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Studies of the Heterogeneity of *Streptomyces griseus* Protease

I.* Polyacrylamide Gel Electrophoresis of Commercial Pronase-P, Derived From *Streptomyces griseus* K1

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The enzyme composition of commercial Pronase-P has been studied by electrophoresis on polyacrylamide.

The enzyme has been separated into altogether fourteen protein bands: eight with protease activity, five with LNA activity, and finally one for which no substrate has been found. There are also indications of other minor enzyme fractions.

Four protein bands with protease activity are shown to be inhibited by EDTA. One of these bands with activity against BAME is also shown to be inhibited by STI and ovomucoid. The composition of different Pronase-preparations is compared.

The results are discussed in relation to previous investigations.

Since the isolation of an extracellular proteolytic enzyme from *Streptomyces griseus* by Nomoto and Narahashi,[1,2] the commercial preparation, sold as Pronase, has been widely applied for the hydrolysis of many types of proteins. Because of the general lack of substrate specificity [3,4] several investigations have been concerned with the homogeneity of the enzyme preparation. Hiramatsu and Ouchi [5] have reported the separation of the highest commercial purity preparation (Pronase-P) into four active fractions by starch zone electrophoresis. There were two principal fractions with activity both at neutral and alkaline pH against casein, and two smaller fractions, one with activity against leucylglycine, while the other was an exclusively neutral protease that could be totally inhibited by EDTA, but not by DFP or potato protease inhibitor.

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Abbreviations used: EDTA, ethylenediaminetetraacetate, DFP, diisopropylfluorophosphate. CM-cellulose, carboxymethyl cellulose, DEAE-cellulose, diethylaminoethyl cellulose. TEAE-cellulose, triethylaminoethyl cellulose. TCA, trichloroacetic acid, BAEE, *N*-benzoyl-L-arginine ethyl ester, BAME, *N*-benzoyl-L-arginine methyl ester. LNA, L-leucyl-β-naphthylamide. PNPA, *p*-nitrophenylacetate. STI, soybean trypsin inhibitor.

The heterogeneity of the commercial product was further established by Nomoto *et al.*,[6] who again obtained four active components by precipitation with ammonium sulfate and chromatography on CM-cellulose and DEAE-cellulose. These fractions were called STP-A, -B, -C, and -D. STP-A was the main component and was considered by them to be identical with the enzyme they had previously isolated in pure form.[1,2]

Narahashi and Yanagita [7] reported the separation of Pronase into four protein peaks by chromatography on CM-cellulose. By testing these four peaks for activity they concluded Pronase to be made up by six different enzymes: two proteases inhibited by EDTA, two proteases inhibited by DFP, and finally two peptidases, of which one had leucine aminopeptidase activity and the other carboxypeptidase activity. Essentially the same results were recently reported by Trop and Birk.[8] Further separation results, based on the above-mentioned CM-cellulose separation, were reported in 1968 by Narahashi *et al.*[9]

By consecutive chromatography on CM-cellulose, phosphate-cellulose, and Sephadex G50, Wählby *et al.*[10–12] further purified the two DFP inhibited proteases and isolated three different fractions. One of them was active against BAME, and the other two were active against PNPA. Hydrolysis of the three (^{32}P) DFP-treated fractions showed them all to have the same amino acid sequence around the reactive serine group. The present paper describes the separation of Pronase-P by polyacrylamide gel electrophoresis into fourteen protein bands: eight with protease activity and five with LNA activity, and relates these separation results to previous data.

MATERIALS AND METHODS

The commercial protease powder, Pronase-P, was obtained from the Kaken Chemical Company, Tokyo, Japan (lot number 511324 and lot number 611735), and from Calbiochem, Los Angeles, California (lot number 109080). If not otherwise specified, lot number 511324 was used. The preparations were stored at −20°C and were used directly without any pretreatment.

BAME, *N*-benzoyl-L-arginine, LNA, ammonium sulfate, *N*-(1-naphthyl)-ethylenediamine dihydrochloride, STI, and hen egg white ovomucoid were all purchased from Sigma Chemical Co., St. Louis, Mo. The soybean trypsin inhibitor was the three times crystallized grade. 1 mg inhibiting about 1 mg of trypsin, while 1 mg of the ovomucoid preparation inhibited about 0.9 mg of trypsin.

Acrylamide, *N*,*N*-methylene-bis-acrylamide, and *N*,*N*,*N*′,*N*′-tetramethylenediamine were obtained from Eastman Organic Chemicals, New York.

