Regulation of Acid Phosphatase Synthesis in Baker's Yeast Protoplast by Phosphate Compounds

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The regulation of acid phosphatase synthesis by various phosphate compounds was examined in Baker's yeast protoplasts. Synthesis was repressed by inorganic phosphate and phosphomonoesters. Phosphomonoesters were hydrolysed by a small amount of non-specific acid phosphatase present in the protoplast membrane. The inorganic phosphate that was liberated and incorporated into protoplasts probably repressed acid phosphatase synthesis. Phosphodiester, such as 3', 5'-cyclic AMP, 3', 5'-cyclic CMP and 3', 5'-cyclic GMP, promoted acid phosphatase synthesis. The effect of 3', 5'-cyclic AMP was not to overcome hexose repression, because high hexose did not repress acid phosphatase synthesis. 3', 5'-cyclic AMP did not overcome repression of the enzyme synthesis by inorganic phosphate. From these observations 3', 5'-cyclic nucleotides probably had some effect on the yeast acid phosphatase-synthesizing system but the exact role of the nucleotides is obscure.

Saccharomyces yeast has two non-specific acid phosphatases. One is repressible and the other is non-repressible by inorganic phosphate,^{1,2)} although activity of the latter enzyme is about 130 times less than that of the former one under inorganic phosphate-deficient condition.²⁾ Extensive studies on the expression of genes controlling the acid phosphatase synthesis have been made.^{1,3~6)}

The acid phosphatases are located on the surface of the yeast cell,^{7~9)} which may indicate the possibility of adaptation to outer nutritional environment. It is known that yeast protoplasts when cultured in liquid, osmotically stabilized medium containing a suitable source of energy secrete most of the compounds normally present in the cell wall. Acid phosphatase, invertase, melibiase and mannan peptides are released into the medium.^{10~12)}

Since little is known about the physiological role and the mechanism of the acid phosphatase synthesis in Baker's yeast, it is interesting to examine whether or not adaptation of acid phosphatase synthesis in the yeast protoplasts occurs in media containing various phosphate compounds such as phosphomonoesters and

phosphodiester, because no reports focused on this subject have been found.

This communication describes the effect of inorganic phosphate, phosphomonoesters and phosphodiester on the acid phosphatase synthesis and shows that inorganic phosphate and phosphomonoesters repress acid phosphatase formation but some of the phosphodiester promote the enzyme synthesis. The implications of these findings with respect to the regulation of acid phosphatase formation in yeast protoplasts are discussed.

MATERIALS AND METHODS

Organism. Baker's yeast (*Saccharomyces cerevisiae*, IAM 4419) was used throughout this work.

Media and cultural conditions. The basal medium contained, in 1 liter, 10 g of glucose, 1.05 g of $(\text{NH}_4)_2\text{SO}_4$, 0.6 g of KCl, 0.5 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 2.74 g of $\text{Na}_2\text{-citrate} \cdot (3/2)\text{H}_2\text{O}$, 0.1 g of $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$, 500 μg of H_3BO_3 , 400 μg of $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 200 μg of $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 200 μg of $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, 200 μg of $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 100 μg of KI, 40 μg of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 2 mg of inositol, 400 μg of calcium pantothenate, 400 μg of pyridoxine-HCl, 400 μg of thiamine-HCl and 2 μg of D-biotin. The pH was adjusted to 5.2. The basal medium was supplemented with KH_2PO_4 at a growth-limiting concentration (0.021 g per liter). All cultures

were incubated at 30°C with shaking. The phosphate-deficient cells cultured for 24 hr were harvested, washed twice with distilled water, and suspended at a concentration of 2×10^8 cells per milliliter in 33 mM Tris-HCl buffer (pH 7.5) containing 0.64 M KCl.

Preparation of protoplasts. Three milligrams of the cell wall-lysing enzyme "Zymolyase" (Kirin Brewery Co., Ltd., Takasaki, Gumma, Japan) and 150 mg of cellulase "Onozuka" (Kinki Yakult Co., Ltd., Nishinomiya, Hyogo, Japan) were added to 5 ml of the buffered cell suspension and digestion was allowed to proceed at 30°C with shaking of 44 rpm. Practically complete conversion of cells into protoplasts was achieved after 1 hr incubation, which was confirmed by observation with an electron microscope.¹³⁾

Cultivation of protoplasts. If not otherwise stated, the protoplasts were washed twice with 0.8 M mannitol and suspended at a concentration of 2×10^8 cells per milliliter of 0.8 M mannitol. The protoplast cultivation was started by adding an equal volume of P2 medium which contained 2 $\times$ the basal medium, 132 mM sodium aspartate and 0.8 M mannitol. Hereafter the final protoplast cultivation medium will be designated as P1 medium. Protoplasts were cultivated at 30°C with shaking of 44 rpm. When indicated 0.8 M mannitol was replaced by 0.64 M KCl as osmotic stabilizer.

