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Characteristics of a Proteinase of a *Trichosporon* Species Isolated from Dungeness Crab Meat

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ABSTRACT

GRONINGER, HERMAN S., JR. (U.S. Bureau of Commercial Fisheries Technological Laboratory, Seattle, Wash.), AND M. W. EKLUND. Characteristics of a proteinase of a *Trichosporon* species isolated from Dungeness crab meat. Appl. Microbiol. **14:**110–114. 1966.—The proteinase of a *Trichosporon* species was partially purified by dialysis, ammonium sulfate fractionation, and Sephadex G-100 gel filtration. A 170-fold purification of the enzyme with a 1.4% recovery of the activity was achieved. The proteinase was separated into a major component and possibly two minor components by starch gel electrophoresis. The *p*H optimum of the enzyme was 5.8 to 6.2. It was active against casein, hemoglobin, and crab protein substrates, but inactive against bovine serum albumin, lysozyme, and benzoylarginine ethyl ester. It was slightly activated by 10 mM cysteine, 0.1 mM ethylenediaminetetraacetic acid, and 0.1 mM Co^{++}. There was slight inhibition by 10 mM Co^{++} and 0.1 mM phenylmethylsulfonylfluoride, and total inhibition by 1 mM *p*-chloromercuribenzoate. The proteinase was completely inactivated by heating at 60 C for 10 min.

Proteolytic activities of yeasts have not been studied extensively. The enzymes do not appear to be as common in yeasts as are those that act on polysaccharides. Yeasts usually do not play a role in the spoilage of protein foods such as fresh fishery products.

Vorbeck and Cone (8) studied an intracellular proteinase from a *Trichosporon* species and determined some of the properties of the partially purified enzyme. Lenney (4) showed that there were two proteinases formed by *Saccharomyces cerevisiae*.

Recently it was shown that yeasts grew quite well on crab meat after radiation pasteurization had inactivated most of the original flora. A yeast, tentatively identified as *Trichosporon* species, was isolated from Dungeness crab meat. It was shown that this isolate attacked gelatin, casein, and crab protein (3).

The significance of proteolytic activity of yeasts in relation to the overall quality change caused by their growth on fishery products is difficult to evaluate. A knowledge of the properties of the enzyme(s) involved, however, would give a basis for an estimate of the contribution of proteolysis. The objectives of the present work were to determine some of the environmental conditions required for proteinase formation by the *Trichosporon* isolate, and to partially purify and study some of the properties of the proteinase.

MATERIALS AND METHODS

Organism. The yeast culture used was a *Trichosporon* species that was isolated from Dungeness crab by Eklund et al. (3). Stock cultures were maintained by periodic transfer on malt-agar (3.0% malt extract, 1.5% agar) slants with interim storage at 5 C.

Media and growth conditions. Mass cultures of the yeast were grown at *p*H 6.0 in a medium composed of the following (per liter): glucose, 10 g; yeast extract, 1 g; peptone (Difco), 20 g; $MgSO_4$, 0.5 g; Fe_2SO_4, 0.1 g; and KH_2PO_4, 2 g. The glucose and $MgSO_4$ plus Fe_2SO_4 were sterilized separately and added to the other ingredients at the time of inoculation. For cell production, 400 ml of this medium was inoculated with a 24- to 36-hr broth culture and incubated on a wrist-action shaker at 21 C for 36 to 48 hr. The cells were harvested by centrifugation and were washed with distilled water at 5 C. The washed cells were frozen and stored at −12 C for not more than 4 months, or they were used immediately.

Chromatography on Sephadex G-100. Approximately 15 g of Sephadex G-100 (Pharmacia Fine Chemicals, Inc., New Market, N.J.) was suspended in 1 liter of 0.5 M NaCl in 0.02 M acetate buffer (*p*H 5.0) and allowed to swell for 12 hr. The columns were packed by pouring the gel into the column with the

bottom stopcock closed, and the column was filled with buffer to a height of 4 to 5 cm. After 2 to 3 cm of gel had settled, the stopcock was opened, and the gel was allowed to settle by gravity until a column of about 75 by 1.8 cm was obtained.

