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Purification and Properties of Proteolytic Enzymes from Thermophilic Actinomycetes

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The enzymes isolated from two selected cultures of thermophilic actinomycetes—*Thermomonospora fusca* (A 29) and *Thermoactinomyces vulgaris* (A 60)—possess proteolytic activity. The enzymes were purified more than 35- to 40-fold and showed three bands each upon cellulose acetate electrophoresis at several *p*H values. Based upon Sephadex gel filtration, molecular weights of 21,500 and 23,800 were calculated for the active peaks of the enyzmes. The purified enzymes lysed heat-killed cells of gram-positive and gram-negative bacteria, mycobacteria, and fungi and also hydrolyzed casein. The enzymes were most active between a temperature range of 60 and 70 C and *p*H 8.0 and 9.0, and were significantly inhibited by potassium permanganate, potassium ferricyanide, and iodine.

A few reports have been made on bacteriolytic and proteolytic activities of thermophilic actinomycetes. Kosmachev (5) observed that some strains of thermophilic actinomycetes isolated from high-altitude soil lysed killed bacterial suspensions, and Lawrence (Diss. Abstr., p. 480, 1957) and Mizusawa et al. (8) reported on the proteolytic activity of thermophilic actinomycetes. The latter investigators worked with enzymes of *Streptomyces rectus* var. *proteolyticus* that could hydrolyze casein, and fractionated the proteinases on CM Sephadex. We previously described (3) thermophilic actinomycetes producing extracellular enzymes that lysed heat-killed bacterial cells and hydrolyzed proteins. The present communication describes purification and properties of the enzymes from two selected cultures.

MATERIALS AND METHODS

Thermomonospora fusca (A 29) and *Thermoactinomyces vulgaris* (A 60) were selected from several organisms which gave good results. Cultures were maintained as described earlier (3).

Preparation of culture filtrates. A series of 250-ml flasks, each containing 100 ml of modified McCarty medium (7, 7*a*), were inoculated with the slant cultures and incubated for 48 to 72 hr on a reciprocal incubator shaker maintained at 50 C. The cells were then removed by filtration and the filtrates were stored at 4 C.

Assay procedure. Except where specifically mentioned, assays of proteolytic enzyme activity of both crude and isolated enzymes were performed by using a suspension of heat-killed cells of *Escherichia coli* as substrate. A stock suspension in 0.025 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer at *p*H 9.0 and showing an optical density of 0.5 (0.6 mg, dry weight, per ml of washed cells) at 540 nm was prepared and stored at 4 C. For the assay, 0.5 ml of the enzyme preparation or Tris buffer (as a control) was mixed with 4.5 ml of the stock substrate solution in a test tube (12 by 100 mm). The turbidity of the tube was immediately determined with a Klett-Summerson colorimeter at 540 nm. The tube was then placed in a water bath (50 C), and the turbidity was again determined after exactly 1 hr. The unit of proteolytic activity was defined as the change in absorbancy of 0.001 at 540 nm of a heat-killed suspension of *E. coli* in 1 hr at 50 C. Specific activity was defined as the number of units per milligram of protein. Protein was estimated by the method of Lowry et al. (6).

Gel filtration through Sephadex gels. The enzymes were fractionated by gel filtration through Sephadex gels, and their molecular weights were determined by the technique described by Andrews (1). The columns (1.5 by 50 cm) of Sephadex G-50 and G-100 were prepared and washed with 0.1 M KCl in 0.025 M Tris buffer at *p*H 7.2. A 2-ml sample of each enzyme containing 20 mg of protein was added to the top of the column and was washed with the KCl-Tris solution at a flow rate of 30 ml/hr. Samples of 5 ml were collected; protein concentration of each was determined, and those fractions containing protein were assayed for proteolytic activity. The molecular weights of the enzymes were determined by comparing their effluent volumes with those of several proteins of known molecular weights.

Electrophoresis. Electrophoresis on cellulose acetate was conducted by use of Toshniwal's electrophoresis chamber and glass plates as support for the strips

(Toshniwal Bros. Pvt. Ltd., Bombay, India). The strips, 2.5 by 12 cm, were soaked overnight in buffer before use. After they were lightly blotted, a 10-μlitre sample of each enzyme preparation, obtained by acetone precipitation followed by dialysis, and active protein from gel filtration followed by dialysis, was applied to the strips. The strips were placed in the chamber with the origin located in the center, and a current of 1.5 to 2 ma per strip was applied for 2 hr. The strips were stained with 0.02% aqueous nigrosin. Experiments were conducted with the following buffers at a concentration of 0.05 M: Veronal (*p*H 7.0, 8.0, and 9.0), citrate-sodium phosphate (*p*H 5.0 and 7.0), and sodium carbonate-bicarbonate (*p*H 10.6).

