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Release of Proteinase from Mycelium of *Mucor hiemalis*

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When *Mucor hiemalis* NRRL 3103 was grown in soybean medium, only a small fraction of the proteinase produced by the organism appeared in the culture filtrate, whereas the bulk of the enzyme was bound to the mycelial surface. Optimal *p*H of the proteinase ranged from 3.0 to 3.5. Inclusion of sodium chloride or other ionizable salts in the growth medium, however, resulted in the liberation from the mycelium of the loosely bound enzyme as it was formed. Maximal release of proteinase was achieved at a sodium chloride concentration of 0.5 M. The loosely bound proteinase was eluted also from intact resting mycelium by ionizable salts but not by water or by nonionizable substances. The amount of enzyme eluted from the mycelium depended upon the concentration of sodium chloride up to 0.3 M. Since liberation took place rapidly even at 0 C, a loose ionic linkage must exist rather than a biochemical binding of the enzyme to the mycelium. The recovery of proteolytic activity from repeated salt extractions was greater than that originally detected in the intact mycelium, possibly owing to unmasking of more active enzymes or functional groups. Further proteinase activity was released when salt-extracted mycelium was ruptured. Part of the proteinase thus observed was firmly attached to the cell fraction, and part of it appeared in the supernatant fluid. These conditions implied the presence of intracellular or firmly attached proteinase which could be partially released.

Localization of enzymes on the surface of a cell has attracted increasing attention by investigators because of its importance in understanding the biochemical and physiological properties of microorganisms. In recent years, several reports described enzymes liberated from the cell surface of microorganisms. β-Penicillinase of *Bacillus cereus* was bound to the cell and was liberated by high salt concentrations (7, 8). Proteinase produced by *Micrococcus freudenreichii* was also found to be cell-bound and could be released by sodium chloride (3). Elution of acid phosphatase from the surface of *Saccharomyces mellis* cells by potassium chloride and other salts was reported by Weimberg and Orton (10). They also observed (11) that acid phosphatase and invertase of *Saccharomyces cerevisiae* and *S. fragilis* were located outside the cell wall but were bound to the cell. Penicillinase of *Bacillus subtilis* liberated from cell or envelope by an enzymatic process was discussed by Kushner and Pollock (2). Malamy and Horecker (4) found that when cells of *Escherichia coli* were converted to spheroplasts with ethylenediaminetetraacetate and lysozyme, the surface-located alkaline phosphatase could be released into the medium. This same method was used to liberate other surface-located enzymes of *E. coli*, such as ribonuclease (5), acid phosphatase, cyclic phosphodiesterase, and 5'-nucleotidase (6).

Relatively few studies have been made upon the liberation of enzymes from the cell surface of molds, despite the fact that some of them have been intensively examined. The enzymes in these studies are often prepared by extracting cultures on solid media, such as wheat bran. The resulting preparations probably contain enzymes of both extracellular and cell-bound character. Generally, in studies dealing with extracellular enzymes found in culture filtrates, the extent of autolysis and the ratio of enzyme activity in culture filtrate to the cell-bound activity are not discussed; therefore, there is little evidence that they are truly extracellular.

During the studies of enzymes produced by *Mucor hiemalis* NRRL 3103, a mold used in Chinese cheese (sufu) fermentation (9), the mold grew surprisingly well on liquid medium of soybean meal or wheat flour, but the yield of proteolytic enzymes in the culture filtrate was not significant. Therefore, a study to locate the proteinase and to determine conditions for release of the enzyme was undertaken.

MATERIALS AND METHODS

M. hiemalis NRRL 3103 was maintained on slants of potato-dextrose-agar at 4 C. Before each experiment, the organism was transferred to a potato-dextrose-agar slant and was incubated at 25 C for 7 days. A spore suspension for inoculation was prepared by adding sterilized distilled water to each slant and shaking the culture vigorously for 1 min. The flasks containing 2% soybean meal in water were sterilized at 121 C for 20 min; they were then inoculated with spore suspension and incubated on a reciprocating shaker at 25 C. After 72 hr of growth, the mycelia were harvested by filtration, washed with distilled water, and then pressed dry. Portions of wet mycelium were then weighed and either were suspended in buffer of appropriate *p*H for assay of total proteinase activity or were eluted with desired solutions.