Casein was of Hammarsten grade, and was obtained from E. Merck AG, West Germany.

Borate buffers for incubation and elution were prepared from a stock solution of 0.2 M boric acid-KOH solution by adjustment to the required pH with HCl or KOH, followed by dilution with deionized water.

All other reagents were of analytical grade and deionized; distilled water was used throughout as well as self-adjusting micro pipettes.

Spectrophotometric measurements were made in a Zeiss PMQII spectrophotometer, equipped with a controlled temperature sample holder. Densitometric measurements were made with a Vitatron densitometer.

Enzyme assays

a. *Proteolytic activity* was measured essentially according to the method of Kunitz.[13] The incubation system consisted of 3.0 ml of 3 % casein solution, adjusted to either pH 7.5 or 10.0, and 3.0 ml of 0.15 M borate buffer at a corresponding pH. The reaction was started by adding 500 μl of eluate solution. The reaction mixture was incubated at 50 ± 0.1°C for 4 h. The reaction was stopped by adding 3.0 ml of 10 % TCA. Thereafter the absorption of the supernatant was measured at 280 mμ.

b. *Esterase activity.* Activity against BAME was measured, using the method described by Schwert and Takenaka.[14] The assays were carried out in a thermostatted cuvette at 25°C. The reaction mixture consisted of 3.0 ml of 0.33 mM substrate in 0.15 M borate buffer, pH 7.5; 0.01 M $CaCl_2$. The reaction was started by adding 100 μl of eluate solution, and followed at 253 mμ for 5 – 10 min. The reaction was linear until about 80 % of the substrate was consumed, and there was no spontaneous decomposition of the substrate under the conditions of the assay.

c. *Peptidase activity.* Activity against LNA was determined according to the method of Goldbarg and Ruthenburg.[15] The reaction was started by adding 100 μl of eluate to 1.0 ml of substrate solution (1.37 mM LNA in 0.15 M borate buffer, pH 7.5; 0.01 M $CaCl_2$), and the mixture was incubated at 40 ± 0.1°C for 50 min.

Inhibition experiments

a. *Treatment with EDTA.* Pooled fractions of corresponding bands from different electrophoretic runs were dialysed at room temperature against 0.03 M borate buffer, pH 8.0; 5×10^{-4} M EDTA. Reference blanks were samples dialysed against 0.03 M borate buffer, pH 8.0; 0.03 M $CaCl_2$, and tested for activity against casein, pH 7.5, and activity against BAME.

Inhibition studies of activity against LNA were performed at room temperature on unfractionated Pronase-P. 0.5 mg enzyme was dissolved in 50 ml 0.15 M borate buffer, pH 7.5; 10^{-3} M EDTA. Residual activity was determined after 1 h. Enzyme dissolved in 0.15 M borate buffer, pH 7.5; 0.03 M $CaCl_2$, was used as reference.

b. *Treatment with STI or ovomucoid.* The same pooled fractions as used in the EDTA-inhibition experiments were incubated in 0.15 M borate buffer, pH 7.5; 0.01 M $CaCl_2$, containing STI or ovomucoid at a concentration of 10^{-4} M for 1 h at room temperature. Remaining proteolytic activity was determined against casein, pH 7.5, and against BAME.

Polyacrylamide gel electrophoresis

a. *Preparative method.* The gels were cast in Pyrex tubes with an inner diameter of 12 mm and a length of 10 cm. Polyacrylamide gels were prepared according to Hjertén.[16] T and C values are specified in the legend to the figure for each separation.

The separations were performed in a cold room at 3 – 5°C. The gel tube was cooled in the lower buffer over the entire length of the gel. Continuous buffer systems were used, and all gels were preflushed for at least 4 h.* Samples of 1.4 mg Pronase dissolved in 100 μl of sucrose stabilized electrophoresis buffer were applied to the preflushed gels. The separations were run at 9 – 14 mA/cm^2 and 8 – 10 V/cm for 8 – 18 h. After the separation, the gels were extruded and either stained or cut into slices with a hair. The procedure used was simply advancing the gel out of the Pyrex tube with a micrometer screw, 1.5 mm at a time, wind a hair around the gel and cut off. The reproducibility of the obtained 1.5 mm slices was within ± 5 %, judged by their weight. The slices were transferred to polyethylene tubes, homogenized with a glass rod and extracted with 2.0 ml of the

* Preflushing of the gels had a marked influence on both the migration speed and the total recovery of enzymatic activity, probably due to removal of ammonium persulfate and unpolymerized monomers.

appropriate buffer by gentle shaking on a "wrist action" shaker for 3 h at room temperature.

b. *Analytical method.* Gels used for evaluating suitable separation conditions, for densitometric measurements, and for comparison of different Pronase preparations, were prepared in 5 mm glass tubes (inner diameter). Gels were preflushed for half an hour, and 5 – 10 µl of buffer containing 0.1 mg enzyme was applied. Both buffer reservoirs were immersed in icewater. These gels as well as the preparative ones were stained in a solution of Amido black in 10 % TCA overnight, followed by washing with 10 % acetic acid and storage in 10 % acetic acid.

