EVIDENCE FOR THE PRESENCE OF MEMBRANE-BOUND FORMS OF ACID PROTEASE IN ASPERGILLUS ORYZAE

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SUMMARY

While approximately 85% of the cell-bound acid protease of Aspergillus oryzae were recovered in the soluble fraction upon disruption of cells, the rest of the enzyme was found to be present tightly associated with the membranes. Two forms of membrane-bound enzyme, which were solubilized with Triton X-100, were similar to the external acid protease found in culture medium in that they had an optimum pH at 3.2, activated trypsinogen at pH 3 and lost their activity upon treatment with 5.1 mM sodium dodecylsulfonate. However, they differed in their hydrophobic properties (i.e. aggregation in the absence of Triton X-100 and activation by the detergent) from both the cell-bound, soluble form and the one excreted into culture medium.

Penicillinase synthesized by a strain of Bacillus licheniformis has been shown to pass through a cell-bound state before release (1). Lampen and his coworkers (2) showed that in B. licheniformis, the extracellular enzyme penicillinase is present in three forms: a well characterized hydrophilic form excreted into culture medium, a hydrophilic form present in a periplasmic vesicle fraction and a hydrophobic form associated with the cell membrane. The last form has been highly purified and characterized (3, 4). Recently, α-amylase of Bacillus amyloliquefaciens has also been shown to be present in association with the cell membrane. The membrane-bound form differed from the one excreted into the culture medium in sedimentation behavior and in electrophoretic mobility (5).

The data presented in this communication indicate that part of the cell-bound acid protease of Aspergillus oryzae is present tightly associated with the membranes and that the membrane-bound forms are distinguished from the enzyme found in the culture medium by their hydrophobic properties.

Table I

DISTRIBUTION OF CELL-BOUND ACID PROTEASE ACTIVITY IN A. ORYZAE

Fraction	Activity	Specific activity (units/mg protein)	Distribution (%)
500 - 105,000 x g pellet	1,682	1.57	15.2
105,000 x g supernatant	8,600	14.80	84.2

One hundred and twenty grams of saline-washed wet mycelia were used in the experiment. Details are described in Materials and Methods.

MATERIALS AND METHODS

Aspergillus oryzae (strain 365-U-64-1) was grown at 28°C on a reciprocal shaker in 500-ml Sakaguchi flasks containing 100 ml of the culture medium (pH 3.0) consisting of 4 g of glucose, 1.5 g of corn steep liquor (Corn Products Co., U.S.A.), 1 g of meat extract (Kyokuto Seiyaku Kogyo Co., Japan), 1 g of Hyflo Super-Cel (Jones-Manville Co., U.S.A.), and 5 mg of the disfoaming agent CB442 (Nippon Oil and Fats Co., Japan). After growing for appropriate periods of time at 28°C, mycelia of A. oryzae were harvested by filtration and washed thoroughly with saline (0.9% NaCl). The mycelial mat was ground in a mortar with silica sands (40-60 mesh, 3 g/g of wet mycelia) at 0°C and the mixture was then extracted with 4 volumes of 50 mM sodium acetate buffer, pH 5.8, containing 5 mM $MgCl_2$, 0.5 mM EDTA and 0.25 M sucrose. The mixture was centrifuged at 500 x g for 5 min and the supernatant was then centrifuged at 105,000 x g for 120 min. The pellet obtained was used as membranes in this study. Acid protease A_1 and A_2 of A. oryzae were obtained as described previously (6).

Acid protease was assayed as described previously (6). One unit of acid protease activity was defined as the amount of enzyme that developed the color equivalent to 1 μmol of tyrosine per min. Trypsinogen-kinase activity was determined as described by Hofmann and Shaw (7). Protein was determined by the method of Lowry et al. (8).

RESULTS

When the mycelia of A. oryzae at mid-log phase of growth (65 hr growth) were examined for their content of acid protease activity, more than 80% of the total acid protease activity associated with cells were recovered in the supernatant solution after centrifugation of the disrupted cells (Table I). This type of the enzyme appeared to be weakly associated with the cells, as compared with the form which remained with membranes after these treatments. This last form was not released from membranes by repeated washing with saline. The membrane-bound form comprised approximately 15% of the total activity associated with the cells.

Table II

SOLUBILIZATION OF MEMBRANE-BOUND ACID PROTEASE WITH TRITON X-100

	Protein (mg)	Activity (units)	Specific activity (units/mg protein)
Starting membranes	22.5	93.5	4.2
Triton X-100 extraction	22.2	(195.0)*	(8.8)*
Cold acetone washing			
Supernatant	7.1	36.3 (100.5)	5.1 (14.2)
Pellet	14.2	4.7 (40.0)	0.3 (2.8)

* Values in parentheses represent activities measured in the presence of 0.7% Triton X-100.

Starting membranes (500 - 105,000 x g pellet, 22.5 mg protein) suspended in 22.5 ml of saline containing 0.7% Triton X-100 were incubated at 37°C for 60 min, and the mixture was then centrifuged at 105,000 x g for 120 min. To the supernatant solution were added 2 volumes of cold acetone (-20°C) and the precipitate formed was collected by centrifugation at 10,000 x g for 15 min. The pellet was washed twice with cold acetone and then suspended in 3.0 ml of 50 mM sodium acetate buffer, pH 5.2. The mixture was centrifuged at 105,000 x g 120 min, and the resultant pellet was suspended in 3.0 ml of the same buffer.

