The intracellular localization of acid and alkaline phosphatases in *Aspergillus oryzae* A 1-5 was studied cytochemically with light and electron microscopy. Two different acid phosphatases were present, one (acid phosphatase I) having a pH optimum of 4.0 and the other (acid phosphatase II) of 5.5. They were shown to be localized at different sites in the fungal mycelium under this experimental condition; acid phosphatase I was demonstrated to be present at the surface of cell wall, whereas acid phosphatase II both at the surface of cell wall and at the cytoplasmic membrane. It was demonstrated cytochemically that excess amount of inorganic phosphate in growth medium promoted the formation of acid phosphatase I but repressed that of acid phosphatase II. Alkaline phosphatase was found to be localized in nucleoli and unknown electron-dense bodies in the cytoplasm. It was also seen at the surface of cell wall and at the cytoplasmic membrane.

Acid and alkaline phosphatases have been reported to occur in fungi, such as, Aspergilli (1-12), Neurospora (13-15), Penicillium (16), and Fusarium (17, 18). In Aspergilli, it was found that at least two phosphatase activities of different pH optima were present in both acid and alkaline ranges (2, 4, 5, 9, 10). The cytochemical demonstration of the activities of phosphatases in fungi has been made by a few workers (19-25). However, the exact intracellular localization of phosphatases in fungal mycelia at electron microscope level is not always well under-
stood (26), and very little is known about the cytochemical localization of multiple forms of phosphatases in fungi, each having different pH optimum and biochemical characteristics.

The present investigation was carried out in an attempt to clarify the intracellular localization of acid and alkaline phosphatases in mycelia of *Aspergillus oryzae* and further to differentiate cytochemically the localization of two different acid phosphatases. The changes in the activities of two acid phosphatases in mycelia grown under different culture conditions were also investigated cytochemically at light and electron microscope levels.

**MATERIALS AND METHODS**

*Organism.* Aspergillus oryzae A 1–5 was used throughout this work.

*Medium and cultivation.* Three loopfuls of conidia were inoculated into a 500-ml culture flask containing 50 ml of basal medium composed of 50 g of glucose, 20 g of Polypepton (Daigo Eiyo Chemical Co., Osaka), 1 g of KH₂PO₄, 0.5 g of MgSO₄·7H₂O, 0.1 g of CaCl₂·2H₂O, and 1 liter of tap water (pH 5.5). High phosphate medium contained 5 g of KH₂PO₄. The flasks were shaken (7 cm/stroke, 120 rpm) at 30°.

*Preparation of intracellular enzyme solution.* Mycelia collected by filtration of the culture broth were washed with deionized water and crushed with quartz sand in a porcelain mortar with a pestle. The crushed mycelia were suspended in deionized water. The suspension was centrifuged at 8,000 × g for 20 min and the supernatant fluid was used as the enzyme solution.

*Measurement of phosphatase activity.* Phosphatase activity was measured by a modification of the method of Torrani (27) using disodium p-nitrophenyl phosphate (p-NPP) (Nakarai Chemical Co., Kyoto) as substrate. The reaction mixture consisted of 1 ml of the enzyme solution, 3 ml of buffer solution, and 1 ml of substrate solution (0.02 M p-NPP). The buffers used were 0.1 M glycine-HCl buffer (pH 3.0), 0.1 M β,β'-diethylglutaric acid-NaOH buffer (pH 3.0–7.5), 0.1 M tris (hydroxymethyl) aminomethane (Tris) buffer (pH 7.5–8.0), and 0.1 M diethanolamine-HCl buffer (pH 8.0–10.0). The reaction was carried out at 40° for 10 min and stopped by adding 5 ml of 1.0 M Na₂CO₃ solution. The amount of p-nitrophenol liberated was determined by a Hitachi Model 101 spectrophotometer as an absorbancy at 400 nm. The enzyme activity was expressed by µmol of p-nitrophenol liberated per min per g dry weight of mycelia.

*Dry weight measurement.* Mycelia obtained after filtration of the culture broth were dried at 105° and the dry weight of mycelia per culture flask was measured.

*Determination of glucose.* Glucose was determined by the method of Sumner using 3,5-dinitrosalicylic acid (28).