RESULTS

a. *Separation results.* The electrophoretic separation of Pronase in veronal buffer at a pH of 8.0 is shown in Fig. 1. The upper part of the figure is a histogram, showing the amounts of enzymatic activity * recovered, while the lower

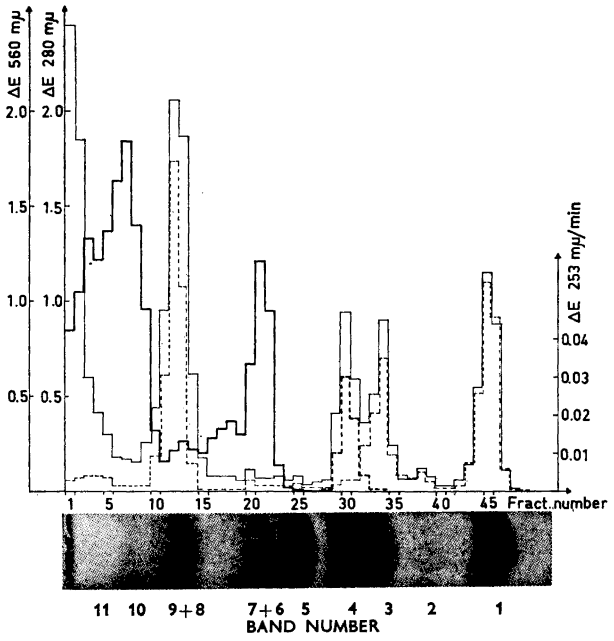

Fig. 1. Small preparative polyacrylamide gel electrophoresis of Pronase-P, performed at pH = 8.0 in 0.038 M veronal buffer, containing 0.012 M Ca^{2+}. Gel characteristics: T = 7.5 % and C = 5.0 %. Time for separation: 8 h at 8 – 10 V/cm and 9 mA/cm² (cathodic migration from the left to the right). The gel was cut into 1.5 mm thick fractions and extracted at pH = 7.5 with an 0.03 M borate buffer, containing 0.01 M Ca^{2+}. The upper part is a histogram, showing enzymatic activities found in the different gel fractions. The lower part shows a stained gel from a duplicate run. ———, activity against casein at pH = 7.5. – – –, activity against casein at pH = 10.0. ▬▬▬, activity against LNA. - - -, activity against BAME (only fractions 29 – 32).

* It has to be pointed out that caseinolytic activities as well as the LNA-activities, reported here for major bands, are measured beyond the linearity of the methods. These conditions have been chosen by purpose in order to clearly expose minor components.

part is a photograph of a gel made in a duplicate run. There is a close correspondence between areas of stained protein and enzymatic activity.

The protein concentration in the eluate of the fractions was too low to be accurately determined, and the amount of staining in each fraction is therefore the only indication of the protein concentration. However, this staining, estimated by visual comparison and by densitometry on analytical gels, was constant in intensity in comparable runs.

Table 1. Type of enzymatic activity, related to the bands from the electrophoretic separation shown in Fig. 1.

Band number	Substrate			
	Casein pH 7.5	Casein pH 10.0	LNA	BAME
1	+	+		
2	+	+		
3	+	+		
4	+			+
5				
6			+	
7			+	
9+8	+	+	+	
10			+	
11	+		+	

The activity of the stained bands is summarized in Table 1. Bands 1, 2, and 3 appear to be closely related, in that all three have activity against casein at both alkaline and neutral pH, but do not show any activity against the two synthetic substrates LNA and BAME.

Band 4 is the only band with activity against BAME, but in contrast to bands 1 – 3 it does not show any activity against casein at an alkaline pH. In band 5, no activity was detected against either casein, LNA, or BAME. Under the conditions used in this separation, only one band can be distinguished in the area marked 7 + 6, but in the smaller analytical gels and in other buffers in the preparative scale, this region can be resolved into two bands. Both bands 6 and 7 show activity only against LNA. The band marked 9 + 8, which appears to be homogeneous in this separation, can under other conditions be resolved into two bands. 9 + 8 show activity against casein at both neutral and alkaline pH. Some minor activity against LNA is often found in this region, but the results vary from run to run and are without significance at this stage of investigation.

