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Investigation of the Protease Forming Ability of *Serratia marcescens* and One of its Mutant Strains

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The ability of eleven different strains of bacteria to form proteolytic enzymes was investigated. *Serratia marcescens*, SBL-strain was found to be amongst the best producers of these enzymes. The strain was cultivated in 10 litre batches and it was demonstrated that only extracellular enzymes were formed. By means of UV-radiation a good protease forming, unpigmented mutant strain was obtained. The enzyme from the mutant strain was purified and concentrated in the same way as that from the natural strain. The mutant strain produced a proteolytic enzyme with a pH-optimum of 5.4 on milk-agar-plates and had an abnormal high tolerance against merthiolate. Studies were also made of the pH-dependence and temperature stability of the enzyme formed by the mutant strain.

In the last few years considerable importance has been placed upon proteolytic enzymes as tools for studies on the structure of proteins and for investigation of split products of biological or biochemical interest. Since the specificity of proteolytic activity towards different proteins can vary considerably among different enzymes the main purpose of this investigation has been to look for an enzyme system with as unspecific an activity as possible, which might be useful in tissue culture technique.

I. INVESTIGATION OF THE ABILITY OF SOME DIFFERENT MICROORGANISMS TO PRODUCE PROTEOLYTIC ENZYMES

The formation of proteolytic enzymes in the following strains was investigated: 1, *Serratia marcescens*, SBL-strain;* 2, *Serratia marcescens*, U 18; 3, *Serratia marcescens*, strain from Karolinska Institutet, Stockholm; 4, *Serratia rubidae*, SBL-strain; 5, *Streptococcus faecalis*, 16908, SBL-strain; 6, *Streptococcus faecalis*, M 19, SBL-strain; 7, *Streptococcus faecium*, $A_2$1, SBL-

* The strain stored at our laboratory for reference use.

strain; 8, *Streptococcus mitis*, SBL-strain; 9, *Proteus vulgaris*, U 26, SBL-strain; 10, *Lactobacillus casei*, SBL-strain; 11, *Proteus mirabilis*, SBL-strain.

Materials and methods

Media. 1, Medium 59, v. Hofsten and Tjeder.[1] 2, Tryptone-medium, 0.2 % with addition of 40 mg/l $CaCl_2$, Mac Donald *et al.*[2] 3, Medium 63 with addition of gelatin and peptone.[3] 4, Nutrient Agar (slant tubes) SBL 541493. 5, Nutrient Agar (Deep agar tubes) SBL 541494. 6, Nutrient Broth, Difco B 3. 7, Trypticase-Soy (TS), BBL 11768. 8, AC-medium, Bleiweiss and Zimmerman, Difco B 3 A.[4] 9, NZ-case, synthetic medium, Bleiweis and Zimmerman.[4] 10, Murakami's medium, Murakami *et al.*[5] 11, Tomato Juice Agar, Difco B 31. 12, Micro Assay Culture Agar, Difco B 319. 13, Glucose-Yeast Extract-Milk-medium, Brandsaeter and Nelson.[6] 14, Skim milk, Brandsaeter and Nelson.[6] 15, Peptonized milk, Difco B 35. 16, Micro Inoculum Broth, Difco B 320. 17, Modified Briggs Medium, Brandsaeter and Nelson.[6] 18, Mills' Medium, Mills *et al.*[7]

Culture methods. Serratia-strains cultivated on agar slants were used to inoculate 5 ml nutrient broth tubes which were incubated at 30°C. After 24 h 1.25 ml of each culture was transferred to flasks containing 25 ml medium. The outgrowth of each strain was tested in the media 1 – 3 and 10 at 25°C with agitation. Tests for growth and enzyme production were made. For the cultivation of *Serratia rubidae* nutrient broth was used as the enzyme-forming medium. Murakami's medium with bovine albumin as enzyme-inducing component was tested on the four *Serratia*-strains but cultivation was done in nutrient broth. Growth at 25°C with agitation as well as growth at 30°C without agitation were tested. Tests for determination of proteolytic activity, growth and pH were taken after 24 and 48 hours.