* Determination of inorganic phosphate.* Inorganic phosphate was determined by the method of Nakamura (29).
Cytochemical demonstration of phosphatases. Acid and alkaline phosphatases in fungal mycelia were demonstrated cytochemically by the deposition of lead phosphate as the reaction product by the methods of Gomori (30) modified by Barka and Anderson (31) and of Mayahara et al. (32).

For electron microscopy, the collected mycelia were washed with chilled deionized water and fixed in 2% (v/v) glutaraldehyde in 0.1 M sodium cacodylate buffer at pH 7.2 at 1-4°C for 60 min for acid phosphatases and for 15 min for alkaline phosphatase. The fixed mycelia were then washed with the same buffer.

For the demonstration of phosphatases, the fixed mycelia were incubated at 30°C for 60 min in the following incubation media: The Barka and Anderson's medium used for acid phosphatases was composed of 10 ml of 0.1 M Tris-maleate buffer (pH 4.0 and 5.5), 10 ml of 1.25% disodium β-glycerophosphate (β-GP) (Sigma Chemical Co., St. Louis, Mo.) (or 1.86 % p-NPP), 10 ml of deionized water and 20 ml of 0.2% lead nitrate. The final pH was adjusted to 4.0 and 5.5 with either NaOH or HCl solution. The modified Mayahara's medium used for alkaline phosphatase was composed of 3 ml of 0.1 M Tris-HCl buffer (pH 9.5), 2 ml of 2.16% β-GP, 1 ml of 0.02 M MgSO₄·7H₂O, and 4 ml of saturated alkaline lead citrate solution. The final pH was adjusted to 9.5 with either NaOH or HCl solution.

After incubation, the mycelia were washed with chilled deionized water, and were postfixed in 1 % OsO₄ in 0.1 M cacodylate buffer at pH 7.2 at 1-4°C for 2 hr.

The mycelia were dehydrated in a graded series of ethanol solution and embedded in Epon 812 (33). Thick (1 μ) and ultrathin sections for light and electron microscopy, respectively, were prepared by cutting with glass knives on a Porter-Blum MT-1 ultramicrotome.

Thick sections were observed with light microscope without staining. Ultra-thin sections were stained with 1 % uranyl acetate, and examined and photographed with a Hitachi HU-125E electron microscope.

The preparations which were incubated in the substrate-free or lead-free medium were also studied as controls.

RESULTS

Mycelial growth and phosphatase activities

As shown in Fig. 1, mycelial growth expressed as dry weight per culture flask reached its maximum level around 3 days of culture and thereafter decreased gradually. Glucose almost disappeared within 3 days, and inorganic phosphate diminished and reached its minimum level at 2-3 days. Most mycelia of this culture were not aggregated into pellets but dispersed in fibrous state.

pH dependency of the intracellular phosphatase activity is shown in Fig. 2. The maximum activity of acid phosphatase was detected between pH 4.0 and 5.0.

The previous studies (10, 34) revealed that acid phosphatase produced by A. oryzae A 1-5 was separated by chromatograph into two components with optimum activity at pH 4.0 and 5.5, and that the former (acid phosphatase I) was
Fig. 1. Changes in mycelial growth, glucose, and inorganic phosphate during the course of cultivation. *A. oryzae* A 1–5 was cultured with shaking in 50 ml of basal medium at 30°. Mycelial dry weight (---), glucose (—△—), inorganic phosphate (———).

Fig. 2. Intracellular acid and alkaline phosphatase activities at various incubation periods. *A. oryzae* A 1–5 was cultured in basal medium for 1 day (—●—), 2 days (———), and 3 days (—△—). Broken lines (———) at acid range (pH 3.0–7.0) and that (———) at alkaline range (pH 7.5–10.0) indicate the enzyme activities in the presence of NaF (0.001 M) and MgSO4 (0.01 M), respectively.
inhibited completely by sodium fluoride while the latter (acid phosphatase II) slightly. It seems reasonable, therefore, to conclude that acid phosphatase activity (solid line in Fig. 2) is a sum of the two acid phosphatases I and II, and that the activity detected in the presence of sodium fluoride (broken line in Fig. 2) corresponds to acid phosphatase II.