Band 10 is active against LNA only. Band 11 cannot be evaluated as to its substrate specificity because of its proximity to the point of sample application, and the fairly large amount of evidently heterogeneous material that does not move during the separation.

To roughly estimate the amount of activity recovered from the gels by the elution procedure used, a determination of the hydrolysis of BAME by Pronase-P was compared with the amounts of activity recovered from a gel. All of the recovered activity was found in band 4, and amounted to about 30 % of that applied. Further elution of the gel resulted in higher recoveries.

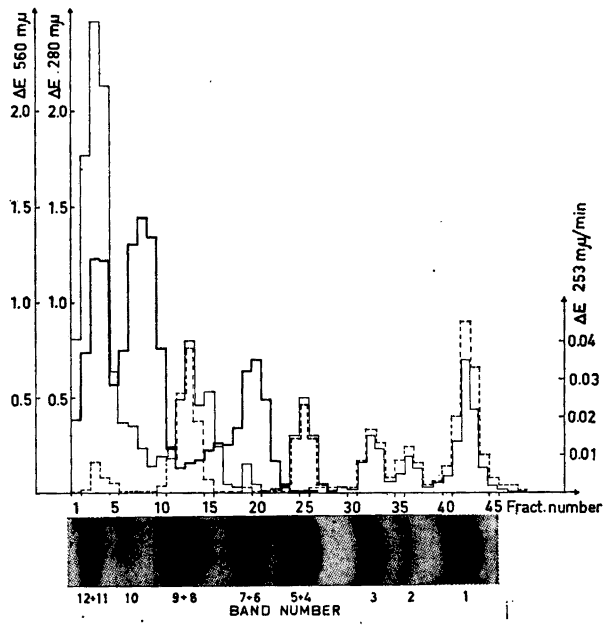

Fig. 2. Small preparative polyacrylamide gel electrophoresis of Pronase-P, performed at pH = 6.8 in 0.38 M borate buffer, containing 0.01 M Ca^{2+}. Gel characteristics: T = 7.5 % and C = 5.0 %. Time for separation: 8 h at 8 – 10 V/cm and 9 mA/cm² (cathodic migration from the left to the right). The gel was cut into 1.5 mm thick fractions and extracted at pH = 7.5 with a 0.03 M borate buffer, containing 0.03 M Ca^{2+}. The upper part is a histogram, showing enzymatic activities found in the different gel fractions. The lower part shows a stained gel from a duplicate run. ———, activity against casein at pH = 7.5. – – –, activity against casein at pH = 10.0. ▬▬▬, activity against LNA. - - -, activity against BAME (only fractions 23 – 27).

In order to investigate the possibility of bands originating from buffer-enzyme artifacts, separations were also done in another buffer system at a different pH. Fig. 2 and Table 2 show the results of a gel electrophoresis run, using borate buffer at pH 6.8 and a T = 7.5. C = 5 polyacrylamide gel. In this separation, essentially the same activity and band pattern as in Fig. 1 and Table 1 are obtained, which speaks against assumptions considering the stained protein bands as artifacts.

Fig. 2 also shows that bands 4 and 5 have merged together and now appear as a single intensely stained band with the enzymatic activity of only "pure" band 4. Further and more interesting, bands 8 and 9 show some evidence of

Table 2. Type of enzymatic activity, related to the bands from the electrophoretic separation shown in Fig. 2.

Band number	Substrate			
	Casein pH 7.5	Casein pH 10.0	LNA	BAME
1	+	+		
2	+	+		
3	+	+		
5+4	+			+
7+6			+	
9+8	+	+	+	
10	+		+	
12+11	+	+	+	