Sephadex G-50 and G-100, cytochrome *c*, gamma-globulins, and ribonuclease were obtained from Pharmacia Fine Chemicals, Inc., New York, N.Y. Insulin was purchased from Boots Drug Co.; *p*-chloromercuribenzoate (PCMB) and serum albumin were from L. Light and Co.; soybean trypsin inhibitor, lysozyme, and crystalline trypsin were purchased from Nutritional Biochemical Corp., and ovalbumin was from British Drug Houses, England.

RESULTS

Purification of the enzymes. The filtrate at 4 C was subjected to acetone precipitation. A 1.5 volume of acetone, precooled to −15 C was added slowly with vigorous stirring. After 24 hr, the precipitate was removed by centrifugation at 1,600 × *g* for 30 min. The precipitate was dissolved in 0.025 M Tris buffer, *p*H 9.0, and dialyzed against the same buffer (0.0025 M) for 24 hr at 4 C (two changes).

Gel filtration. The enzymes were purified by gel filtration. Acetone-precipitated, dialyzed enzyme preparations of A 29 and A 60 could be resolved into three protein peaks each (Fig. 1). Proteolytic activity of each peak was assayed after its protein concentration was brought to 200 μg/ml. In both of the organisms peak B was active. Molecular weights of enzyme fractions were determined by comparison of their effluent volumes with those of several proteins with established molecular weights. The molecular weights of the proteins used in standardizing the columns were taken from the literature as follows: cytochrome *c*, ribonuclease, lysozyme, and trypsin (12); soybean trypsin inhibitor, ovalbumin, serum albumin, and gamma-globulins (1); and insulin (2). A curve was plotted as effluent volume against molecular weight for each grade of Sephadex (Fig. 2). The curve can be extrapolated to include the experimental points for the effluent volumes of the enzymes. The molecular weights of the active peaks of A 29 and A 60 enzymes were 21,500 and 23,800, respectively.

The results of a typical purification of the lytic enzymes are summarized in Table 1. A 35- to 40-fold purification of the enzymes was achieved.

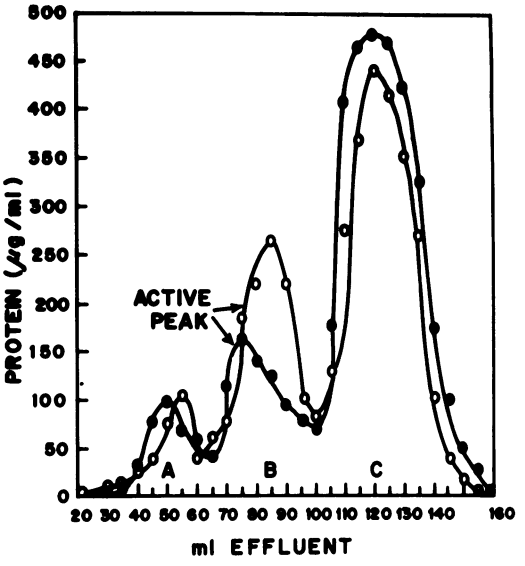

FIG. 1. *Purification of A 29 and A 60 enzymes on a Sephadex G-100 column. A 2-ml amount of acetone-precipitated enzyme was applied to a column (1.5 by 50 cm), and the enzyme was washed through with 0.025 M Tris buffer (pH 7.2) at a rate of 30 ml/hr. Fractions containing protein were assayed for proteolytic activity. Symbols: ○, A 29; ●, A 60.*