Streptococcus-strains. From agar slants and deep agar tubes cells were transferred to tubes containing 5 ml nutrient broth and to tubes containing 5 ml Trypticase-Soy-medium. After 24 h 0.25 ml of the culture was transferred to 5 ml AC-medium and after another 24 h the culture was transferred to NZ-case. The inoculum was 1 % of the total volume of medium. The growth temperature was 37°C and each strain was grown with or without agitation. According to the method of Bleiweis and Zimmerman,[4] 50 % of the culture volume was changed with fresh medium after 24 h and the growth continued. By this means the cells were maintained in log-phase to reach maximum enzyme formation. Tests were taken when the medium was changed. The cultivations were run at pH 7, 6.0, and 5.5. In the last two pH's the medium was not exchanged. The most active enzyme producer *Streptococcus faecalis* 16908 and *Streptococcus faecalis* M 19 were also tested at pH 7 without changing the medium.

Proteus vulgaris. Tests for enzyme formation were negative and therefore this bacterium was not used further.

Proteus mirabilis. From agar slants and deep agar tubes, cells were transferred to 5 ml Trypticase-Soy-medium. After 24 h 1.25 ml was inoculated into 25 ml Mills' medium and medium 63 supplemented with gelatin and peptone. Incubation at 37°C was made with and without agitation. Tests for enzyme formation, pH, and bacterial density were taken after 24 and 48 h.

Lactobacillus casei. Streak plates were made on Tomato Juice Agar and Micro Assay Culture Agar. After 48 h at 30°C and 37°C, respectively, the cells were transferred to 3 ml each of the media glucose-yeast extract-milk, skim milk, peptonized milk and micro inoculum broth. They were incubated at 30°C and 37°C for 48 h, then 1.25 ml from each tube was transferred to 25 ml of Briggs medium. Different incubation times were tried before enzyme tests were taken.

Enzyme tests. For determination of proteolytic activity the following three methods were used: viscosimetry, Kunitz' method modified by Rydén and von Hofsten and the agar plate method.[8-11] Agar- or agaroseplates, diameter 14 cm, containing hemoglobin, milk or albumin were prepared as follows:

Hemoglobin plates. 1 g agar or agarose was dissolved in 50 ml boiling buffer-solution (see buffer-solution). 25 ml 4 % bovine hemoglobin was mixed with 25 ml buffer solution (see buffer solution). The two solutions were carefully mixed with each other.

Milk plates. 1 g agar or agarose was dissolved under boiling in 50 ml buffer-solution. 50 ml 3 % fat-free dry milk (Semper) in distilled water was added.

Albumin plates. 1 g agar or agarose was dissolved under boiling in 50 ml buffer-solution after which the mixture was cooled to 80°C. A solution containing 5 ml 10 % bovine albumin in 50 ml buffer solution was added to the agar solution. The resulting solution was heated instantly to 87°C. 3 mm holes were made in the agar plates into which the test materials were placed. The enzyme activity was demonstrated by clear zones formed around the holes. The logarithm of the diameter of the zones is directly proportional to the actual enzyme activity. The tests were carried out at pH 5, 6, 7, and 8. Each plate contained 35 ml agar or agarose and 14 holes per plate were made and filled with 20 μl test solution.

The bacterial cultures were centrifuged. To the supernatants merthiolate was added to a final concentration of 0.01 %. Then the supernatants were put into the holes. The plates were incubated at 30°C for the *Serratia*-strains and at 37°C for the other organisms. After a defined time the plates were read. At the same time corresponding tests were carried out with NBC Trypsin (1:300), crystallized trypsin, and subtilisin.

Buffer solutions. 0.1 M phosphate buffer was made up to the various pH's used except for pH 5, where 0.1 M citrate-phosphate-buffer was employed. The buffer solutions were prepared according to Augustinsson.[12] To all plates merthiolate was added to a final concentration of 0.01 %.