The proteinase activity was measured according to the hemoglobin digestion method described by Anson (1). The reaction was carried out in 1 ml of 1% denatured hemoglobin in 0.05 M citrate buffer of desired *p*H, with 1 ml of sample tested at 38 C for 30 min. The reaction was then stopped by the addition of 3 ml of 5% trichloroacetic acid, and the nondigested hemoglobin was removed by filtration. The amount of split products remaining in solution was determined spectrophotometrically at 280 mμ.

One unit of proteinase is defined as the amount of enzyme that forms 1 μmole of tyrosine per hr at 38 C.

RESULTS

Effect of pH on activity and stability of the proteinase. Although a trace amount of proteinase was detected in culture filtrate of *M. hiemalis* NRRL 3103 grown in 2% soybean meal in water, enzyme activity was greatly increased when sodium chloride was incorporated into the medium. Comparison of enzyme activity at various *p*H values showed that a maximal activity occurred at *p*H 3.0 to 3.5 (Fig. 1). Portions of culture filtrate were adjusted to *p*H values ranging from 2.0 to 8.0. After 20 hr at 25 C, the solutions were readjusted to *p*H 3.0. The amount of enzyme activity recovered as measured at *p*H 3.0 differed with the various *p*H treatments. As shown in Fig. 2, the enzyme was fairly stable between *p*H 3.0 and 5.0, was not so stable at *p*H above 5.0, and lost its activity almost completely at neutral or alkaline *p*H.

Effect of sodium chloride concentration and other reagents on the production of proteinase as an extracellular enzyme. To illustrate the effect of sodium chloride quantitatively, the mold was grown in 2% soybean medium containing various concentrations of sodium chloride. Proteolytic activity was found in the culture filtrates over a range of sodium chloride concentrations (Fig. 3), and the optimal concentration for maximal yield of enzyme was 0.5 M. The amount of enzyme was