The activity of acid phosphatase I was predominant in this culture (Fig. 2). That of acid phosphatase II was almost negligible after 1 day of culture, with a relatively large amount of inorganic phosphate still remaining, but the enzyme with its maximum activity between pH 5.5 and 6.0 was detected after 2 days of culture which was very poor in inorganic phosphate (Fig. 1).

Intracellular alkaline phosphatase was also produced in this culture. This enzyme has a pH optimum of 9.0 to 9.5 and was activated by the presence of Mg$^{2+}$ ions (Fig. 2).

**Effect of inorganic phosphate on the formation of acid phosphatases**

The effect of higher content of inorganic phosphate in the medium was studied on the formation of intracellular acid phosphatases I and II. As shown in Fig. 3, activities of both acid phosphatases I and II were detected in fungal mycelia cultured in the basal medium. However, after the cultivation for 3 days in high-phosphate medium, the activity of acid phosphatase I per mycelium was

![Fig. 3. Effect of inorganic phosphate on the formation of acid phosphatases. A. oryzae A 1–5 was cultured for 3 days in basal medium (---○---) and high-phosphate medium (—●—). Broken lines (---○---, —●—) indicate the enzyme activities in the presence of NaF (0.001 M).]
greatly enhanced but that of acid phosphatase II, as shown by the experiment with sodium fluoride, was almost undetectable. In this culture broth, a large quantity of inorganic phosphate (ca. 14 μmol/ml) was still present.

**Cytochemical demonstration of acid phosphatases by light microscopy**

Preliminary examinations were made with a light microscope to localize cytochemically two different acid phosphatases. Mycelia cultured for 3 days in the basal medium were used throughout this cytochemical work, unless otherwise stated. As shown in Fig. 4b and 4c, the reaction products from the two enzymes were deposited at different sites of the cells when the fungal mycelia were incubated at pH 4.0 and 5.5 in the incubation medium using β-GP as substrate. Reaction products at pH 4.0 showed a line of granules on the cell wall (Fig. 4b), whereas those at pH 5.5 were found on both the outer and inner sides of the cell wall and also at the septal portion (Fig. 4c). The control preparation incubated in the substrate-free medium showed no reaction product (Fig. 4a). The present results suggest that the different pH of incubation media can distinguish cytochemically two acid phosphatases in the fungal cells.

**Cytochemical demonstration of acid phosphatase I by electron microscopy**

The ultrastructural localization of two acid phosphatases was investigated by cytochemical electron microscopy. To demonstrate acid phosphatase I activity in the cell, the mycelia were incubated at pH 4.0, where the activity of acid phosphatase II was scarce (Fig. 2), in the incubation medium using β-GP as substrate. Dense deposits of the reaction product were observed at the surface of cell wall but not at the cytoplasmic membrane or in the cytoplasm (Fig. 6). The enzyme activity was completely inhibited when the mycelia were incubated at pH 4.0 in the presence of 0.01 M sodium fluoride (Fig. 7). The same results were obtained when p-NPP was used as substrate. No reaction product was found in the control preparations which were treated with the incubation medium devoid of either the substrate or lead nitrate (Fig. 5).

**Cytochemical demonstration of acid phosphatase II by electron microscopy**

When the mycelia were incubated at pH 5.5 in the incubation medium using β-GP as substrate, dense deposits of the reaction product were found localized both at the surface of cell wall and at the cytoplasmic membrane (Fig. 8), which seemed to result from activities of the two types of acid phosphatase. When the specimens were incubated in the presence of sodium fluoride to show the enzyme activity that corresponds to acid phosphatase II, the reaction products were seen at the same sites as those observed without the inhibitor (Fig. 9), but they appeared to be reduced slightly. No reaction product was found in the cytoplasm. The same results were obtained with p-NPP used as substrate. Small amounts of reaction products were rarely seen in the space between the cell wall and the cytoplasmic membrane (Fig. 9).
Fig. 4. Light micrographs of fungal mycelia incubated in substrate-free medium (a) and in β-GP medium at pH 4.0 (b) and pH 5.5 (c) without NaF for reaction product deposition by acid phosphatases. Note the difference of reaction product deposition between b and c.

