Occurrence of Acid Phosphatase- isozymes Repressible by Inor- ganic Phosphate in Rice Plant Cell Cultures

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Received September 18, 1972

The phenomena of controlled formation of alkaline phosphatases by inorganic phosphate have been observed in *Escherichia coli*^{1,2)} and *Bacillus subtilis*,³⁾ whereas the bacterial acid phosphatases are not repressed by inorganic phosphate. On the other hand, the acid phosphatases of *Aspergillus* species^{4~6)} have been reported as repressible enzymes. It is also found that *Euglena gracilis*^{7,8)} and *Neurospora crassa*⁹⁾ produce the acid or alkaline phosphatases, which are involved in constitutive and induced (repressible) enzymes.

In higher plants, increased yields of acid enzymes in phosphate-deficient medium have been reported with tomato leaves,¹⁰⁾ germinating barley seeds¹¹⁾ and tobacco callus tissues.¹²⁾

The present communication is concerned with syntheses of different type of the intracellular soluble acid phosphatase isozymes occurring in rice plant cell cultures in response to the phosphate concentration in a medium.

The rice plant callus cells were derived from the seed of variety Nipponkai, *Oryza sativa* L. subsp. *Japonica* and were routinely grown in Murashige and Skoog's inorganic salt medium¹³⁾ supplemented with 2,4-D (2 mg/liter), thiamine-HCl (5 mg/liter), inositol (100 mg/liter) and sucrose (30 g/liter) (MS medium). The stock culture was grown in the same medium containing 80% alcohol-soluble fraction of commercial yeast extract (5 g/liter, Ebiosu P2G). The cells (4 g of wet weight) precultured for a week in the same medium were inoculated in a 500 ml Sakaguchi flask containing 120 ml

medium. The flasks were incubated for the specified length of time at 28°C in the dark on a reciprocal shaker.

After the incubation, the cells were harvested by filtration through double layers of gauze and weighed for the growth determination. The washed cells were homogenized in a Potter-Elvehjem homogenizer in cold water. The brei was centrifuged in the cold at $600 \times g$ for 10 min and the residue was washed three times with distilled water by centrifugation. All the supernatants were combined and further centrifuged at $28,000 \times g$ for 20 min to give the cytoplasmic fraction.

For the enzyme assay, 15 μ moles of phosphate ester, 250 μ moles of acetate buffer (pH 5.5) and an appropriate amount of enzyme solution in a total volume of 1 ml were incubated for 10 min at 37°C.

The reaction was stopped by the addition of 1 ml of 10% trichloroacetic acid, and any insoluble material was removed by centrifugation. Inorganic phosphate liberated was determined by the method of Nakamura *et al.*¹⁴⁾ One unit of activity was defined as the amount of enzyme which liberating 1 μ mole of inorganic phosphate per hr under the conditions indicated above. Acid phosphatase isozymes were separated by polyacrylamide disc electrophoresis as described by Davis.¹⁵⁾ Electrophoresis was carried out for 1.5 hr at 3 mA per gel. The enzyme activity was stained according to the procedure described by Ogita,¹⁶⁾ using α -naphthylphosphate and Fast Blue B salt.

Densitometric tracing of the enzyme bands was carried out by a Ozumor-82 (Yamato) with a 520 m μ filter. The activity of each isozyme was determined as the rate of the stain intensity to the total activity applied to the gel. When 4 g of the initial cells were incubated for a week, the total enzyme activity in the cytoplasm was 1.5×10^3 units using α -naphthylphosphate as the substrate. The electrophoretic pattern showed 3 main activity bands, isozymes 1, 2 and 3, as shown in Fig. 1 (a).

Endo *et al.*¹⁷⁾ have observed that acid

FIG. 1. Densitometric Patterns of Acid Phosphatase Isozymes in the Cytoplasmic Fraction.

The origin is at the left end and the enzyme migration was toward the + electrode.

(a) initial cells precultured in a MS medium for 7 days, (b) 5-day-old cells in a phosphate-deficient medium, (c) 4-day-old cells after adding 150 μ moles of inorganic phosphate to (b), (d) 5-day-old cells in a phosphate-rich medium (450 μ moles phosphate/120 ml medium), (e) 11-day-old cells in a phosphate-rich medium, (f) 18-day-old cells in a phosphate-rich medium.

phosphatase zymograms of the leaves of cultivated rice plant, Japonica type, by starch gel electrophoresis showed 3 main activity bands and 2 other minor bands, and have analyzed the genetic systems specifying these zymograms. From the intensity of the activity stain, these 3 major bands might correspond to those of isozymes 1, 2 and 3 in Fig. 1 (a).