Assay of acid phosphatase formed by protoplasts. One ml of protoplast suspension was centrifuged at $2000 \times g$ for 5 min and the resultant supernatant fluid was assayed for acid phosphatase activity by the method of Torriani.¹⁴⁾ The reaction mixture contained, 0.12 M sodium acetate buffer (pH 4.0), 16 mM *p*-nitrophenylphosphate (NPP) and the enzyme, in a final volume of 0.5 ml, and was incubated at 37°C. The reaction was terminated by adding a mixture of 1 ml of 1 M Tris-HCl (pH 8.5) containing 0.4 M K_2HPO_4 and 0.5 ml of water. In determining the activity of the acid phosphatase on substrates other than NPP, the inorganic phosphate released was determined by the method of Nakamura and Mori.¹⁵⁾ One unit of enzyme was defined as the amount of enzyme required to produce 1 μ mol of *p*-nitrophenol (NP) per hr at 37°C.

Glucose-1-phosphate (g-1-p) determination. Two ml of g-1-p-containing solution and 0.5 ml of 15% H_2SO_4 were added to a test tube, which was then covered loosely with a glass stopper, and the tube was heated for 7 min in a boiling water bath. After the tube is cooled to 20°C, 0.5 ml of a mixture of 0.4% amidole-8% $NaHSO_3$ and 0.5 ml of 3.3% ammonium molybdate were added successively to it, and then the reaction mixture was adjusted to 5 ml with water and left at 20°C. After 20 min the color developed was read at 740 nm with a Beckman DB spectrophotometer.

Chemicals. NPP was purchased from Daiichi Chemical Co., Ltd., and g-1-p, glucose-6-phosphate (g-6-p) and β -glycerophosphate from Boehringer (Manheim, F.R.G.). Adenosine 5'-monophosphate (AMP), adenosine 5'-diphosphate (ADP), adenosine 5'-triphosphate (ATP), 3', 5'-cyclic AMP, 3', 5'-cyclic CMP, 3', 5'-cyclic GMP, 3', 5'-cyclic UMP, 2', 3'-cyclic AMP, 2', 3'-cyclic CMP, 2', 3'-cyclic GMP, 2', 3'-cyclic UMP and 3', 5'-cyclic dibutyryl AMP were obtained from Sigma Chemical Co., St. Louis, Mo. Uridine 5'-monophosphate (UMP) and inosine 5'-monophosphate (IMP) were gifts from Yamasa Shoyu Co., Ltd., (Choshi, Chiba, Japan).

RESULTS

Acid phosphatase-synthesizing system using protoplasts

Preliminary experiments were performed to test whether acid phosphatase was formed by protoplasts in the P1 medium with or without 0.8 M mannitol as osmotic stabilizer. In the former condition acid phosphatase was produced, but, in the latter case, it was not because

FIG. 1. Acid Phosphatase Formation by Yeast Cells and Protoplasts.

Methods of yeast cell culture and making protoplasts were described in the text. Intact cells and protoplasts were suspended at 1×10^8 per ml in P1 medium. One ml samples of the incubation mixture were centrifuged at $2000 \times g$ for 5 min and the acid phosphatase activity of the resultant supernatant fluid was measured.

○, intact cells; ●, protoplasts.

of the lysis of the protoplasts. The amount of acid phosphatase released into the medium by intact cells was less than 8% of that observed with protoplasts (Fig. 1). Yeast alkaline phosphatase which is known to be localized inside protoplasts⁷⁾ was not observed in the P1 medium (data not shown), indicating that protoplasts were scarcely lysed throughout the course of the incubation. Since both of the acid phosphatases present in the cell wall and secreted in the medium gave the maximum enzymic activity at pH 4.0~4.3 (data not shown), the acid phosphatases could be the same with regard to enzymatic properties. Therefore, the protoplast system was shown to be a good system for determining the acid phosphatase synthesis in yeast cells.