Fig. 5. The control preparation incubated in substrate-free medium. Note the absence of reaction product. The same result was obtained in the preparation incubated in lead-free medium. The bars in this and subsequent electron micrographs indicate 1 μ. 
Fig. 6–7. Electron micrographs of fungal mycelia incubated at pH 4.0 in the absence (Fig. 6) and presence (Fig. 7) of NaF for acid phosphatase I using β-GP as substrate. Reaction product was seen at the surface of cell wall in the absence of NaF but the enzyme activity was completely inhibited by the inhibitor.
Fig. 8-9. Electron micrographs of fungal mycelia incubated at pH 5.5 in the absence (Fig. 8) and presence (Fig. 9) of NaF for acid phosphatase II using β-GP as substrate. Reaction product was seen both at the surface of cell wall and at the cytoplasmic membrane irrespective of NaF. Very small amount of reaction product was also seen in the periplasmic space (arrow).
Fig. 10-11. Light micrographs of fungal mycelia incubated at pH 4.0 for acid phosphatase I (Fig. 10) and pH 5.5 in the presence of NaF for acid phosphatase II (Fig. 11). Mycelia cultured for 3 days in a high-phosphate medium were used. Most of the mycelia were strongly positive in activity at pH 4.0, but not at pH 5.5.
Reaction products of both acid phosphatases I and II were not detected in any of the fungal cells cultured in the basal medium for 3 days, but 30-40% cells remained unstained. This would be attributed to the presence of physiologically different cells (e.g., aging of cells) in such mycelial mass at the stationary phase as cultured under these conditions (Fig. 1).

Cytochemical studies on the effect of inorganic phosphate in growth medium on two acid phosphatase activities

As previously noted (Fig. 3), the activity of acid phosphatase I was significantly greater in fungal cells cultured in a high-phosphate medium than in the basal medium, whereas that of acid phosphatase II was very scarce. Such changes in two acid phosphatase activities in mycelia were examined cytochemically. Most of fungal cells grown in a high-phosphate medium displayed very strong activities after incubation at pH 4.0 for acid phosphatase I as shown in Fig. 10 by light microscopy and in Fig. 12 at electron microscope level. On the contrary, the reaction product at pH 5.5 with sodium fluoride for acid phosphatase II was not definitely seen in the light microscopy (Fig. 11). This was confirmed by electron microscopy; almost all of the fungal cells were not reactive (Fig. 13) though a small amount of reaction product was observed at the surface of cell wall and at the cytoplasmic membrane of a few fungal cells (Fig. 14).

Cytochemical demonstration of alkaline phosphatase by electron microscopy

As in the case of acid phosphatases, when the mycelia were incubated at pH 9.5 using β-GP as substrate, the reaction product by alkaline phosphatase was localized at the surface of cell wall and at the cytoplasmic membrane of some fungal cells (Figs. 15 and 16) though the activity was usually very weak. Reaction products were also invariably found in the nucleoli (Fig. 17) and in the unknown electron-dense bodies (Fig. 18), which could be distinguished from the nucleoli by the absence of nuclear membrane and chromatin.

DISCUSSION

Cytochemical observations on N. crassa (21) and A. niger (24) provided informations as to the intracellular localization of phosphatases. In centrifuged hyphae of Neurospora, acid phosphatase was positive in cytoplasm and cell wall as stained by Gomori’s reaction, and in mitochondria and nuclei as stained by Seligman’s method with α-naphthyl phosphate and Diazo-Blue B. Acid phosphatase in A. niger was localized at the cell wall, mitochondria, microsome, and cell sap. The present studies with electron microscopic cytochemistry on A. oryzae mycelium showed acid phosphatases at the surface of cell wall and at the cytoplasmic membrane. These results are similar to those in A. terreus observed by Yamamoto et al. (26). In their investigations acid phosphatase in relatively young mycelium was localized on the outside of the cell wall, in the cell wall, and between
Fig. 12–13. Electron micrographs of fungal mycelia incubated at pH 4.0 (Fig. 12) for acid phosphatase I and pH 5.5 in the presence of NaF for acid phosphatase II (Fig. 13) using β-GP as substrate. The same mycelia as in Figs. 10 and 11. Very heavy accumulations of reaction product were present at the surface of cell wall at pH 4.0, but no definite reaction product was seen at pH 5.5.
Fig. 14. Electron micrographs of fungal mycelia incubated at pH 5.5 in the presence of NaF for acid phosphatase II using β-GP as substrate. The same mycelia as in Figs. 10 and 11. Small amount of reaction product was seen at the surface of cell wall and at the cytoplasmic membrane of a few cells.