Results and discussion

The *Serratia*-strains with the exception of *Serratia rubidae* were easy to cultivate and formed large quantities of the red pigment prodigiosin. The proteolytic activity seemed to be proportional to pigment formation and growth. Medium 63 with peptone and gelatin yielded the best cultures after 48 h incubation at 25°C. Strains of *Serratia marcescens* gave the best proteolytic activity. Optimum activity was obtained at pH 8 on milk agar plates after 48 h incubation (Table 1).

Table 1. Proteolytic activity of tested organisms. Enzyme tests carried out at pH 8.0.

Organism	Enzyme activity, zone diam. in mm			Culture conditions	Media number
	Hemoglobin	Milk	Albumin		
Serratia marcescens, SBL	12	14	6	[a]	3
Serratia marcescens, U18	11	13	0	[a]	3
Serratia marcescens, KI	11	12	0	[a]	3
Serratia rubidae [d]	0	–	0	[a]	3
Proteus mirabilis	0	15	8 (pH 7.0)	[b]	16
Streptococcus faecium, A_2I	0	0	–	[c]	7
Streptococcus faecalis 16908	10	15	9	[c]	7
Streptococcus faecalis, M 19	8	8	0	[c]	7
Streptococcus mitis	0	0	–	[c]	7
Lactobacillus casei [d]	0	0	0		–

[a] Agitation, 25°C. [b] Agitation, 37°C. [c] Without agitation at pH 7.0 with media exchange 37°C. [d] *Serratia rubidae* and *Lactobacillus casei* do not form protease and showed a weak growth.

The best cultivation of *Streptococcus*-strains was obtained after a change of medium and cultivation without agitation at 37°C. The best enzyme activity was obtained from *Streptococcus faecalis* 16908 on milk agar plates at pH 8 (Table 1).

Of the *Proteus*-strains studied, *Proteus mirabilis* was found to yield growth after 24 h in Mills' medium with agitation at 37°C. The best enzyme activity was obtained at pH 8 on milk agar plates (Table 1).

Lactobacillus casei gave bad growth in several media. No proteolytic activity could be detected.

II. CULTIVATION OF *SERRATIA MARCESCENS* (SBL-STRAIN) AND PURIFICATION OF ITS PROTEOLYTIC ENZYME

Materials and methods

Serratia marcescens (SBL-strain) was cultivated in 10 litres scale using medium 63 supplemented with gelatin and occasionally peptone. The medium was sterilized in an autoclave at 120°C for 60 min. It was inoculated with 195 ml bacteria suspension and the culture incubated for 60 h at 25°C with an agitation speed of 400 rpm by daytime and 250 rpm by night. The air flow was 1.2 – 1.5 l/min. Antifoam Emulsion FG was added. Tests for determination of enzymatic activity were taken at equal time intervals. pH and growth were measured at the same time. For determination of the number of bacteria at the end of the cultivation, 10-fold dilutions were made in a buffer containing 8 g/l NaCl, 1.89 g/l $Na_2HPO_4.12H_2O$, 0.2 g/l KCl and 0.2 g/l KH_2PO_4, pH 7.3 – 7.4 (dilution buffer). A volume of 0.1 ml culture was spread on Trypticase Soy agar plates. They were read after 24 h incubation at 37°C. Determination of dry weight of cells was made at the same time by desiccation at 105°C for 24 h. The bacterial suspension obtained was centrifuged at 2000 *g* for 1 h and washed two times in the dilution buffer mentioned. After the last washing the supernatant was stored at 4°C and the pellet resuspended in the dilution buffer mentinoed above and also stored at 4°C.

