liberation of acid phosphatase and dissolution of the cell walls.

There is a striking parallelism between the dependence upon the pH of the *R*-value for phosphate esters and the acid phosphatase activity of our baker's yeast. One might be inclined to think that the pH-activity curve reflects the variation with pH of the ability of the substrate to reach the enzyme rather than that of the reaction rate, were it not that a similar pH-activity curve is also obtained with dried yeast[13], where penetration of substrate is no longer a problem.

If one were to speculate upon the physiological function of the acid phosphatase, one could, in view of its location in the cell wall, think in terms of the production of substrates able to enter the cell from surrounding phosphate esters in the medium.

The α-glycerophosphatase in *S. carlsbergensis* (No. 74) bears a close resemblance to the enzyme described in baker's yeast[7,20]. This could be ascertained in a study of its properties, not described here, preceding the experiments on localization. For instance we found the same K_m of approx. 0.01 mole/l as TSUBOI AND HUDSON did for the baker's yeast enzyme[20]. It is entirely located in the unstructured protoplasm of the cell. From immunological experiments with dried yeast SEVAG *et al.*[25] concluded that the enzyme would be located in the cell wall, although this was inconsistent with the lack of activity of intact cells also observed by them. The most plausible explanation would seem to be that the α-glycerophosphatase is located inside the protoplast membrane but can leave the cell in a suspension of dried yeast (with its disrupted membranes), to react with an anti-enzyme in solution.

It is likely that, physiologically speaking, α-glycerophosphatase will be involved in the regulation of the level of α-glycerophosphate in the cell, an important intermediate in the synthesis of triglycerides and phospholipids. Under certain conditions larger amounts of α-GP can also be formed by reduction of dihydroxyacetone phosphate when this reaction is stimulated as an alternative for the regeneration of DPN from DPNH. It is impossible to state whether such a regulation of the α-GP level is an exclusive function of this enzyme or to what extent non-specific phosphatases also capable of hydrolysing α-GP participate, until we know more about the relative sites of the latter enzymes and α-GP in the yeast cell.

S. carlsbergensis (No. 74) contains only one non-specific alkaline phosphatase. It is activated by Mg^{2+}. In some respects it resembles the alkaline phosphatase of bottom yeast studied by HOFFMANN-OSTENHOF *et al.*[27] (inhibition by CN^-, pH of optimum activity towards phenyl phosphate). But our enzyme differs in that it is not inhibited by P_i and can be completely reactivated by Mg^{2+} after dialysis. It is quite possible that alkaline phosphatases are not alike in all yeasts; sometimes it is not found at all in bottom yeast, more than one has been found in baker's yeast, and its content in this yeast depends upon the phosphate concentration of the medium of culture[15].

It has not been possible to designate one particular kind of cell particles as the location of the sedimentable portion of the alkaline phosphatase. There seem to be two possibilities: either the enzyme is contained in more than one kind of particle; as such nuclei, mitochondria, ribosomes and vacuole walls could come under consideration, or, should only one type of particle be the carrier of the enzyme, nuclei and vacuole walls could not qualify for this role. If mitochondria would be the sole carrier, the observed pattern of sedimentation could be explained if they were to

occur in various sizes (younger cells with lighter mitochondria), and some mitochondria would be fragmented during the desintegration of yeast cells or protoplasts. In the case of ribosomes being the sole carrier, one would have to assume an extensive aggregation of these particles upon desintegration.

The change in the pattern of distribution of the alkaline phosphatase in the aging culture is quite surprising. It would appear that the age of the culture can not only determine the amount of enzyme found in the yeast cell[15], but also its location. Various possibilities can be conceived for this shift in location: as the cell grows older, the phosphatase passes from the particles into the cytoplasm, or, second, the amount of enzyme in the cytoplasm increases, while that in the particles remains constant, or increases to a lesser degree, or, third, the enzyme becomes more loosely bound to the particles, so that some passes into the soluble fraction upon desintegration.

Because neither the exact location of the alkaline phosphatase nor its physiological substrate is known, it is not yet possible to speculate upon its function in the metabolism of the yeast cell. As a number of alkaline phosphatases possess phosphotransferase properties, it could be that our enzyme in part also acts as a transferase *in vivo*.

A general impression gained from these experiments is that the non-specific phosphatases exhibit a considerable variability in yeast. This regards their presence in a certain strain, and, if present, their concentration and location in the cell. The determining factors seem to be: the type of the yeast, the age of the culture, and the composition of the growth medium (see also ref. 15).

Another general conclusion we can draw is that further experiments on the localization of enzymes in yeast cannot be expected to yield unequivocal results until more is known regarding the identification and preparation of the structural components of the yeast cell.

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