Dungeness crab muscle extract. A muscle-extract substrate was prepared from crab by blending body muscle for 3 min with two parts (w/v) water in the cold. The cell debris was removed by centrifugation at 20,000 × *g*, and the supernatant fluid was dialyzed against several changes of water over a period of 24 hr.

Starch gel electrophoresis. Starch gel electrophoresis was performed according to the method of Smithies (7) modified by the use of a discontinuous buffer. The gel was prepared with a buffer (*p*H 9.2) composed of 0.054 M tris(hydroxymethyl)aminomethane (Tris), 0.007 M boric acid, and 0.0015 M ethylenediaminetetraacetic acid (EDTA); the electrophoresis was carried out at a 3.3-fold greater buffer strength. A current of 25 ma at a potential of approximately 20 v/cm was applied to the gel for 1.5 hr. A horizontal slice was stained for protein with Buffalo Black NBR.

Analytical procedures. The proteolytic activity of the sonic-treated material and of preparations resulting from subsequent purification steps was determined by a modification of the Anson (1) technique. The assay mixture contained 0.5 to 2.0 ml of enzyme solution, 1.0 ml of substrate, 2.0 ml of buffer, and water to give a total of 7.0 ml. Incubation was for 1 hr at 37 C. Acetate buffer was used below *p*H 5, phosphate buffer between 5 and 7.5, and Tris above *p*H 7.5. The regular substrate was 5% casein (Hammersten); the other substrates tested were used in the concentrations noted in Table 2. After incubation, 3 ml of 10% (w/v) trichloroacetic acid was added, and the mixture was heated at 70 C for 10 min. The solution was centrifuged to remove the precipitated protein, and the absorbancy was measured at 280 mμ. A control was run by incubating enzyme in buffer solution and by adding, at the end of the incubation period, the trichloroacetic acid followed by the substrate. The absorbancy of the control was subtracted from the absorbancy of the assay sample. Specific activity was expressed as micromoles of tyrosine liberated per minute per milligram of protein.

Benzoylarginine ethyl ester activity was determined by use of the method of Rick (6), and protein was determined by the method of Lowry et al. (5).

RESULTS

Proteinase production. The demonstration of proteolytic activity by sonic-treated preparations of cells and the absence of activity in cell-free growth media indicated that the enzyme was intracellular or was bound to the surface of the cell.

A number of media variables were tested, including the addition of casein, dried milk solids, or Ca^{++} to the media. None of these additives appeared to affect significantly the quantity of proteinase formed. Although the yeast grew well at 5 C, there did not appear to be increased proteinase formation compared with that obtained at 21 C. Under the conditions used for cell production, the log-growth phase occurred between 6 and 18 hr after inoculation.

Purification of proteinase. A summary of the steps used to achieve partial purification of *Trichosporon* proteinase is shown in Table 1.

To prepare the cell extract, yeast cells were ruptured ultrasonically for 4 to 5 min at 20 kc/sec at 5 to 20 C. Each batch contained 6 g of cells per 25 ml of 0.02 M phosphate buffer (*p*H 6.0). The mixture was centrifuged at 8,000 × *g* for 20 min to give the crude extract, fraction 1.

Dialysis was introduced to eliminate salts and other low molecular weight materials from the protein mixture. Fraction 1 was dialyzed at 2 C against several changes of distilled water over a period of 12 to 18 hr. Fraction 2 was obtained by centrifugation at 20,000 × *g* for 20 min.

For the first ammonium sulfate precipitation, the *p*H of fraction 2 was adjusted to 7.0 with 0.5 N NaOH, and sufficient $(NH_4)_2SO_4$ was added to give 42.5% saturation (2). After 8 to 12 hr at 2 C, the precipitate was removed by centrifugation at 20,000 × *g* and discarded. Sufficient

TABLE 1. *Typical purification chart for Trichosporon species proteinase*

Fraction	Volume	Protein	Specific activity (units/mg of protein)*	Total units	Recovery
	ml	*mg/ml*			%
1. Crude sonic extract†	1,625	12	0.0126	246	100
2. Dialysate	1,800	7.2	0.0149	193.5	78
3. First $(NH_4)_2SO_4$ precipitate	200	9.6	0.0575	110.7	45
4. First Sephadex G-100 eluate	400	0.075	0.815	24.4	10
5. Second $(NH_4)_2SO_4$ precipitate	30	0.5	1.15	17.4	7
6. Second Sephadex G-100 eluate	36	0.045	2.16	3.5	1.4

* A unit is defined as micromoles of tyrosine liberated per minute.