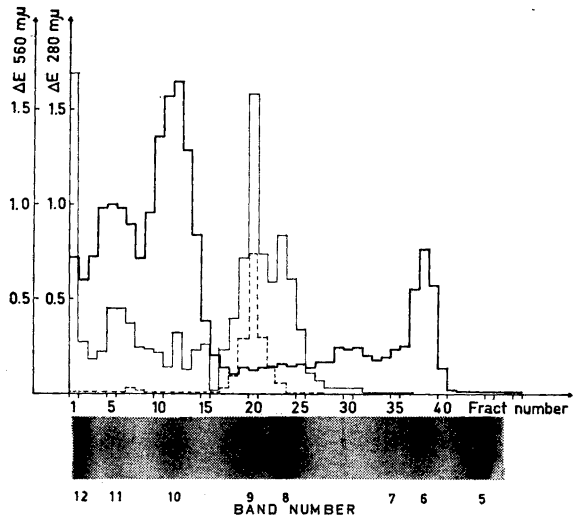

Fig. 3. Small preparative polyacrylamide gel electrophoresis of Pronase-P, performed at pH = 8.2 in 0.038 M veronal buffer, containing 0.013 M Ca^{2+}. Gel characteristics: T = 7.5 % and C = 5.0 %. Time for separation: 17 h at 6 – 8 V/cm and 9 mA/cm² (cathodic migration from the left to the right). The gel was cut into 1.5 mm thick fractions and extracted at pH = 7.5 with a 0.03 M borate buffer, containing 0.03 M Ca^{2+}. The upper part is a histogram, showing enzymatic activities found in the different gel fractions. The lower part shows a stained gel from a duplicate run. ———, activity against casein at pH = 7.5. – – –, activity against casein at pH = 10.0. ▬▬▬, activity against LNA.

separation, particularly on analytical gels (Fig. 7). Band 12, which did not move at pH 8.0, moves together with band 11 in the pH 6.8 buffer.

To achieve a better resolution of the region from band 12 to band 6, a longer separation time and a pH of 8.2 was used. The results are shown in

Table 3. Type of enzymatic activity, related to the bands from the electrophoretic separation shown in Fig. 3.

Band number	Substrate			
	Casein pH 7.5	Casein pH 10.0	LNA	BAME
5				
6			+	
7			+	
8	+			
9	+	+		
10				
11	+		+	

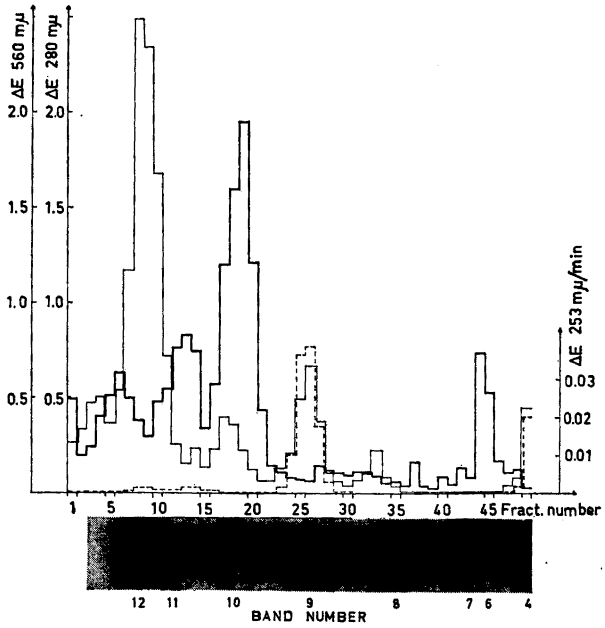

Fig. 4. Small preparative polyacrylamide gel electrophoresis of Pronase-P, performed at pH = 6.8 in 0.38 M borate buffer, containing 0.01 M Ca^{2+}. Gel characteristics: T = 6% and C = 10.0%. Time for separation: 9 h at 8 – 10 V/cm and 8 mA/cm² (cathodic migration from the left to the right). The gel was cut into 1.5 mm thick fractions and extracted at pH = 7.5 with a 0.03 M borate buffer containing 0.03 M Ca^{2+}. The upper part is a histogram, showing enzymatic activities found in the different gel fractions. The lower part shows a stained gel from a duplicate run. ———, activity against casein at pH = 7.5. – – –, activity against casein at pH = 10.0. ▬▬, activity against LNA. - - -, activity against BAME (only fractions 49 and 50).

Fig. 3 and Table 3. Bands 1 – 4 have moved out of the gel. Bands 6 and 7 are separated clearly, and both show activity against LNA. In some of the runs, however, band 7 failed to demonstrate any activity with any of the substrates used. The individuality of the two bands 8 and 9 is also confirmed, band 8 being a neutral protease, while band 9 is a more alkaline protease. Bands 10 and 11 are also clearly separated, and both are active against LNA. Close to band 11, there is also a small, not numbered component with activity against casein at neutral pH. Over all, this region shows minor protease activities that does not conform to the distribution of the LNA activity. Further studies are needed to interpret these minor protease activities.