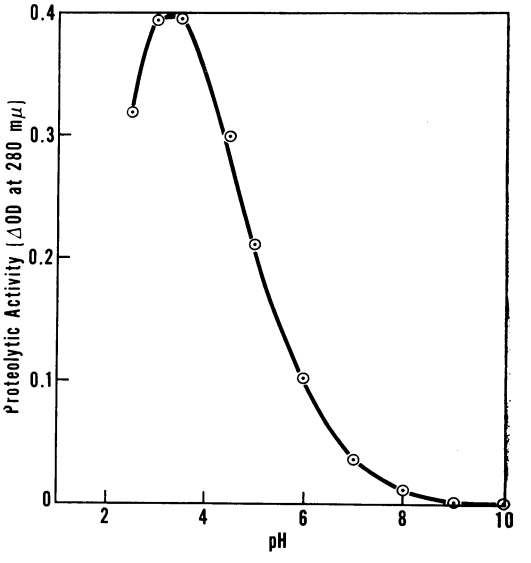

FIG. 1. *pH-activity curve of proteinase produced by Mucor hiemalis NRRL 3103.*

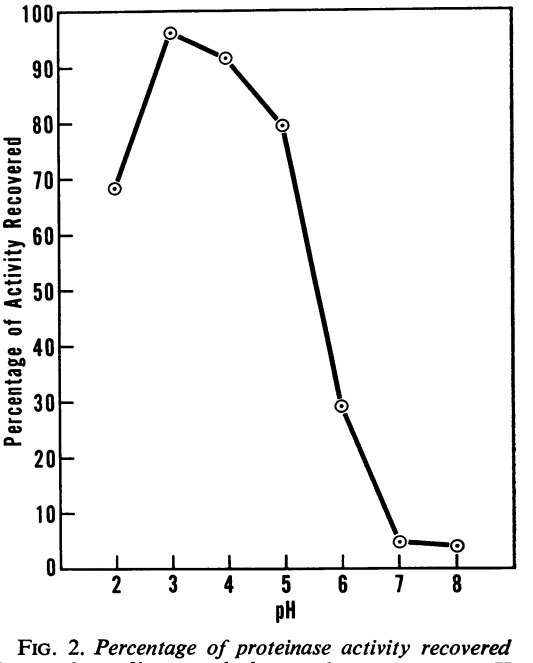

FIG. 2. *Percentage of proteinase activity recovered from culture filtrates which were kept at various pH values for 20 hr at 25 C.*

seven times greater than the maximal activity present in the filtrate of cultures grown in soybean medium with no sodium chloride added. The mold growth was suppressed even at a level of 0.5 M sodium chloride (Fig. 3), but was not

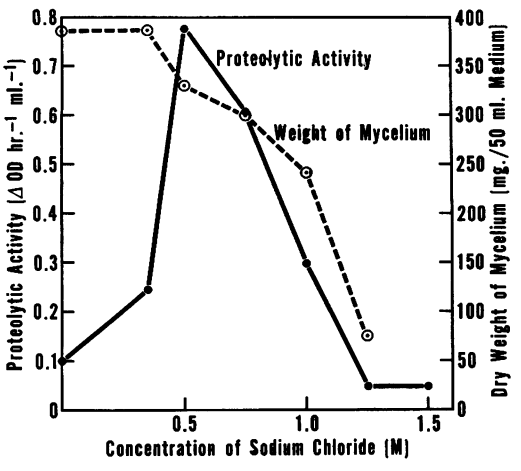

FIG. 3. *Growth of Mucor hiemalis and its extracellular proteinase production as a function of sodium chloride concentration in the medium.*

seriously affected until the salt concentration was above 1.0 M. Therefore, the decrease in enzyme activity with increasing salt concentrations probably was caused by growth inhibition.

Although growth of *M. hiemalis* in soybean medium containing sodium chloride resulted in the appearance of proteinase in the culture filtrate as an extracellular enzyme, the sodium chloride could be replaced by a number of salts: potassium chloride, calcium sulfate, magnesium sulfate, and ammonium sulfate (Table 1). All of these salts are ionized in water. Urea, a nonionizable compound, failed to increase the proteolytic activity in culture filtrate. On the other hand, incorporation of sucrose in the soybean medium increased extracellular proteolytic activity. The combined effects of sodium chloride and sucrose on the growth and extracellular proteinase are illustrated further in Fig. 4. The addition of sucrose to soybean medium greatly stimulated the growth of the organism and slightly increased the amount of proteinase appearing in the culture filtrate. Further increase in enzyme activity was noted in the filtrate of the organism grown in soybean-sucrose medium with the addition of 0.5 M sodium chloride, although growth was suppressed by the addition of this salt. These results suggest that both sucrose and sodium chloride gave rise to the appearance of proteinase in the culture filtrate, but the effect was due to different mechanisms: sucrose stimulated the growth and sodium chloride released the cell-bound enzyme.

Proportion of proteinase activity between culture filtrate and mycelium. To verify the true extracellularity of the proteinase produced by *M. hiemalis*, enzyme activities of culture filtrate as

TABLE 1. *Effect of salts and other reagents on the production of extracellular proteinase by Mucor hiemalis NRRL 3103 on a basal medium of 2% soybean meal*

Supplement (0.5 M)	Unit of enzyme per ml filtrate
None	0.3
KCl	7.0
NaCl	6.4
$CaSO_4$	2.4
$MgSO_4$	4.2
$(NH_4)_2SO_4$	3.0
Sucrose	3.4
Urea	0.3