† Material from 390 g of wet cells. Original activity was 0.053 units per g of wet cells.

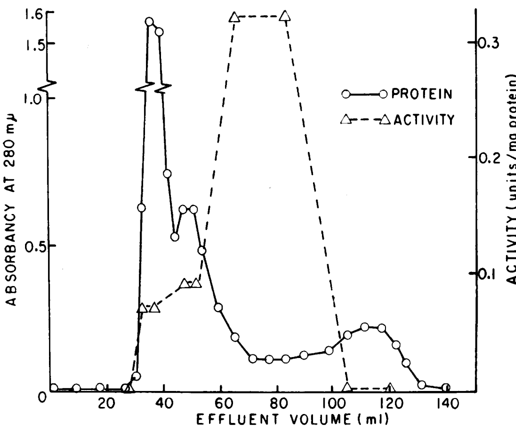

FIG. 1. *Elution profile of fraction 4 from Sephadex G-100 column. Column, 75 by 1.8 cm; load, 77 mg of protein in 8 ml of 0.02 M acetate buffer (pH 5.0) containing 0.5 M NaCl. Eluent, same as above; rate of flow, 0.5 ml/min; volume per tube, 3.0 ml; temperature, 21 C.*

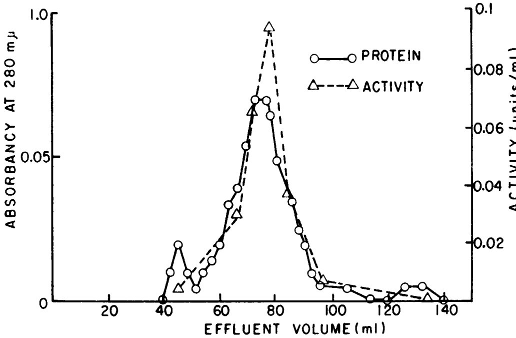

FIG. 2. *Elution profile of fraction 5 from Sephadex G-100 column. Column, 75 by 1.8 cm; load, 3 mg of protein in 6 ml of 0.02 M acetate buffer (pH 5.0) containing 0.5 M NaCl. Eluent, same as above; rate of flow, 0.5 ml/min; volume per tube, 3.0 ml; temperature, 21 C.*

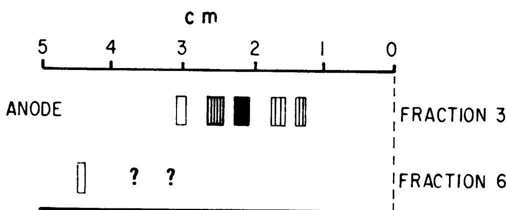

FIG. 3. *Schematic representation of starch gel electrophoresis at two stages of purification. Time, 1.5 hr at 25 ma; temperature, 5 C.*

$(NH_4)_2SO_4$ was added to the supernatant fluid to give 75% saturation. After 8 to 12 hr at 2 C, the precipitate was collected by centrifugation at 20,000 × *g*; it was dissolved in sufficient cold distilled water to give approximately 8 mg/ml of protein and was dialyzed at 2 C against several changes of 0.5 M NaCl in 0.02 M acetate buffer (*p*H 5.0) over a period of 24 hr. The final product was designated fraction 3.