In the cells incubated in a phosphate-deficient medium, it can be seen that the electrophoretic pattern of the cytoplasmic enzyme showed a remarkable change. The ratio of isozyme 1 to other isozymes apparently decreased at the 2nd day as shown in Fig. 1 (b). When the same amount of phosphate equivalent to that of the original MS medium was added at the 5th day, the pattern was restored as shown in Fig. 1 (c). When the cells were transferred in a phosphate-rich medium (450 μ moles/120 ml, 3 times higher concentration than that of the MS medium), isozyme 3 decreased at first followed by isozyme 2 (Fig. 1 (d)). After the 11th day, almost one band of isozyme 1 was observed (Fig. 1 (e)). But after long culture periods (at the 18th day) isozymes 2 and 3 appeared again

and increased, probably due to derepression (Fig. 1 (f)). To obtain more quantitative informations, the change of the activity of each isozyme was determined using α -naphthyl-phosphate as the substrate. The results are shown in Fig. 2. In the case of Fig. 2 (A), the total activity rapidly increased as the time proceeded; whereas isozyme 1 increased scarcely in parallel with the cell growth. The pattern was apparently inconsistent with that of Fig. 1 (b). This might be due to the fact that isozymes 2 and 3 were derepressed and increased markedly.

In the case of Fig. 2 (B), the total activity was repressed contrary to the above results. While the synthesis of isozyme 1 was coincided with the cell growth, that of isozyme 3 was remarkably repressed. The above results suggested that isozyme 1 was constitutively synthesized despite the existence of phosphate, but the formation of isozymes 2 and 3 was controlled by phosphate and that of isozyme 3 was more sensitive to phosphate. The phosphate concentration in the phosphate-deficient medium remained almost constant in both cytoplasm and medium, while in the case of the phosphate-rich medium, phosphate

FIG. 2. Time Course of Acid Phosphatase Activity and the Growth of Cells in Phosphate-deficient and -rich Media.

(A) phosphate-deficient medium, (B) phosphate-rich medium (450 μ moles phosphate/120 ml medium)

●—●, total activity; ○—○, activity of isozyme 1; □—□, activity of isozyme 2; △—△, activity of isozyme 3; ▲—▲ growth (wet weight), ×—×, inorganic phosphate in the cytoplasm; ×—×, inorganic phosphate in the medium.

in the medium was incorporated rapidly into the cells within 5 days, and it was secreted into the medium again after the 9th day. Intracellular phosphate increased up to about the 10th day, after which phosphate decreased rapidly owing to its secretion into the medium.

The increment of activity in the phosphate-deficient medium was inhibited completely for at least 4 days by the addition of cycloheximide (2 μ g/ml) and the addition of phosphate also showed similar results.

It is conceivable that such a phosphate-phosphatase isozymes regulatory system will play an important role in the phosphorus metabolism of the rice plant callus cells. Further study is now in progress to investigate the functional and structural differences among these isozymes.

The authors thank Mr. M. Hirooka for his technical assistance in part of this experiment. Thanks are also due to Mr. K. Matsui, Ebiosu Pharmaceutical Industry for providing the yeast extract.

REFERENCES

- 1) T. Horiuchi, S. Horiuchi and D. Mizuno, *Nature*, **183**, 1529 (1959).
- 2) A. Torriani, *Biochem. Biophys. Acta*, **38**, 460 (1960).
- 3) C. Anagnostopoulos, *Federation Proc.*, **19**, 48 (1960).
- 4) Y. Ohta, K. Ikeda and S. Ueda, *Appl. Microbiol.*, **16**, 973 (1968).
- 5) T. R. Shieh, R. J. Wodzinski and J. H. Ware, *J. Bacteriol.*, **100**, 1161 (1969).
- 6) T. Komano, The Annual Meeting of Japanese Biochemical Society, Sendai, 1971, p. 615.
- 7) C. A. Price, *Science*, **135**, 46 (1962).
- 8) J. J. Blum, *J. Cell Biol.*, **24**, 223 (1965).
- 9) A. Toh-e and T. Ishikawa, *Genetics*, **69**, 339 (1971).
- 10) E. J. Hewitt and P. Tatham, *J. Exptl. Bot.*, **11**, 367 (1960).
- 11) R. Bianchetti and M. L. Sartirana, *Biochem. Biophys. Acta*, **145**, 485 (1967).
- 12) K. Ueki and S. Sato, The Second Symposium for Plant Tissue Culture, Kyoto, 1970, p. 40.
- 13) T. Murashige and F. Skoog, *Physiol. Plantarum*, **15**, 473 (1962).
- 14) M. Nakamura, K. Yamazaki and B. Maruo, *Nippon Nogetikagaku Kaishi*, **24**, 197 (1951).
- 15) B. J. Davis, *Ann. New York Acad. Sci.*, **121**, Art. 2 404 (1964).
- 16) Z. Ogita, *SABCO J.*, **2**, 91 (1966).
- 17) T. Endo, B. B. Shahi and C. Pai, *Japan. J. Genetics*, **46**, 147 (1971).