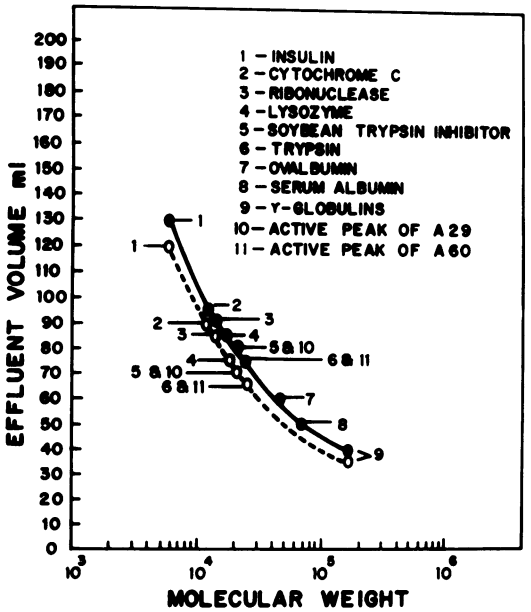

FIG. 2. *Determination of the molecular weight of the A 29 and A 60 enzymes by gel filtration on Sephadex G-50 and G-100 columns (1.5 by 50 cm) calibrated with proteins of known molecular weights. Symbols: ○, G-50; ●, G-100.*

TABLE 1. *Summary of purification data for A 29 and A 60 enzymes*

Step	Organism	Units/ml[a]	Total units	Specific activity[b]	Purification (fold)	Yield (%)
Dialyzed growth liquor	A 29	500	70,400	400	1	100
	A 60	480	54,250	350	1	100
After acetone (1.5 vol) precipitation	A 29	450	50,400	7,200	18	71
	A 60	425	36,700	5,250	15	67
After gel filtration	A 29	475	36,800	16,000	40	52
	A 60	440	29,400	12,250	35	41

[a] The unit of proteolytic activity is defined as the change in absorbancy of 0.001 at 540 nm of a heat-killed suspension of *E. coli* in 1 hr at 50 C.

[b] Expressed as units per milligram of protein.

Electrophoresis. Acetone-precipitated enzyme preparations, followed by dialysis, were concentrated under vacuum until the protein concentration was 10 mg/ml. A 10-μliter amount was applied to cellulose acetate strips. The pattern of protein bands obtained with both the organisms was similar; at *p*H 7.0 and below, two bands migrated towards the cathode and one remained at the origin. At *p*H 8.0 and above, two bands migrated towards the anode and one remained at the origin. Best separations of both the enzyme fractions were obtained with Veronal buffer, *p*H 7.0 (Fig. 3). The activity of the protein bands was tested by running, at *p*H 7.0, two strips simultaneously for each enzyme. One of the strips was stained, and the corresponding protein bands on the unstained strips were eluted in 0.5 ml of Tris buffer and assayed for proteolytic action. In the case of both organisms, the band nearest the cathode, at *p*H 7.0, was active (Fig. 3).

To correlate the results of electrophoresis of acetone-precipitated, dialyzed enzymes and Sephadex gel filtration, the active peak (obtained by gel filtration followed by dialysis) of each enzyme was allowed to run on cellulose acetate electrophoresis strips in Veronal buffer, *p*H 7.0. The active proteins from Sephadex gel filtration corresponded to those obtained by electrophoresis of acetone-precipitated enzymes (Fig. 3).

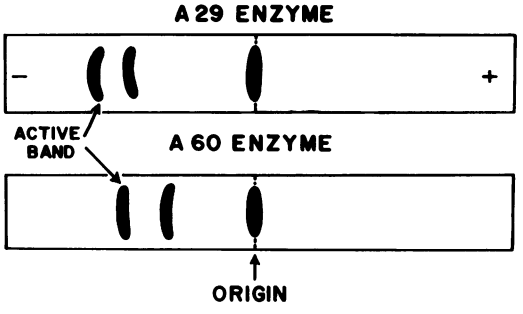

FIG. 3. *Drawing of the appearance of the electrophoresis of A 29 and A 60 enzymes on cellulose acetate strips (Veronal buffer, pH 7.0). A 10-μlitre amount of each enzyme solution was applied in a streak at the origin. A current of 1.5 to 2 ma was applied to each strip for 2 hr. Stained with nigrosin.*