An investigation was made to determine the amount of cell-bound proteolytic enzymes. An aliquot of the washed centrifugate was agitated and treated with ultrasonic waves at an energy of 20 kc/s for 3 × 1 min and also for 10 × 1 min both at + 4°C. Then the samples were centrifuged at 10 000 *g* for 30 min. The purification of the proteolytic enzyme was carried out according to von Hofsten *et al.*[13] To 1 litre of the supernatant 10 g Whatman cellulose powder was added after which 700 g ammonium sulphate was dissolved under agitation. The solution was stored for 24 h at + 4°C, then it was filtered through a sintered glass filter. The cellulose powder was washed with 50 ml 50 % ammonium sulphate solution. The pale pink coloured cellulose powder was agitated in 250 ml 50 % ammonium sulphate solution and stored at + 4°C. Then the cellulose suspension was packed in a column. A clear eluate was obtained. The cellulose was sucked to dryness, then suspended in 25 ml 0.1 M Tris-HCl, pH 8.0, packed in another column and the enzyme was eluted with the buffer. The eluent was recirculated until it was clear. More Tris-HCl was added and eluted. The total volume of the eluate was measured and solid ammonium sulphate was added to a final concentration of 0.4 g/ml. The solution was stored for 24 h at + 4°C. The resulting precipitate was sedimented by centrifugation at 10 000 *g* for 30 min. The centrifugate was suspended in 25 ml distilled water, then dialyzed first against distilled water and after that against 0.05 M Tris-HCl, pH 8.0. The dialysate contained a dark red precipitate which was separated by centrifugation at 10 000 *g* for 30 min. The supernatant received was concentrated by sequestering the water from the dialysis bags. Part of the concentrate was gel filtered on Sephadex G 75 in 0.1 M phosphate buffer, pH 7.2.

Results

The changes in growth, protease activity, and pH during the cultivation appears in Fig. 1. The number of bacteria at the end of the cultivation was

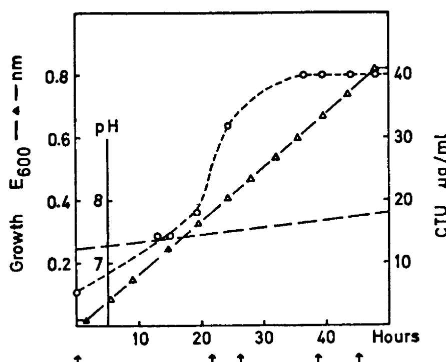

Fig. 1. Growth, protease activity and pH of *Serratia marcescens* culture in 10 litre culture. (○), Protease (CTU = Comparative Trypsin Units); – – – pH; (↑) Addition of 1 ml antifoam.

3.0×10^9/ml and the cell density was 1.25 g/l. Microscopic investigation showed that the cells which had been treated with ultrasonic waves were destroyed but no proteolytic activity was obtained. This result indicates that only extracellular enzymes have been formed. The result from the purification appears in Fig. 2. Two extinction peaks were obtained and the proteolytic activity was present in only one of them.

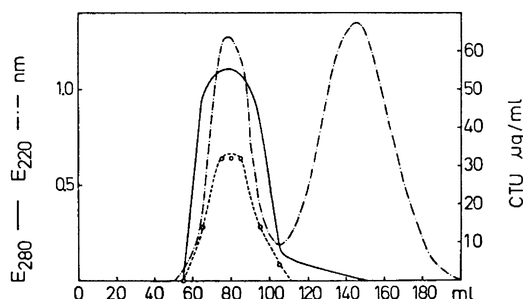

Fig. 2. Extinction diagram and protease activity obtained by gel filtering on Sephadex G75 of enzyme solution from *Serratia marcescens*. (○) Protease (CTU = Comparative Trypsin Units).