Effect of inorganic phosphate on acid phosphatase formation

Protoplasts of Baker's yeast were cultivated in media containing various amounts of inorganic phosphate at 30°C and the activity of the acid phosphatase synthesized was determined. Results shown in Fig. 2 indicated that the total activity of acid phosphatase secreted in

FIG. 2. Effect of Inorganic Phosphate on Acid Phosphatase Formation by Yeast Protoplast.

Protoplasts were incubated in the P1 medium containing KH_2PO_4 as indicated.

●, zero; ○, 0.5 mM; △, 1 mM; □, 5 mM.

the medium increased as the concentration of inorganic phosphate was reduced, and that in the medium free of phosphate the highest level of acid phosphatase was produced. These results were consistent with the observation by Schmidt *et al.*,⁹⁾ and Van Rijn *et al.*¹⁶⁾ that the acid phosphatase was derepressed in a medium whose concentration of inorganic phosphate was low.

Effect of phosphomonoesters on acid phosphatase formation

Table I shows that all of the phosphomonoesters such as NPP, g-1-p, g-6-p, AMP, IMP, UMP, ADP, ATP and β -glycerophosphate repressed the acid phosphatase formation.

TABLE I. EFFECT OF PHOSPHOMONOESTERS AND PHOSPHODIESTERS ON ACID PHOSPHATASE FORMATION BY YEAST PROTOPLAST

Effector	Acid phosphatase ^c	
	(Units/ml)	(%)
Phosphomonoesters ^a		
None	14.8	100
g-1-p	0	0
g-6-p	0	0
AMP	0	0
IMP	0	0
UMP	0	0
ADP	0	0
ATP	0	0
β -Glycerophosphate	0	0
NPP	0	0
Phosphodiester ^b		
None	10.1	100
3', 5'-Cyclic AMP	13.6	134
3', 5'-Cyclic CMP	13.6	134
3', 5'-Cyclic GMP	14.3	142
3', 5'-Cyclic UMP	10.5	103
3', 5'-Cyclic dibutyryl AMP	10.7	106
2', 3'-Cyclic AMP	11.6	115
2', 3'-Cyclic CMP	9.4	93
2', 3'-Cyclic GMP	11.2	110
2', 3'-Cyclic UMP	10.8	107

^{a, b} Concentrations of phosphomonoesters and phosphodiester^b were 5 mM and 2.5 mM, respectively.

^c Three hr cultured protoplast suspension was centrifuged at $2000 \times g$ for 5 min and the acid phosphatase activity of the supernatant was measured.

FIG. 3. Liberation of NP and Inorganic Phosphate from NPP during Protoplast Cultivation.

Protoplasts were incubated with 1 mM (-----) or 4 mM (—) of NPP in the PI medium and the liberated NP (●) and inorganic phosphate (▲) in the culture supernatant were determined.

Since it was reported that intact yeast cells are impermeable to phosphorylated sugars,¹⁷⁾ it was postulated that each phosphomonoester is degraded to a phosphate moiety and a non-phosphate moiety by a small amount of acid phosphatase present in the protoplast membrane, then the phosphate moiety liberated is taken into protoplasts and affects the phosphatase synthesis. In order to confirm the above hypothesis, protoplasts were cultivated in the PI medium containing NPP, and then NP and inorganic phosphate in the medium were determined. Figure 3 shows that NP liberated from 4 mM NPP in the medium increased linearly for the first 1.5 hr, then the rate of NP liberation slowed down. On the other hand inorganic phosphate was not observed in the medium for the first 1 hr. Since it was released parallel with the NP, it must have been taken up by the protoplasts. Figure

3 also shows that NP was stoichiometrically liberated from 1 mM NPP, indicating that NP was scarcely incorporated into protoplasts. The fact that the hydrolysis of the NPP to NP and inorganic phosphate by protoplast suspensions began from zero time of the incubation suggested that some acid phosphatase activity was present in the membrane of protoplasts. Van Rijn *et al.*¹⁸⁾ shows that secreting protoplasts always contain a small amount of acid phosphatase, firmly bound, even after lysis. In fact, we confirmed that a low level of acid phosphatase activity (0.01~0.05 units/ml) was present in the protoplasts at the initiation of the incubation. This protoplast-bound acid phosphatase is known to be a precursor of the exocellular enzyme, and both of the acid phosphatases are exactly the same with regard to enzymatic and stability properties.¹⁸⁾ Therefore, the substrate specificity of secreted acid phosphatase for phosphomonoesters was examined. As is shown in Table II, acid phosphatase hydrolyzed phosphomonoesters without particular specificity. Both the profile