For the first gel filtration, fraction 3 was placed on a Sephadex G-100 column and eluted with

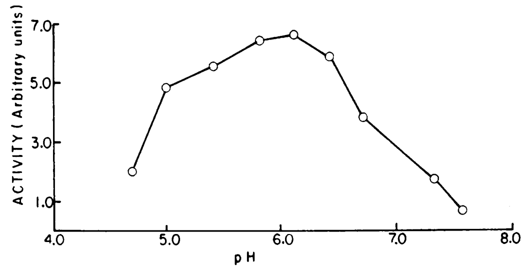

FIG. 4. *Effect of pH on the activity of fraction 6. Assay system containing 0.023 mg of enzyme protein and 50 mg of casein substrate.*

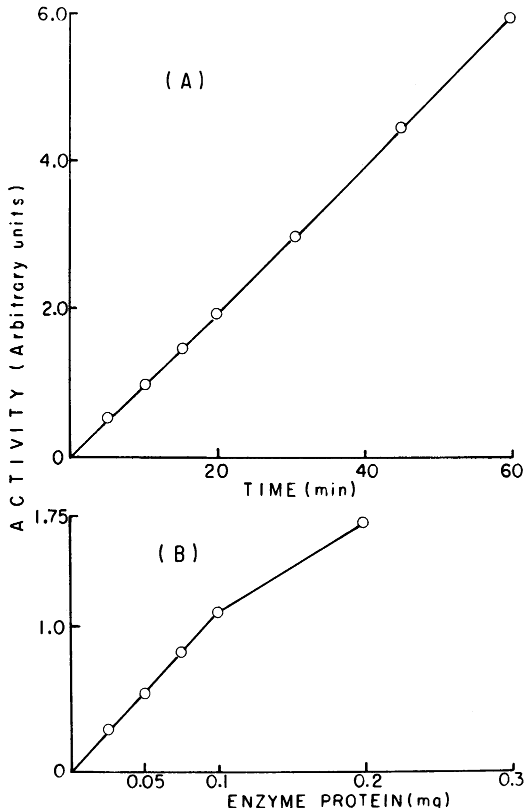

FIG. 5. *Linearity of proteinase hydrolysis of casein. Curve A shows the activity as a function of time. The amount of enzyme used was 0.023 mg of protein. Other assay conditions were the same as those described in the text. Curve B shows the effects of increasing concentration of enzyme; the incubation time was 1 hr.*

0.5 M NaCl in 0.02 M acetate buffer (*p*H 5.0). The elution profile is shown in Fig. 1. The highest specific activity was eluted between 66 and 84 ml of elution volume. The eluates from a number of runs were pooled to give fraction 4.

The second ammonium sulfate precipitation was carried out by treating fraction 4 with sufficient $(NH_4)_2SO_4$ to give 90% saturation. After 8 to 12 hr at 2 C, the precipitate was collected by centrifugation at 20,000 × *g*; it was dissolved in a minimal amount of cold distilled water and was dialyzed at 2 C against several changes of 0.5 M NaCl in 0.02 M acetate buffer (*p*H 5.0) for a period of 24 hr. The product was designated fraction 5.

For the second gel filtration, fraction 5 was placed on a Sephadex G-100 column and eluted with 0.5 M NaCl in 0.02 M acetate buffer (*p*H 5.0). The elution profile is shown in Fig. 2. The highest specific activity was eluted between 72 and 80 ml of elution volume. This is fraction 6, the material used in the property studies.

To determine purity, fraction 6 was dialyzed against water, concentrated about fivefold by treatment with Sephadex G-25, and fractionated by starch gel electrophoresis. Staining indicated the presence of one major component and perhaps several minor ones that migrated toward the anode; no cathode migrating components were detected. In Fig. 3, fraction 6 is compared to fraction 3 in which at least five anode migrating components are evident.

Properties. The enzyme exhibited a *p*H optimum at 5.8 to 6.2. There was little activity below 4.7 and above 7.55 (Fig. 4).

The proteolytic activity was a linear function of enzyme concentration and of time up to 1 hr of incubation (Fig. 5).

The effect of casein concentration in the assay mixture is presented in Fig. 6. Saturation of the enzyme was obtained with approximately 25 mg of casein per tube.