As shown in Table II, the membrane-bound acid protease could be solubilized by treatment with Triton X-100. The total amount of activity recovered in the supernatant solution exceeded the activity which could be measured in the starting membranes. Treatments with deoxycholate, Tween 80, phospholipase A, and snail gut extract were also effective in solubilizing the membrane-bound enzyme. After removal of Triton X-100 from the solubilized enzyme by washing with cold acetone, part of the enzyme became insoluble and was sedimented after centrifugation, and the rest of the activity remained soluble (Table II). The data suggested the presence of two enzymatic forms in the membranes with different solubilities. The acid protease activity in this pellet was again solubilized by Triton X-100 treatment. The presence of two molecular forms of the membrane acid protease was further indicated by gel filtration in Bio-Gel A-15m. The

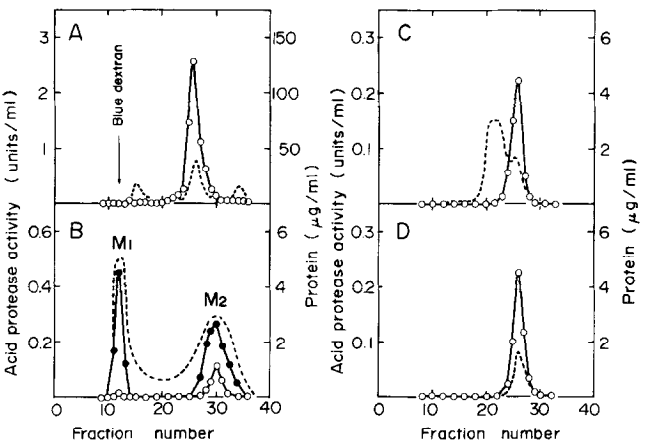

Fig. 1. Gel filtration of the membrane-bound acid protease of A. oryzae in Bio-Gel A-15 m. All experiments were carried out using the same column of Bio-Gel A-15 m (1.52 x 55 cm) equilibrated with 50 mM sodium acetate buffer, pH 5.2. The column was developed with the same buffer at a flow rate of 5 ml/hr at 4°C and fractions of 3 ml were collected. In experiments shown in A, C and D the buffer contained 0.1% of Triton X-100. A. Triton X-100 extract obtained as described in Table II (1.5 mg protein). B. Precipitate formed upon addition of cold acetone to active fractions obtained in A (0.35 mg protein). C. Peak fractions (M_1) obtained in B were concentrated in Diafilter G-10T (Bioengineering Co., Ltd., Tokyo), treated with 0.7% Triton X-100 at 37°C for 10 min and applied to the column (50 μg of protein). D. Peak fractions (M_2) obtained in B were treated as described in C and applied to the column (30 μg of protein). —o—, Acid protease activity; —•—, acid protease activity measured in the presence of 0.7% Triton X-100; -----, protein.

Triton-extracted enzyme gave a single activity peak at fraction number 26 when subjected to gel filtration in the presence of Triton X-100. (Fig. 1A). However, after removal of the detergent, the enzyme gave two peaks on gel filtration: an aggregated form with low specific activity eluted in the void volume of the column (designated M_1) and a form with low molecular weight eluted at fraction number 30 (designated M_2) (Fig. 1B). Both forms were eluted from the column at the same rate when developed in the presence of Triton X-100 (Figs. 1C and 1D).

As shown in Table II and in Fig. 1, the membrane-bound forms of acid protease were strongly activated in the presence of 0.7% Triton X-100. The activation was approximately 9- and 3-fold for M_1 and M_2, respectively,

Table III

SUMMARY OF CATALYTIC PROPERTIES OF A. ORYZAE ACID PROTEASES

Acid protease form	Activation by Triton X-100[a]	Optimum pH (casein as substrate)	Activation of trypsinogen (nmol/min/unit of enzyme)[b]
M_1	8.5	(3.2)[e]	(1.0)
M_2	3.0	3.2	1.1
Cell-bound, soluble[c]	0.9	3.0	1.1
External[d]	1.0	3.0	1.1
A_1	1.0	3.0	1.4
A_2	0.9	3.0	1.1

a) The ratio of activity in the presence of 0.7% Triton X-100 relative to that in the absence of the detergent.
b) Values represent amounts of trypsin formed at pH 3 and 37°C.
c) 105,000 x g supernatant in Table I.
d) Culture filtrate at 65 hr growth.
e) Values in parentheses were obtained in the presence of 0.05% Triton X-100.

while the cell-bound, soluble enzyme, external enzyme (in culture filtrate) and acid protease A_1 and A_2 (6) were unaffected by the detergent (Table III). Both forms of the membrane-bound enzyme, like other internal and external enzymes, had an optimum pH at around 3 and activated trypsinogen (Table III), indicating that all of these acid protease forms have similar catalytic properties.

DISCUSSION

The results presented provide evidence that part of the cell-bound acid protease of A. oryzae is present in association with membranes. The two forms of the membrane-bound enzyme differ in their hydrophobic properties from the soluble enzymes found in culture medium and in cells, although all of these enzyme fractions have similar catalytic properties. The data suggest that at least part of the membrane-bound acid protease might be an intermediate in the process of acid protease secretion.

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