III. CULTIVATION OF *SERRATIA MARCESCENS* MUTANT STRAIN AND PURIFICATION OF ITS PROTEOLYTIC ENZYME

Materials and methods

Attempts were made with UV-radiation to obtain a mutant capable of producing higher proteolytic activity. A 24 h nutrient broth culture was centrifuged and the cells suspended in the dilution buffer and six 10-fold dilutions were made. The three last dilutions were irradiated with a UV-lamp (Westinghouse sterillamp 782 L-20, 14 W) at a distance of 50 cm for 10, 20, 45, and 60 sec, in Petri dishes with 10 ml in each dish. They were slowly rotated during the test. Photoreactivation was avoided by covering the dishes with aluminum foil after the UV-treatment. UV-irradiation for 60 sec yielded about 2 % survivors, for 45 sec about 50 %, and for 20 sec about 80 %. Irradiation for a shorter time than 10 sec had no effect. Many white colonies were found when after irradiation

Table 2. Protein- and pH-effect on growth and protease activity of *Serratia marcescens*, natural and mutant strains. Protease activity measured after incubation during 24 h at 30°C.

Organism	Medium [a]	Growth ($E_{600\ nm}$)[b] 48 h culture	pH[c] 48 h culture	Protease activity, milk-agar (diam. mm) pH 8 48 h culture	pH 6 48 h culture
Natural strain	I	0.57	7.8	16	15
	II	0.92	8.4	11	7
	III	0.66	7.4	14	13
	IV	1.02	6.5	12	12
Mutant strain	I	0.27	7.4	precipitation	6
	II	0.89	8.4	precipitation	0
	III	0.21	7.2	precipitation	precipitation
	IV	0.29	6.2	precipitation	precipitation

[a] See Materials and methods. [b] Tests and blanks diluted 1:5 in dist. water. [c] Original pH = 7.2 (except medium IV with pH 6.0).

0.1 ml suspension was spread on nutrient milk agar plates (3 plates per dilution). At the same time unirradiated cells were spread for control. The plates were incubated for 120 h at 30°C. A mutant was isolated and the optimum culture and enzyme-inducing conditions were investigated. The effect of different proteins and the influence of pH were investigated. Medium 63 supplemented with peptone and one of the following protein sources was used (Table 2). I, Gelatin, Difco, pH 7.2; II, Casein hydrolysate (Casamino Acids, Difco) pH 7.2; III, Casein according to Hammarsten (Merck) pH 7.2; IV, Casein according to Hammarsten (Merck) pH 6.0.

On nutrient-milk agar plates the irradiated colonies were surrounded by a clear zone with a diameter of only 3 mm. The corresponding diameter for the natural strain was 9 mm, although the size of the colonies was similar.

Results

Effect of mercury-compounds. It was found that addition of merthiolate in ordinary concentrations to the mutant strain culture did not inhibit the growth. The tolerance of the mutant strain against merthiolate was compared with the natural strain of *Serratia marcescens* and some other bacteria (Table 3). To Medium 63 supplemented with peptone and gelatin, pH 7.2, different amounts of merthiolate were added. The cultures were incubated for 22 h at 25°C with agitation. A 24 h old Trypticase-Soy-culture was used as inoculum. Measurement of bacterial density was made in a Beckman DB-G spectrophotometer at 600 and 660 nm in a 10 mm cuvette. The medium was used as blank.

The mutant strain was cultured in 10 litres-scale in the same way as described for the natural strain. Medium 63 supplemented with peptone and gelatin pH 7.2 was used as before. The cultivation was run at 25°C with an agitation speed of 300 rpm by day and 200 rpm by night. The air flow was 1.2 – 1.5 l/min by day and 0.5 – 0.6 l/min by night. When necessary Antifoam Emulsion FG was added. The medium was inoculated with 200 ml of a 48 h

Table 3. Effect of mercury-compounds on growth of some bacteria.