Casein was the only good substrate found for this proteinase (Table 2). It did, however, attack

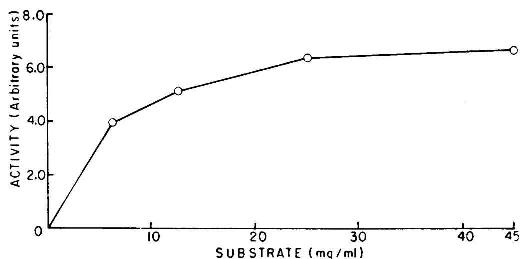

FIG. 6. *Effect of substrate concentration on proteinase activity when assayed for 1 hr at 37 C with 0.023 mg of enzyme protein present.*

TABLE 2. *Substrate specificity of proteinase*

Substrate	Concn	Activity
	mg/ml	%
Casein	7	100
Hemoglobin	7	25
Crab	6.3	50
Bovine serum albumin	7	0
Lysozyme	7	0
Benzoylarginine ethyl ester	1 mM	0

TABLE 3. *Heat inactivation of proteinase**

Heat treatment for 10 min at	Activity remaining
C	%
20	100
50	70
55	23
60	0

* Enzyme was heated in 0.2 M phosphate buffer (*p*H 6.0). Assay system included 0.5 ml of enzyme, 2.0 ml of buffer, 1.0 ml of casein substrate, and 3.5 ml of water. Incubation was for 1 hr at 37 C.

TABLE 4. *Comparison of the properties of yeast proteinases*

Property	*Trichosporon* sp. proteinase	*Trichosporon* sp. proteinase (Vorbeck and Cone, 8)	*Saccharomyces cerevisiae* proteinase B (Lenney, 4)
*p*H optimum	5.8–6.2	5.8	6.2
Activated by	10 mM cysteine 0.1 mM EDTA 0.1 mM Co	5 mM cysteine	1% $CaCl_2$ Not activated by 0.4 mM cysteine or 100 mM cyanide
Inhibition	0.1 mM pCMB* 0.1 mM phenylmethylsulfonyl fluoride Not inhibited by 0.15 mg/ml of ovomucoid	0.01 mM pCMB 0.01 mM *N*-ethylmaleimide 0.1 mM iodoacetic acid	1 mM pCMB 0.15 mg/ml of ovomucoid

* *p*-Chloromercuribenzoate.

crab protein at a rate of about 50% that of casein, and hemoglobin at a rate of about 25% that of casein. It was inactive against lysozyme, bovine serum albumin, and benzoylarginine ethyl ester.

The proteolytic activity with casein as substrate was activated slightly by 1 to 10 mM cysteine, 0.1 to 1 mM EDTA, and 0.1 to 1 mM Co^{++}. There was slight inhibition by 10 mM Co^{++} and 0.1 to 1 mM phenylmethylsulfonylfluoride. *p*-Chloromercuribenzoate was 100% inhibitory at 1 mM concentration. Results indicated that it was a competitive inhibitor.

The proteinase was inactivated completely by heating to 60 C for 10 min (Table 3). When the enzyme was held at a *p*H below 4 or above 8, at 21 C, for periods of 0.5 hr the activity was decreased considerably.

Discussion

There are several factors that could make yeasts potentially important contributors in the spoilage of fishery products. These are their abilities to grow at low temperatures and to compete with other surviving flora after a food has been subjected to a semipreservation method such as radiation pasteurization.

The properties of the proteinase can be considered in regard to its potential activity against substrates such as a protein food. The optimal *p*H, when casein was the substrate, was within the *p*H range of most fishery products. Crab protein was shown to be a substrate. Since the enzyme studied was intracellular or bound to the surface of the cell, proteolysis would probably only occur in the immediate vicinity of the cell, unless the cell was disrupted.

The proteinase from the *Trichosporon* species used in these studies did not have any unusual properties. It did have a number of properties (Table 4) that were similar to the intracellular proteinase from a *Trichosporon* species isolated from cheese by Vorbeck and Cone (8) and the intracellular proteinase B from *S. cerevisiae* studied by Lenney (4). Although the proteinases were only partially purified in each of these studies, the similarity of the properties indicates the probability of like enzymes in different species of yeasts.

Diethylaminoethyl (DEAE) cellulose was used unsuccessfully in initial purification attempts. The enzyme appeared to lose activity on the column, since no activity could be eluted even though large amounts were originally placed on the column. No efforts were made to determine the reason for this difficulty. Other purification materials such as DEAE-Sephadex, Sephadex G-75, Sephadex G-200, and calcium phosphate gel were tested during the purification experiments; however, each was found to be less effective than Sephadex G-100.

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