TABLE II. SUBSTRATE SPECIFICITY OF ACID PHOSPHATASE FOR VARIOUS PHOSPHATE COMPOUNDS

Substrate ^a	Relative activity
NPP	100
AMP	56
ADP	47.5
ATP	17.6
IMP	85
UMP	62.5
g-1-p	103
g-6-p	146
β-Glycerophosphate	82.5
3', 5'-Cyclic AMP	0
3', 5'-Cyclic CMP	0
3', 5'-Cyclic GMP	0
3', 5'-Cyclic UMP	0
3', 5'-Cyclic dibutyl AMP	0
2', 3'-Cyclic AMP	0
2', 3'-Cyclic CMP	0
2', 3'-Cyclic GMP	0
2', 3'-Cyclic UMP	0

^a Substrate specificity was examined by measuring the released inorganic phosphate according to the method described in MATERIALS AND METHODS. Concentration of substrate was 5.2 mM.

of the liberation of NP and inorganic phosphate from NPP and the substrate specificity of the acid phosphatase support the above hypothesis.

Another line of evidence supports the hypothesis. The rates at which KH_2PO_4 and g-1-p

FIG. 4A. Change of the Concentration of G-1-P or Inorganic Phosphate during Protoplast Cultivation. Protoplasts were incubated with 1 mM of G-1-P (—○) or KH_2PO_4 (---○) in the PI medium osmotically supported by 0.64 M KCl and the concentration of the inorganic phosphate (●) and the G-1-P (○) in the culture supernatant were determined.

FIG. 4B. Transferred Culture of Protoplasts Incubated with G-1-P or Inorganic Phosphate to Phosphate-free Medium.

Protoplasts pre-incubated with G-1-P (○—○) or KH_2PO_4 (○---○) for 30 min as is shown in Fig. 4A were washed twice with 0.64 M KCl and transferred to the phosphate-free PI medium and the acid phosphatase activity in the culture supernatant was measured. As a control protoplasts (●—●) which were not incubated with G-1-P nor inorganic phosphate were cultured in the PI medium in the same way.

disappeared when added to protoplasts in the PI medium, supported osmotically by 0.64 M KCl instead of 0.8 M mannitol, were different; the amount of incorporation of KH_2PO_4 was 9×10^{-4} mol per 30 min, whereas that of g-1-p was 1.5×10^{-4} mmol per 30 min (Fig. 4A). Protoplasts incubated either with 1 mM KH_2PO_4 or with 1 mM g-1-p in the PI medium for 30 min were centrifuged, washed twice with 0.64 M KCl, and transferred to the phosphate-free PI medium. The amount of acid phosphatase secreted was determined. The protoplasts which were treated with 1 mM KH_2PO_4 did not produce acid phosphatase (Fig. 4B). On the contrary, the protoplasts treated with 1 mM g-1-p produced acid phosphatase as well as untreated protoplasts. However, if protoplasts were incubated continuously with 1 mM g-1-p in the PI medium, acid phosphatase was not produced (data not shown). These results prove the validity of the above hypothesis that phosphomonoesters regulate the acid phosphatase synthesis through the inorganic phosphate which is derived from phosphomonoesters by hydrolysis and is incorporated into protoplasts.

Effect of phosphodiester on acid phosphatase formation

Table I shows that 3', 5'-cyclic AMP, 3', 5'-cyclic CMP and 3', 5'-cyclic GMP increased the amount of acid phosphatase synthesis by 34 to 42%, whereas other cyclic nucleotides did not. Since yeast acid phosphatase does not hydrolyze the phosphodiester (Table II), 3', 5'-cyclic AMP, 3', 5'-cyclic CMP and 3', 5'-cyclic GMP are probably incorporated intact into protoplasts and give some effects favorable for acid phosphatase formation. 3', 5'-cyclic AMP is known to overcome repressible effect of glucose on the synthesis of β -galactosidase and tryptophanase in *Escherichia coli*,¹⁹⁾ therefore it was examined whether or not yeast acid phosphatase synthesis was repressed by high concentration of glucose. High glucose (5%) did not repress acid phosphatase synthesis, rather it stimulated the enzyme synthesis (Fig. 5).

FIG. 5. Effect of 3',5'-cyclic AMP and Glucose Concentration on Acid Phosphatase Formation in Yeast Protoplasts.

Protoplasts were incubated with 1% glucose (●) or 5% (○) glucose in the presence (—) or absence (-----) of 2.5 mM 3',5'-cyclic AMP. Glucose and 3',5'-cyclic AMP were added at time zero.