By using a higher crosslinked polyacrylamide gel, T = 6.0, C = 10.0, and performing a separation at pH 6.8 for 9 h, it was possible to obtain a better separation in the band 12 – 10 region. The results are shown in Fig. 4 and Table 4. Unfortunately, the gel gets very opaque at C = 10 and the rather high Ca^{2+} concentration used here. Satisfactory photographs of the stained bands can therefore not be produced. Band 5, which migrates in front of band 4, has migrated out of the gel together with bands 1, 2, and 3. Band 8 is rather labile at pH 6.8 and loses most of its activity during the 9 h of electrophoresis. Band 12 is clearly separated from LNA activity and shows activity only against casein at neutral pH.

Table 4. Type of enzymatic activity, related to the bands from the electrophoretic separation shown in Fig. 4.

Band number	Substrate			
	Casein pH 7.5	Casein pH 10.0	LNA	BAME
4	+			+
6			+	
7				
8	+			
9	+	+		
10	+		+	
11	+			
12	+		+	

In the vicinity of band 10, there appears a small component with activity against casein at neutral pH. The same activity can also be recognized in Fig. 2 (fraction numbers 6 and 7) and, as already mentioned, close to band 11 in Fig. 3. Moreover, there appears in this highly crosslinked gel (Fig. 4) two small components with slower migration than band 12, one with activity against LNA, and the other with activity against casein at neutral pH. However, no corresponding protein bands could be detected in the stained gels, and so far, no further attempt to evaluate these smaller components have been made.

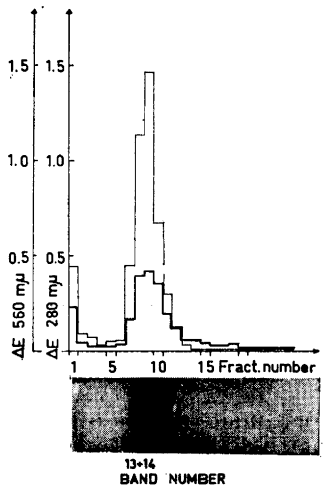

Fig. 5. Small preparative polyacrylamide gel electrophoresis of Pronase-P, performed at pH = 6.8 in 0.38 M borate buffer, containing 0.01 M Ca^{2+}. Gel characteristics: T = 7.5% and C = 5.0%. Time for separation: 10 h at 8 – 10 V/cm and 6.0 mA/cm² (anodic migration from left to the right). The gel was cut into 1.5 mm thick fractions and extracted at pH = 7.5 with a 0.03 M borate buffer, containing 0.03 M Ca^{2+}. The upper part is a histogram, showing enzymatic activities found in the different gel fractions. The lower part shows a stained gel from a duplicate run. ———, activity against casein at pH = 7.5. ▬▬, activity against LNA.

Fig. 5 shows an electrophoretic separation in borate buffer at pH 6.8 of the anod migrating components in Pronase-P. The two bands 13 and 14 move together but can be separated in a higher crosslinked gel (T = 6.0, C = 10.0) at the same pH. Band 13 shows activity against casein at neutral pH, while LNA is the only substrate hydrolyzed by band 14.

Table 5. Inhibitory effect on electrophoretically separated Pronase components.

Band number	% inhibition by		
	EDTA	STI	Ovomucoid
1	0	0	0
3	0	0	0
4	0	100	100
8	100	0	0
9	0	0	0
12	100	0	0
13	100	0	0

b. *Inhibition results.* Some of the Pronase components, active against casein at pH = 7.5, have been treated with EDTA, STI, and ovomucoid. The results, shown in Table 5, indicate that the components belong to at least three different groups: one represented by band 4 and sensitive to STI and ovomucoid, one represented by bands 8, 12, 13 and sensitive to EDTA, and finally one re-

presented by 1, 3, 9 and not sensitive to either EDTA, STI or ovomucoid. The STI sensitive band 4 also shows esterolytic activity against BAME. This activity is also, as expected, completely inactivated by STI or ovomucoid treatment. It is also noteworthy that the bands 1, 3, and 9 in the non-sensitive group are all active against casein at an alkaline pH, while bands 8, 12, and 13 in the EDTA sensitive group only show caseinolytic activity at neutral pH. In addition to these results, all aminopeptidase activity against LNA was inhibited by EDTA treatment. Regarding our data as well as earlier reports [7,9] concerning the EDTA effect on different Pronase activities, it must be stressed that it is not clear whether they should be interpreted as effects on the enzyme stability or on the catalytic center, or as a combination of both these effects. Further studies are in progress to bring about a better understanding of this question.

c. *The composition of different Pronase preparations.* Determination of enzymatic activities against casein, BAME, and LNA on different lots of Pronase indicated that there might be differences in activity from lot to lot.