Action on different test organisms. The purified enzymes were tested for their ability to clear up suspensions of heat-killed cells and cell walls of a variety of microorganisms. Each organism was harvested in the early stationary phase, washed thrice with 0.025 M Tris buffer at *p*H 9.0, and suspended in this buffer to an optical density of 0.5. Cell walls were prepared by a procedure adopted by Ensign and Wolfe (4). To 4.5 ml of cell or cell wall suspension was added 0.5 ml of purified enzyme. The mixture was incubated at 50 C, and the turbidity was followed for 1 hr. Each suspension of cells or cell walls without enzyme was similarly assayed as an autolysis control; in no instance did the change exceed 0.01 absorbancy. The data presented in Table 2 include this correction. Gram-negative bacteria were more susceptible than the gram-positive ones. Of 14 species of gram-negative bacteria tested, suspensions of 5 were cleared up within 15 min, 8 within 30 min, and 1 within 1 hr. *E. coli* was lysed to a maximum extent. Of five species of gram-positive bacteria tested, *Bacillus subtilis* underwent maximal lysis, whereas *Corynebacterium poinsettiae* was least affected; mycobacteria were resistant, with the exception of *Mycobacterium phlei*. Among the fungi, only *Microsporum gypseum* and *Penicillium notatum* were lysed. Cell walls of all the organisms were resistant to the enzymes.

Time course of proteolytic activity on E. coli cells. The rate of lysis of a suspension of heat-killed *E. coli* cells by 0.5 ml of purified A 29 and A 60 enzymes is illustrated in Fig. 4. Change in absorbancy was measured at 10-min intervals for

TABLE 2. *Survey of the proteolytic activity of purified A 29 and A 60 enzymes against various microorganisms*[a]

Organism	Per cent lysis by enzyme from[b]	
	A 29	A 60
	Lysis in 15 min	
Aerobacter aerogenes	93	92
Escherichia coli	95	92
Proteus vulgaris	75	76
Pseudomonas marginata	72	68
Vibrio comma	85	80
	Lysis in 30 min	
Cytophaga hutchinsonii	50	45
Pseudomonas aeruginosa	75	70
Salmonella typhosa	88	88
S. paratyphi	89	80
Shigella dysenteriae	76	78
S. flexneri	75	80
Spirillum serpens	80	78
Xanthomonas begoniae	80	50
	Lysis in 1 hr	
Bacillus subtilis	48	44
Corynebacterium poinsettiae	0	4
Sarcina lutea	30	32
Staphylococcus aureus	2	5
Streptococcus fecalis	10	15
Serratia marcescens	55	52
Mycobacterium phlei	5	4
M. smegmatis	00	0
M. tuberculosis	0	0
Candida albicans	0	0
Saccharomyces cerevisiae	0	0
Microsporum gypseum	40	50
Penicillium notatum	35	32
Trichophyton mentagrophytes	0	0
T. rubrum	0	0

[a] Cell walls of all the test organisms were resistant to the enzymes.

[b] Initial optical density − final optical density/final optical density × 100.

2 hr. The cell lysis was linear up to 65 to 70 min. Hence, 1 hr was selected as an assay time.

Caseinolytic action. Caseinolytic action was assayed with casein as substrate by a procedure adopted from that of Rick (10) in which release of trichloroacetic acid-soluble tyrosine is estimated with Folin Ciocalteu reagent. To 1 ml of a 1% solution of casein in a test tube (20 by 150 mm) was added a suitable sample of purified enzyme preparation diluted in 0.025 M Tris buffer at *p*H 9.0. After incubation at 37 C for 1 hr with occasional shaking, 3.0 ml of 5% trichloroacetic

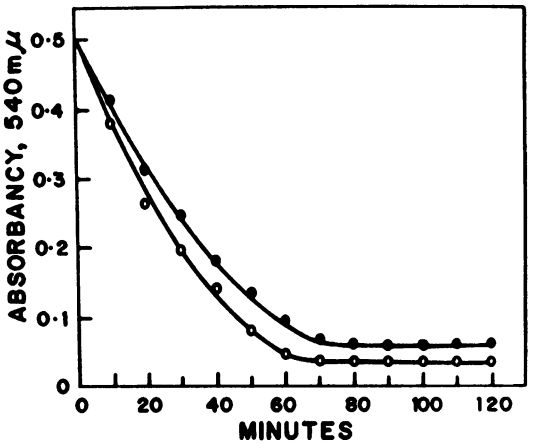

FIG. 4. *Rate of lysis of heat-killed E. coli cells by purified A 29 and A 60 enzymes. Incubation was done at 50 C for 2 hr and change in absorbancy was recorded at 10-min intervals. Symbols:* ○, *A 29;* ●, *A 60.*