3',5'-cyclic AMP did not overcome the inorganic phosphate repression of acid phosphatase synthesis (data not shown).

DISCUSSION

For studying regulation of yeast acid phosphatase synthesis, protoplasts have various advantages; they are devoid of acid phosphatase present in yeast cell surface and protoplasts do not divide, so the concentration remains constant and, under favorable conditions, protoplasts are able to secrete newly formed acid phosphatase.¹¹⁾ In the experimental conditions described here, the cell wall regeneration was not observed by electron microscope (data not shown), so all the amount of acid phosphatase synthesized by protoplasts is considered to be secreted in the medium. In the present study effect of inorganic phosphate, phosphomonoesters and phosphodiester on acid phosphatase formation in Baker's yeast protoplasts is described in detail.

Repression of acid phosphatase formation by inorganic phosphate was confirmed as has

been reported,^{9,16)} and 1 mM phosphate in the P1 medium was enough to repress acid phosphatase synthesis. This result coincided well with that obtained by Van Rijn *et al.*¹⁶⁾ Although *S. cerevisiae* has two kinds of non-specific acid phosphatases, *i. e.*, repressible and constitutive acid phosphatases,^{1,2)} the level of acid phosphatase activity in the repressed condition is very low (Fig. 2), indicating that the amount of constitutive enzyme is negligible in the experimental systems described here even under derepressed condition.

Phosphomonoesters were suitable substrates for non-specific acid phosphatases,²⁾ therefore, phosphomonoesters were expected to derepress or induce acid phosphatase synthesis, since usually induction of enzyme occurs so that substrate can be utilized through hydrolysis of substrates by the induced enzyme. But as it is, phosphomonoesters did not derepress nor induce acid phosphatase synthesis (Table I). The phenomenon was examined in detail. Phosphomonoesters were hydrolyzed by acid phosphatase present in the protoplast membrane very quickly (Fig. 3, Table II) and the inorganic phosphate liberated by the enzyme is incorporated into protoplasts and repressed the acid phosphatase synthesis (Fig. 4A, 4B). Therefore it can be stated that not only inorganic phosphate but also phosphomonoesters regulate yeast acid phosphatase synthesis. Borst Pauwels *et al.*²⁰⁾ reported that radioactive orthophosphate was incorporated into phosphate-deficient yeast cells by active transport, and that ATP, GTP and UTP were the compounds phosphorylated primarily. The radioactive nucleotide triphosphates were detected as early as 1 sec after addition of ³²P at 1°, suggesting that phosphate incorporation is very rapid. They did not show the radioactive inorganic phosphate in the yeast cells in the experiment, nevertheless, it was a very short period incorporation experiment. If incubated a little longer, inorganic phosphate could have been detected in the cell. The results seem to be consistent with our results that NP was observed in the NPP-containing medium for the

1 hr incubation of protoplasts whereas inorganic phosphate was not (Fig. 3). The inorganic phosphate incorporated probably represses the expression of acid phosphatase structural gene (*phoE*)⁶⁾ through holorepressor composed of inorganic phosphate and aporepressor, a gene product of *phoR* and *phoU*.⁴⁾

Phosphodiesterases such as 3', 5'-cyclic AMP, 3', 5'-cyclic CMP and 3', 5'-cyclic GMP promoted acid phosphatase synthesis by 34 to 42%. The other cyclic nucleotides did not (Table I). The effect of 3', 5'-cyclic AMP was not an overcoming of hexose repression, because high glucose (5%), which represses invertase formation in the yeast protoplasts under the experimental conditions described here (data not shown), promoted acid phosphatase synthesis as compared with low glucose (Fig. 5). High glucose (5%) may decrease the level of inorganic phosphate in the protoplasts by converting the inorganic phosphate into hexose- or glycerol-phosphate, because, as an enzyme source, 0.2% hexose was shown to be sufficient for the biosynthesis of invertase, a glycoprotein enzyme in yeast.²¹⁾ 3', 5'-cyclic AMP also promoted the acid phosphatase synthesis in high glucose medium, suggesting 3', 5'-cyclic AMP and high glucose differ in its effect on acid phosphatase synthesis (Fig. 5). 3', 5'-cyclic AMP, 3', 5'-cyclic CMP and 3', 5'-cyclic GMP may have some effect on the yeast acid phosphatase-synthesizing system directly or indirectly. But it is obscure how the nucleotides affect the phosphatase synthesis, and the exact role of these nucleotides will be clarified if the cell-free acid phosphatase-synthesizing system is established.

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