Fig. 15–16. Electron micrographs of fungal mycelia incubated at pH 9.5 for alkaline phosphatase using β-GP as substrate. Reaction product was present at the surface of cell wall and at the cytoplasmic membrane.
Fig. 17-18. Electron micrographs of fungal mycelia incubated at pH 9.5 for alkaline phosphatase using β-GP as substrate. Note reaction products in the nucleoli (Fig. 17) and in the unknown electron-dense bodies in the cytoplasm (Fig. 18).
the cell wall and the cell membrane. In addition, the vacuolar membrane was positively stained in relatively old mycelium, which may be due to the diffusion of the enzyme in old fragile structures of the cells. In the present study on *A. oryzae*, however, the reaction product was not deposited at or inside the vacuole-membrane of relatively young mycelium cultured for 3 days. It may be considered from these observations that acid phosphatases in the mycelium of *A. oryzae* are localized at the surface structures such as cell wall and cytoplasmic membrane. Such surface localization of acid phosphatases was also reported in bacteria (35) and yeast (36–39).

The acid phosphatase is considered to be the “marker enzyme” for lysosomes, and possible presence of lysosomes or lysosome-like particles in fungi is suggested by cytochemical studies (40–45). In *A. niger*, small granular particles within the cytoplasm were shown to possess phosphatase activity (24), but in the mycelium of *A. oryzae* no particulate localization of acid phosphatase could be recognized in the present investigations at electron microscope level.

With respect to the localization of alkaline phosphatase, ZALOKAR (21) observed its activity in the supernatant cytoplasm and nucleoli of the centrifuged hyphae of *N. crassa* as stained by Gomori’s and Seligman’s reactions. NAGASAKI (24) reported that the enzyme in young hyphae of *A. niger* is present mainly in nuclei, while that in old hyphae is dispersed in the cytoplasm. The present cytochemical study in *A. oryzae* also revealed that alkaline phosphatase is found in nucleoli as in the case of such fungi as *N. crassa* and *A. niger*, and of higher organisms. In addition, we found activities of alkaline phosphatase at the surface of cell wall and at the cytoplasmic membrane and also in the unknown granular structures within the cytoplasm. The deposits of alkaline phosphatase reaction product occupied the nucleoli or the unknown structures in some fungal cells (Figs. 17 and 18), but in other cells the deposits were restricted to the cell surface or the membrane (Figs. 15 and 16). It is likely that such differences in the cytochemical localization patterns of alkaline phosphatase among the fungal cells may be related to the physiological state of the cells.

Biochemical studies by SAKURAI and SHIOTA (10) showed the presence of two different acid phosphatase in *A. oryzae*. The present cytochemical studies were also able to differentiate the two acid phosphatases; acid phosphatase I is present at the surface of cell wall, whereas acid phosphatase II both at the surface of cell wall and at the cytoplasmic membrane (Figs. 6–9). Furthermore, different behavior of the enzymes in elution pattern (46), showing that acid phosphatase I was eluted in a very small amount when the fungal mycelium was soaked in KCl solution, but acid phosphatase II in a considerable amount, would suggest the possibility of a different association of the enzymes with cell structures.

Activities of two acid phosphatases in this fungus depend on the concentration of inorganic phosphate in the growth medium (Figs. 2 and 3). The present study revealed that the biochemically detected enzyme activities were correlated with
the intensities of the deposits cytochemically reacted in microscopical preparations. A similar phenomenon was reported in A. terreus by YAMAMOTO et al. (12), who demonstrated by electron microscopic cytochemical procedures that inositol promotes the formation of acid phosphatase at the periplasmic space.

As already mentioned, we found a different localization of reaction products for two acid phosphatases in the vicinity of cell wall and no cytochemical reaction for the enzymes in the cytoplasm under this experimental condition. Unfortunately, we have scarce information on the permeation of substrate or capture reagent through a fixed fungal cell wall or membrane. Therefore, much further work will be necessary to confirm whether acid phosphatase I is located at the membrane or whether two acid phosphatases in the cytoplasm.

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1975 Phosphatases in *Aspergillus oryzae* 249