acid was added, and the mixture was filtered through Whatman no. 1 filter paper. A 0.5-ml amount of filtrate, 10 ml of 0.5 N NaOH, and 3.0 ml of dilute phenol reagent (diluted 1:3 with distilled water) were mixed in a 50-ml flask. At 10 min after addition of phenol reagent, the absorbancy of the mixture at 540 nm was determined in a Klett-Summerson colorimeter. A blank prepared by adding trichloroacetic acid before incubation was similarly assayed, and the assay readings were corrected for the blank values. The caseinolytic unit was defined as the amount of enzyme which hydrolyzed casein at such an initial rate that the amount of hydrolytic products formed per minute had the same optical density of reaction with the phenol reagent as 1 mmole of tyrosine. For both enzymes, a curve was plotted as increase in absorbancy of tyrosine released against time. The curve was linear up to 100 to 110 min for the two enzymes. However, 1 hr was selected as an assay time to maintain the constancy with cell lysis. Specific activity was expressed as units per milligram of protein. The specific activities of A 29 and A 60 were 2,831 × 10^{-4} and 3,944 × 10^{-4} units, respectively.

Effect of pH on enzyme activity and stability. The effect of *p*H on proteolytic activity was determined by the use of acetone-dried heat-killed *E. coli* cells suspended in 0.025 M citrate-sodium phosphate buffer (*p*H 3.0 to 7.0), 0.025 M Veronal buffer (*p*H 7.5 to 9.0), and 0.025 M sodium carbonate-bicarbonate buffer (*p*H 9.5 to 10.0). The enzymes of A 29 and A 60 were active over a *p*H range of 6.0 to 10.0, with maxima at *p*H 8.5 and *p*H 9.0, respectively (Fig. 5). The *p*H stability of the enzymes was determined by exposing 0.5-ml

amounts of the enzymes (200 μg/ml) for 2 hr at 30 C to buffers at various *p*H values. Samples were mixed with 4.5 ml of *E. coli* cells in 0.025 M Tris buffer and assayed for proteolytic activity at *p*H 8.5 and 9.0 for A 29 and A 60 enzymes, respectively. The enzymes of A 29 were most stable between *p*H 7.0 and 10.0, and the enzymes of A60 were most stable between *p*H 7.0 and 9.0, but they were least stable between *p*H 3.0 and 5.0 (Fig. 6).

Effect of temperature on enzyme activity and stability. Proteolytic activity was determined by incubating enzymes with the substrate for 1 hr over a temperature range of 40 to 90 C (Fig. 7). Both the enzymes were active between 60 and 70 C. The stability of the enzymes with respect to temperature was determined by exposing 0.5-ml samples of the enzymes (200 μg/ml) in 0.025 M Tris buffer at various temperatures. Samples were removed after 1 hr and assayed for proteolytic activity. Although the thermal stability falls with increase in temperature, the enzymes retained 40 to 60% of their activity at 80 and 90 C (Fig. 8).

Effect of inhibitors on the proteolytic activity. The effect of some inhibitors upon proteolytic activity is presented in Table 3. Maxima of 34% and 67 to 72% inhibition were observed with oxidizing agents like iodine and $KMnO_4$, respectively, at 10^{-3} M. Iodine at 10^{-4} M and $KMnO_4$ at 10^{-5} M showed only 5 to 7% and 9 to 17% inhibition, respectively. Reducing agents such as KCN and sodium thioglycolate had almost negligible effect at 10^{-3} M, but L-cysteine and potassium ferricyanide inhibited 4 to 23% and 43% of the enzymatic activity at this concentration. Metal chelating agents such as ethylenediaminetetraacetate, sodium citrate, and sodium oxalate had negligible effect on the proteolytic activity of

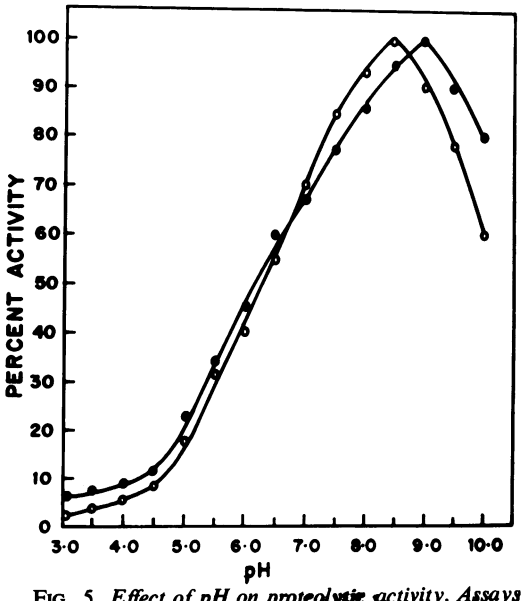

FIG. 5. *Effect of pH on proteolytic activity. Assays were carried out at 50 C. Symbols: ○, A 29; ●, A 60.*