Organism	Conc. Hg^{2+} mM	g/l	Hg-compound	Inhibition %	Reference
Serratia marcescens					
Natural strain	0.246	0.05	(merthiolate)	97	SBL-strain, natural strain used in the investigation
Mutant strain	8.85	1.79	(merthiolate)	100	SBL-strain mutant used in the investigation
Mutant strain	4.425	0.90	(merthiolate)	83	SBL-strain mutant used in the investigation
Mutant strain	2.2	0.45	(merthiolate)	29	SBL-strain mutant used in the investigation
Mutant strain	1.106	0.225	(merthiolate)	19	SBL-strain mutant used in the investigation
E. coli	0.246	0.05	(merthiolate)	97	SBL-strain, natural strain used in the investigation.
E. coli	0.008	0.0016		100	Cooper and Mason [14]
E. coli	0.06	0.0119		100	Cook and Steel [15]
B. subtilis	1	0.201	*p*-mercuri-benzoate	97	Falcone *et al.*[16]

culture. Tests were taken at equal time intervals for determination of enzyme activity, growth, and pH. The number of bacteria and the cell mass were determined at the end of the cultivation. The culture was stopped by adding 0.01 % merthiolate. The bacteria were centrifuged and washed. The supernatant was sterilized by filtration to avoid further growth. The centrifugate was diluted in the dilution buffer. Both supernatant and centrifugate were stored at +4°C.

Preliminary purification of protease from the mutant strain. The protease produced by the mutant strain was purified and concentrated in the same manner as described for the natural strain except that the enzymes produced by the mutant strain were precipitated with ammonium sulphate only. 1000 ml culture supernatant was precipitated with 700 g $(NH_4)_2SO_4$. The precipitate was sedimented by centrifugation and dissolved in 10^{-3} M phosphate buffer, pH 7.5. The proteolytic activity was found in this solution. The dissolved centrifugate was dialyzed first against distilled water and then against 10^{-3} phosphate buffer, pH 7.5. All the activity was recovered in the dialysate, which was concentrated by ultrafiltration. One ml concentrate from the natural and mutant strain was applied to a Sephadex G 100 column (Fig. 3). The pH- and temperature-dependence of the protease from the natural and mutant strain was tested with the supernatants from the respective 10 litre scale cultures. As substrate milk agar pH 4–9 was used and the proteolytic assay was carried out as described in part I. The pH-dependence can be seen in Fig. 5. The temperature dependence of the enzyme activity was tested after exposure to 0°, 20°, 30°, 37°, 60°, and 100°C for 5, 10, 15, 30, 60 min, and 24 h. After the treatment the solutions were rapidly cooled and placed in

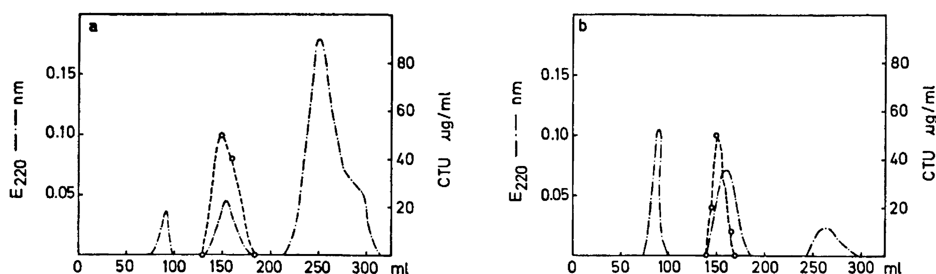

Fig. 3. Extinction and proteolytic activity obtained by gel filtering on Sephadex G100 of a concentrated enzyme solution from *Serratia marcescens.* (O) Protease (CTU= Comparative Trypsin Units). (a) Mutant strain; (b) natural strain.

holes in the agar plates. The plates were incubated for 24 h at 30°C before reading. As an alternative test cooled enzyme solutions (0°C) were placed on the substrate plates after which they were incubated at the various temperatures given above for 24 h before reading. The temperature 100°C could not be tested in this case.