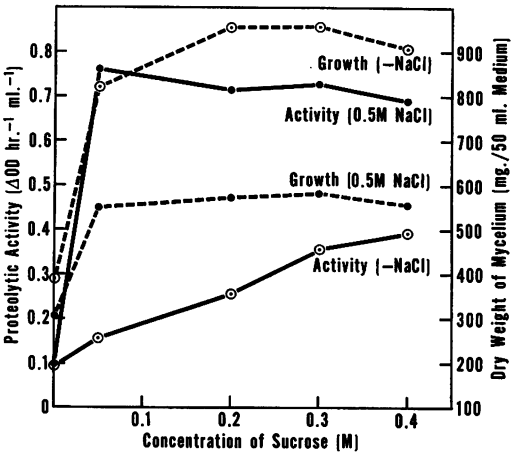

FIG. 4. *Combined effect of sucrose and sodium chloride on the growth and extracellular proteinase production of Mucor hiemalis.*

well as of mycelium were measured. Only one-third of the enzyme appeared in the culture filtrate when *M. hiemalis* was grown in soybean medium (Table 2). Addition of sucrose to the medium enhanced the total enzyme production, but the fraction of enzyme activity in the culture filtrate remained the same as that of soybean alone. When sodium chloride was added to the medium containing either soybean or soybean and sucrose, however, more than 90% of the enzymes appeared in the culture filtrate. In other experiments, even less enzyme activity occurred in the culture filtrate of the growth medium without sodium chloride. Therefore, the proteinase produced by *M. hiemalis* was mainly bound to the mycelium, and sodium chloride released the enzyme from the mycelium as the enzyme was formed. The total proteinase activity from filtrate and mycelium of *M. hiemalis* grown in soybean medium with sodium chloride greatly exceeded that of the organism grown in

TABLE 2. *Percentage of proteinase activity in culture filtrates of Mucor hiemalis grown on a basal medium of 2% soybean meal with various supplements*

Supplement	Proteinase activity (units)		Proteinase in filtrate
	Filtrate	Mycelium	
			%
None	26.6	57.3	31.7
NaCl, 0.5 M	456.5	21.6	95.5
Sucrose, 0.2 M	170.2	340.0	33.3
NaCl, 0.5 M, plus sucrose, 0.2 M	465.5	37.4	92.4

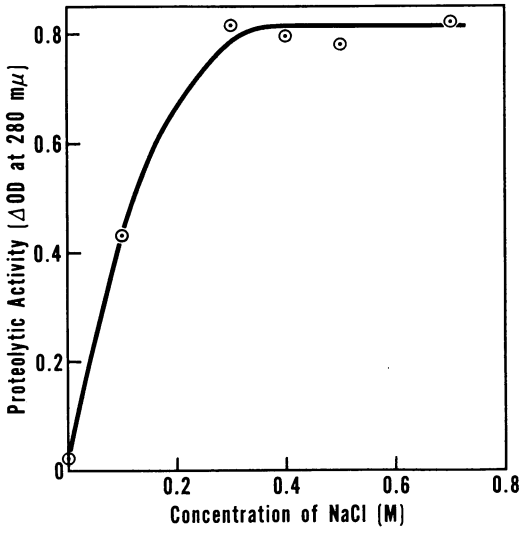

FIG. 5. *Effect of various concentrations of sodium chloride on the elution of proteinase from intact resting mycelium of Mucor hiemalis.*

soybean medium without sodium chloride, although this salt suppressed the growth of the organism. Apparently, as soon as the mycelium surface was free from proteinase the organism responded and produced more enzyme protein.

Elution of proteinase by extracting mycelium with salt solutions. Since proteinase produced by *M. hiemalis* was released from growing cultures by salts, it seemed likely that the enzyme might also be eluted from resting mycelium by salts. Three-day-old mycelium of *M. hiemalis* grown in 2% soybean meal and 0.2 M sucrose was harvested, washed with water, and pressed dry. Portions of weighed mycelium were suspended either in water or in sodium chloride for 1 hr. Proteolytic activity was found in the sodium chloride supernatant fluid but not in the water supernatant fluid. The amount of enzyme released from the mycelium depended upon the concentration of sodium chloride up to 0.3 M (Fig. 5). A time study of elution by sodium chloride demonstrated that the liberation of cell-bound enzyme took place very rapidly at 25 C. Mycelium suspended in 0.3 M sodium chloride for several hours did not release more enzyme than mycelium suspended for 20 to 30 min. Although release of enzyme also occurred rapidly at 0 C, the amount of enzyme liberated was only about 90% of that released at 25 C. Solutions of potassium chloride, magnesium sulfate, and ammonium sulfate also released proteinase (Table 3) as in the growing culture. Glucose, sucrose, and urea did not release appreciable amounts of the enzyme.