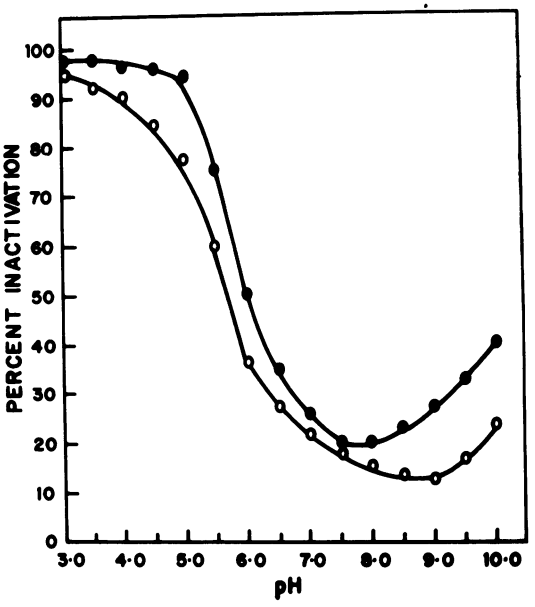

FIG. 6. *Effect of pH on the stability of purified proteolytic enzymes. Enzymes were assayed in Tris buffer, pH 8.5 and pH 9.0 (for A 29 and A 60 enzymes, respectively) at 50 C. Symbols: ○, A 29; ●, A 60.*

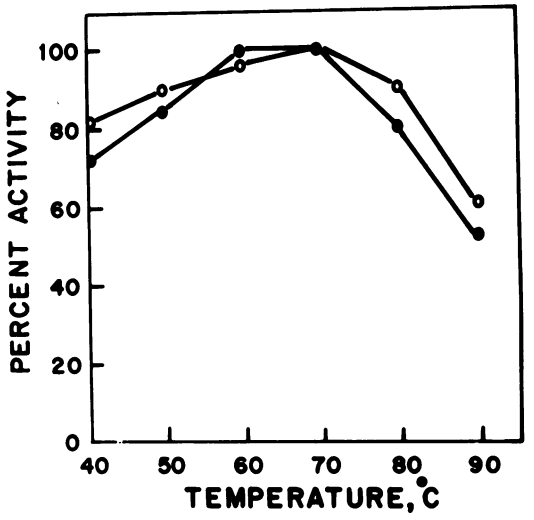

FIG. 7. *Effect of temperature on proteolytic activity. Enzymes were assayed in Tris buffer. Symbols: ○, A 29 ●; A 60.*

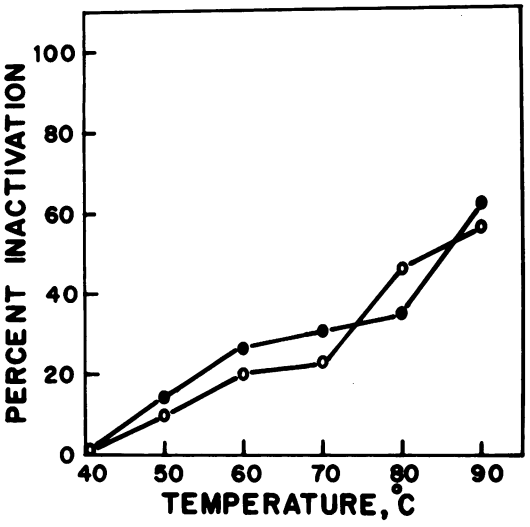

FIG. 8. *Thermal stability of the proteolytic enzymes. Symbols:* ○, *A 29;* ●, *A 60.*

both the enzymes at 10^{-3} M. Among the sulfhydryl inhibitors, iodoacetic acid inhibited only 3 and 12% of the enzymatic activity, respectively, whereas PCMB inhibited 24 and 27% of the activity, respectively, at 10^{-3} M. Sodium lauryl sulfate and semicarbazide had only 15 to 34% and 16 to 20% inhibitory effect, respectively, at 10^{-3} M.