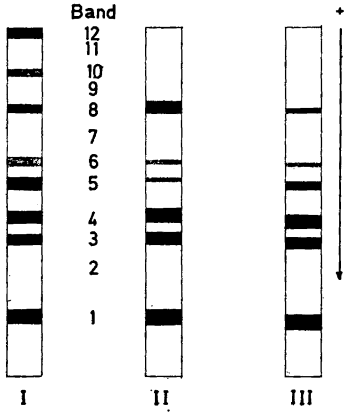

Fig. 6. Analytical polyacrylamide gel electrophoresis of Pronase-P preparations I, II, and III (see Materials) at pH = 8.0 in 0.038 veronal buffer, containing 0.012 M Ca^{2+}. Gel characteristics: T = 7.5 % and C = 5.0 %. Time for separation: 3 h at 10 V/cm and 15 mA/cm² (cathodic migration downwards as indicated by an arrow).

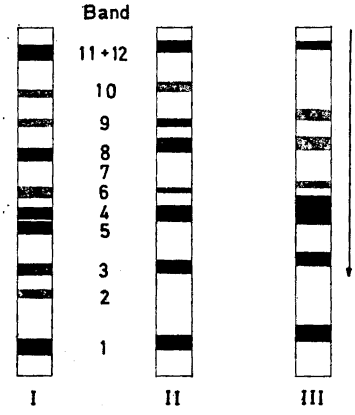

Fig. 7. Analytical polyacrylamide gel electrophoresis of Pronase-P preparations I, II, and III (see Materials) at pH = 6.8 in 0.38 M borate buffer, containing 0.01 M Ca^{2+}. Gel characteristics: T = 6.0 % and C = 10.0 %. Time for separation: 2 h and 10 min at 10 V/cm and 12 mA/cm² (cathodic migration downwards, as indicated by an arrow).

Differences between batches of Pronase were also shortly mentioned by Nomoto *et al.*[6] Electrophoretic separations of three different lots (numbers 109080 and 511324 obtained in 1962, and number 611734 obtained in 1965) were undertaken by the analytical method, in order to examine whether these differences in activity were due to the occurrence of new bands, or to difference in quantity of the bands already described. Figs. 6, 7 and 8 show that bands 1 – 14 are

present in all the three Pronase lots examined, and that there are no detectable new bands. However, the quantities of several bands were found to vary considerably. Band 12, for example – the major constituent of lot 511324 – was only found as a weak band in lots 109080 and 611735. Another example:

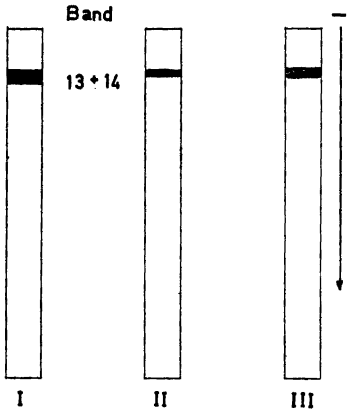

Fig. 8. Analytical polyacrylamide gel electrophoresis of Pronase-P preparations I, II, and III (see Materials) at pH = 6.8 in 0.38 M borate buffer, containing 0.01 M Ca^{2+}. Gel characteristics: T = 7.5% and C = 5.0%. Time for separation: 3 h at 10 V/cm and 12 V/cm^2 (anodic migration downwards as indicated by an arrow).

the intensity of band 10 is very low in lot 109080. These results make it probable that the above-mentioned differences in activity between different Pronase preparations are due to differences only in the amount of the components.

Table 6. Distribution of the total staining of Pronase-P on the electrophoretically separated protein bands.

Band number	% staining
1	11
2	2
3	8
4	15
5	12
6+7	7
8	8
9	3
10	3
11	1
12	25
13+14	5
Total	100

The relative amount of each stained protein band in lot 511324 was determined on analytical gels with a densitometer. Table 6 gives the figures, which are averages from three separations.

DISCUSSION

The enzyme preparation Pronase-P is in this investigation found to be far more heterogeneous than earlier reported.[5–12] As many as fourteen protein bands have been electrophoretically separated on polyacrylamide gels, and enzymatic activity has been correlated to thirteen of these bands. The origin of the bands have not been investigated, and whether they all are produced by the microorganism itself or if they, in part, are formed during the time of industrial fermentation, purification, or storage, is beyond the scope of this study. It is, however, important to establish that the different Pronase-P preparations tested so far, all show the same patterns of stained bands when separated under varied conditions, and that this picture of heterogeneity is not altered by altering the separation conditions.