Recovery of proteinase activity from repeated salt extraction and ruptured mycelium. A 250-mg amount of wet mycelium was suspended in 5 ml of 0.3 M sodium chloride for 30 min at 25 C and was then centrifuged. After removing the supernatant fluid for enzyme assay, the mycelium sample was resuspended in another 5 ml of 0.3 M sodium chloride for a second elution, and then two more elutions were made in the same manner. About 70% (Table 4) of the measurable proteinase activity of the intact mycelium was recovered from the first sodium chloride eluate, an appreciable amount of enzyme was found in the second eluate, and a small amount of enzyme was also recovered from third and fourth extractions. The total proteinase from the sodium chloride extractions was nearly 140% of that originally detected in the mycelium.

Further release of proteinase from salt-extracted mycelium was observed after sonic disruption of the mycelium. The four-times salt-extracted mycelium was washed once with water; it was then suspended in 5 ml of 0.3 M sodium chloride and subjected to a Branson 20-kc Sonifier for 15 min. After centrifugation at 23,000 × *g* for 15 min, the proteinase activity of supernatant

TABLE 3. *Elution of proteinase from mycelium of Mucor hiemalis by salts and other reagents*

Reagent	Concn	Enzyme recovered from 200 mg of wet mycelium
	M	*units*
NaCl	0.3	18.7
KCl	0.3	19.7
$MgSO_4$	0.3	16.3
$(NH_4)_2SO_4$	0.3	13.0
Glucose	0.6	2.7
Sucrose	0.6	1.3
Urea	0.6	0.6

TABLE 4. *Liberation of proteinase by salt elution from intact and ruptured mycelium*

Treatment	Fraction for enzyme assay	Proteinase (units) per 250 mg of wet mycelium
Suspended mycelium in buffer solution	Intact mycelium	20.6
Suspended intact mycelium in 0.3 M NaCl at 25 C for 30 min, followed by centrifugation	Supernatant fluid	14.4
Same sample subjected to second NaCl extraction	Supernatant fluid	8.7
Same sample subjected to third NaCl extraction	Supernatant fluid	3.4
Same sample subjected to fourth NaCl extraction	Supernatant fluid	2.2
Suspended mycelium in buffer solution after fourth NaCl extraction	Mycelium	2.4
Sample of four-times salt-extracted mycelium subjected to sonic oscillation for 30 min, followed by centrifugation	Residue	7.2
	Supernatant fluid	8.7

fluid was measured. The residue was washed with water and then suspended in buffer solution for enzyme assay.

After salt extraction four times, the mycelium showed low proteinase activity (Table 4), but significant amounts of enzyme were in the supernatant fluid and residue of the ruptured mycelium. Obviously, some of the cell-bound proteinase cannot be released by salt but becomes accessible to the substrate after the structure is ruptured.

DISCUSSION

It is well-known that the great majority of molds produce appreciable amounts of proteolytic enzymes, which may be cell-bound or extracellular according to the type of organism producing them. *M. hiemalis* is used for sufu fermentation, in which the substrate, tofu, has an extremely high content of protein (60% on dry basis). Therefore the production of proteinase by this organism is expected.

When the organism was grown in a medium with no salt, a small fraction of proteinase appeared in the culture filtrate, but most of the proteinase remained attached to the mycelium. When the mold was grown in a salt medium, not only was a large fraction of enzyme activity recovered from the culture filtrate, but also a great increase in total enzyme activity was observed. Apparently, only a certain amount of proteinase could be associated with the mycelial surface; continual removal of proteinase from mycelial surface by salt made it possible for the organism to synthesize more of the enzyme protein.