TABLE 3. *Effect of inhibitors on the proteolytic activity of the purified A 29 and A 60 enzymes*[a]

Agent tested	Molarity	Per cent inhibition	
		A 29	A 60
None (control)		0	0
KCN	10^{-2}	4.5	9.1
	10^{-3}	2.3	1.2
EDTA[b]	10^{-2}	3.4	4.6
	10^{-3}	0	1.2
$KMnO_4$	10^{-3}	67	72
	10^{-4}	50	55
	10^{-5}	17	9.1
$K_3Fe(CN)_6$	10^{-3}	43	43
	10^{-4}	13	15
Sodium-citrate	10^{-2}	3.4	4.6
	10^{-3}	0	1.2
Sodium-oxalate	10^{-2}	0	1.2
	10^{-3}	0	0
Sodium-lauryl sulfate	10^{-3}	34	15
	10^{-4}	14	9.1
Sodium-thioglycolate	10^{-3}	12	4.6
	10^{-4}	0	0
Sodium-thiosulfate	10^{-3}	9	4.6
	10^{-4}	1	9.1
Iodoacetic acid	10^{-2}	14	9.1
	10^{-3}	12	3
PCMB	10^{-2}	36	38
	10^{-3}	24.5	27
	10^{-4}	12	7
L-Cysteine	10^{-3}	23	4.6
	10^{-4}	12	3
Iodine	10^{-3}	34	34
	10^{-4}	5.6	7
Semicarbazide	10^{-3}	16	20
	10^{-4}	5.6	9.1

[a] A 0.5-ml amount of enzyme solution in 0.025 M Tris buffer (*p*H 9.0) was mixed with 0.1 ml of each inhibitor solution, and its activity was determined after 30 min of incubation at 30 C.

[b] Ethylenediaminetetraacetate.

DISCUSSION

Extracellular proteinases of relatively high thermal stability have been reported in recent years. Williamson et al. (13) reported that proteinases of *Streptococcus lactis* retained 32% of the enzymatic activity after 60 min at 98 C, and that this activity was inhibited by PCMB. Mizusawa et al. (8) showed that proteinases of thermophilic *Streptomyces* species 1689 retained 60% of the activity after 30 min at 80 C in 0.01 M $CaCl_2$, and the activity was inhibited by PCMB. Ensign and Wolfe (4) reported that a cell wall lytic and proteolytic enzyme from a myxobacter-AL-1 retained 35% of the activity after 2 hr at 60 C, but the enzyme was insensitive to PCMB. A 29 and A 60 enzymes in the present study retained 40 to 45% of their activity after 10 min at 90 C; A 29 showed 24 and 67% inhibition by PCMB and $KMnO_4$, respectively, while A 60 showed 27 and 72% inhibition by PCMB and $KMnO_4$, respectively.

According to Montague (9), a proteolytic enzyme may attack cell walls under some conditions. He showed that trypsin had almost no effect on intact cell walls of *Streptococcus faecalis*; but after partial lysis by lysozyme, the cell walls were attacked rapidly. Ensign and Wolfe (4) reported that the AL-1 enzyme from a myxobacter possessed both cell wall lytic and proteolytic properties. Even by employing extremely sensitive techniques like gel filtration, density gradient centrifugation, ultracentrifugation, and electrophoresis on polyacrylamide gel or cellulose acetate, separation of the cell wall lytic and proteolytic enzymes was not achieved. In the present study, purification of A 29 and A 60 enzymes was done by gel filtration, and separation was achieved by electrophoresis. Electrophoresis at several *p*H values, as well as gel filtration through Sephadex G-50 and G-100, gave a single active protein for each enzyme. These were taken as the criteria for the purity of the

enzymes. It is interesting that the purified enzymes having only proteolytic activity caused 44 to 48% lysis of heat-killed cells of *B. subtilis*. It is possible that the cell walls of *B. subtilis* possess some substrate for these enzymes, but this aspect was not studied.

Mizusawa et al. (8) observed maximal proteolytic action at 50 C, *p*H 10.6, and at 80 C, *p*H 8.0. A protease from *Mucor pussilus* (11) exhibited optimal proteolytic activity at about 55 C; the enzyme was irreversibly destroyed at 65 C with 90% loss of activity and was stable between *p*H 3.0 and 6.0. A 29 and A 60 enzymes were most active at 60 to 70 C and stable between *p*H 7.0 and 10.0.

Thus, several proteolytic enzymes of microbial origin have been reported which differ from each other in their sensitivity to inhibitors, and in their *p*H and thermal stabilities. The differences in their modes of action and the products of lysis can only be elucidated by comparative experiments.

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