The mutant protease showed an acid pH-optimum on milk agar. At pH 6 a clear zone of 13 mm and 11 mm diameter was formed after 24 h incubation at 30°C with the mutant and natural strain, respectively. Merthiolate added to the milk agar inhibited the growth of the natural strain but had no effect on the mutant strain. The investigation of optimum growth and enzyme formation is shown in Table 2. Medium 63 supplemented with peptone and gelatin, pH 7.2, gave the largest protease activity for both the natural and mutant strain. In other experiments it was found that casein hydrolysate yielded better growth than gelatin in this medium. A pH close to neutral gave optimal enzyme production from both organisms in spite of the fact that the protease of the mutant strain had an acid pH-optimum and that good growth occurred on milk agar, pH 5.

Table 3 shows the influence of mercury-compounds. 100 % inhibition of the mutant strain required 8.85 mM merthiolate whereas the natural strain

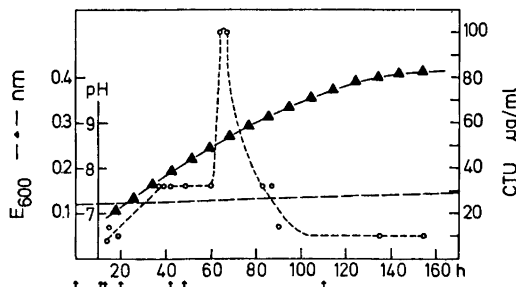

Fig. 4. Growth, protease activity and pH of *Serratia marcescens*, mutant strain in 10 litre culture. (▲), Growth, E_{600} nm (blank and test diluted 1:5 in dist. water); (O), Protease (CTU = Comparative Trypsin Units); — — — pH; (↑) Addition of 1 ml antifoam.

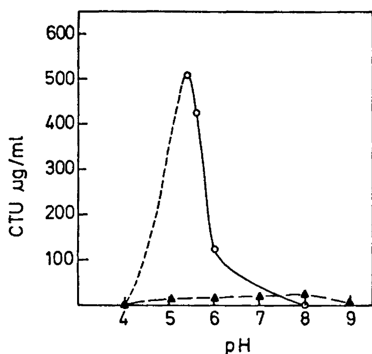

Fig. 5. Protease activity from *Serratia marcescens*, natural and mutant strain as a function of pH on milk agar plates after 24 h at 30°C. (▲) Protease activity, natural strain (CTU = Comparative Trypsin Units); (○) Protease activity, mutant strain.

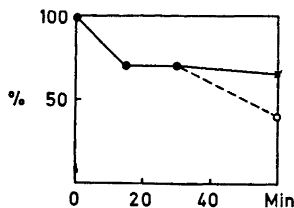

Fig. 6. Remaining activity in % of original activity after heating at 60°C for different periods. (×) Natural strain at pH 8.0. (○) Mutant strain at pH 6.0.

was fully inhibited at 0.246 mM. In comparison with other organisms the mutant strain also showed a much larger tolerance against merthiolate. Fig. 4 shows that the mutant strain grew slower than the natural strain in 10 litre cultures. The protease activity reached its maximum after about 60 h. $(NH_4)_2$-SO_4 precipitation was the most suitable method for separating the proteolytic activity from the medium for both the mutant and natural strain. The yield in this case was 30 % and 40 %, respectively. With gel filter three peaks were obtained at about 90, 150, and 250 ml. All proteolytic activity was found in peak number 2 (about 150 ml), see Fig. 3. The protease from the natural and mutant strains had an optimal activity around pH 8 and pH 5.4, respectively (Fig. 5). Temperature stability studies (Fig. 6) showed that after 15 min at 60°C the activity was reduced by 30 %. After 60 min at 60°, 65 % and 40 % of the proteolytic activity from the natural and mutant strain, respectively, remained. All activity was lost after 5 min boiling. The optimum temperature was 30°C. Attempts to demonstrate intracellular enzymes were negative; only extracellular proteases were found.

Conclusions. The very marked resistance of the mutant strain to merthiolate (Table 3), and perhaps other mercury compounds is being investigated further to detect what mechanism is responsible for this resistance. More extensive work on the purification and characterization of these proteolytic enzymes is in progress.

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