The mycelium-bound proteinase could also be easily eluted by sodium chloride or other ionizable salts from intact resting mycelium. Such nonionizable substances as urea, glucose, and sucrose were unsatisfactory for eluting the proteinase, just as they were unsatisfactory for releasing the proteinase from growing culture. Consequently, the enzyme is probably loosely bound to the mycelial surface by ionic linkage. The liberation of the enzyme by salts might not involve any biochemical reactions, because the elution took place rapidly even at 0 C. Previously β-penicillinase of *B. cereus* (7, 8), proteinase of *M. freudenreichii* (4), and acid phosphatase of *S. mellis* (10) were reported to be cell surface enzymes held by ionic linkage.

Although the release of proteinase by salts took place rapidly and large amounts of enzyme from resting mycelium were eluted by the first salt extraction, small amounts of enzyme were obtained by repeated extractions. Furthermore, the recovery of total enzymatic activity in salt supernatant fluids was greater than was initially found with the mycelium. In this respect, it behaved like acid phosphatase from *S. mellis* (10). The reason for this behavior is not clear, but, as suggested by Weimberg and Orton (10), it could be due to unmasking of more active enzymes and an alteration of the protein structure.

A further yield of proteinase was recovered from the supernatant fluid and residue of the salt-extracted mycelium treated by a sonic oscillator. This finding suggested the presence of intracellular or firmly cell-bound proteinase. The liberation and location of these firmly bound proteinases will have to be determined by future work.

Since proteinase of *M. hiemalis* grown in a low salt medium remained attached to the mycelium, the opportunity was provided not only to study the location of enzyme before its liberation, but also to comprehend the brineing process in sufu fermentation. It is now apparent that the sodium chloride in the brine solution not only retards mold growth and imparts a salty taste to the final product, but also releases the mycelium-bound proteinase, which, in turn, penetrates the molded tofu and acts on the protein.

LITERATURE CITED

1. ANSON, M. L. 1938. Estimation of pepsin, trypsin, papain, and cathepsin with hemoglobin. J. Gen. Physiol. 22:79–89.
2. KUSHNER, D. J., AND M. R. POLLOCK. 1961. The location of cell-bound penicillinase in *Bacillus subtilis*. J. Gen. Microbiol. 26:255–265.

3. MCDONALD, I. J. 1962. Localization of proteinase in cells of a species of *Micrococcus*. Can. J. Microbiol. **8**:785–794.
4. MALAMY, M., AND L. HORECKER. 1961. The localization of alkaline phosphatase in *E. coli* K_{12}. Biochem. Biophys. Res. Commun. **5**:104–108.
5. NEU, H. C., AND L. A. HEPPEL. 1964. The release of ribonuclease into the medium when *E. coli* cells are converted to spheroplasts. Biochem. Biophys. Res. Commun. **14**:109–112.
6. NEU, H. C., AND L. A. HEPPEL. 1964. On the surface localization of enzymes in *E. coli*. Biochem. Biophys. Res. Commun. **17**:215–219.
7. POLLOCK, M. R. 1956. Cell-bound penicillinase of *Bacillus cereus*. J. Gen. Microbiol. **15**:154–169.
8. POLLOCK, M. R., AND K. KRAMER. 1958. Intermediates in the biosynthesis of bacterial penicillinase. Biochem. J. **70**:665–681.
9. WAI, N. 1964. Soybean cheese. Bull. Inst. Chem. Acad. Sinica, Taiwan **18**:75–94.
10. WEIMBERG, R., AND W. L. ORTON. 1965. Elution of acid phosphatase from the cell surface of *Saccharomyces mellis* by potassium chloride. J. Bacteriol. **90**:82–94.
11. WEIMBERG, R., AND W. L. ORTON. 1966. Elution of exocellular enzymes from *Saccharomyces fragilis* and *Saccharomyces cerevisiae*. J. Bacteriol. **91**:1–13.