Unfortunately, our small scale of separation did not permit reelectrophoresis of the separated bands in order to give a full proof of their homogeneity and stability towards the described separation and storage conditions, but as far as we have been able to test, these conditions have not had any influence on the separation results. Borate buffers, for example, have been reported by Cann,[17] and by Parker and Bearu [18] to cause "artifactual" heterogeneity in certain cases of electrophoretical separations. But this does not seem to be relevant for our Pronase-P separations in borate buffer, since we have obtained the very same separation results also in borate-free buffers (Figs. 2, 4). Furthermore, artifactual protein bands or zones could be caused during electrophoresis by high concentrations of ammonium persulfate in the polyacrylamide gels. But this again cannot explain the observed heterogeneity of Pronase, since the separation results from the ordinary ammonium persulfate gels have been reproduced on photopolymerized gels, and here ammonium persulfate is fully replaced by riboflavin. Judged by the band composition of the stained analytical gels, Pronase-P also seems to be stable for more than 72 h in the electrophoresis buffers at the actual separation temperature (3 – 5°C).

We have also examined if our conditions of storage used for the crude Pronase-P preparations (– 20°C in dry state) could result in formation of any new bands, especially as storage in dry state at 4°C has been reported as resulting in loss of activity towards synthetic polypeptides. We have found, however, that storage at – 20°C over a period of three years did not cause any change in band pattern, and did not lower the activity towards LNA, BAME, and casein, provided the sample was kept completely dry.

In addition to the conditions of separation reported above, several other buffer systems and gel concentrations have been tested. None of these separation conditions have led to detection of any new active bands. From all information gathered, we are convinced that the presented results give a valid, highly reproducible picture of the heterogeneity of Pronase-P.

In earlier heterogeneity studies, Narahashi *et al.*,[7,9] followed by Wählby *et al.*,[10–12] and Trop *et al.*,[8] separated Pronase-P into four active protein peaks (I – IV) by using ion exchange chromatography on CM-cellulose. In order to investigate the relationship between these four protein peaks and our electrophoretically obtained protein bands (1 – 14) we have analyzed the fractions

of such an ion exchange separation* on analytical polyacrylamide gels. The A_{280} and BAEE-activity curves of the ion exchange separation are seen to the left in Fig. 9, and the electrophoretically separated bands, corresponding to the ion exchange peaks I–IV, are given to the right. From the figure it is clear that peak I can be separated into bands 14+13, 12+11, 10, 9, 8, 7+6, and some minor bands. Narahashi and Yanagita [7] describe this protein peak as a highly active neutral protease with high LNA-activity "at the tailing edge". They also reported that the neutral protease activity was inhibited by EDTA. This corresponds well with the properties of our protein bands

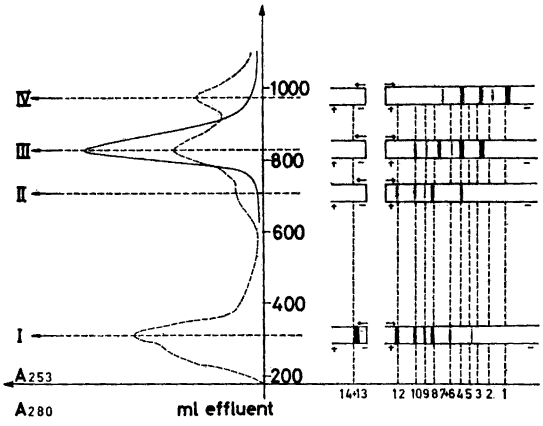

Fig. 9. Ion exchange chromatogram of Pronase-P on CM-cellulose (for conditions of separation see Ref. 10). The four main A_{280} peaks (I–IV) have been electrophoretically analyzed on polyacrylamide gels by both cathodic and anodic migration in 0.38 M borate buffer, pH=7.5; 0.01 M Ca^{2+}. Gel characteristics: T=6.0 % and C=10 %. Time for separation: 2 h at 10 V/cm and 12 mA/cm². – – –, protein: A_{280}. ———, BAEE hydrolyzing activity.

6–8 and 10–14, which all show either LNA or neutral protease activity (see Table 4). All of them are also, in agreement with the results of Narahashi and Yanagita,[7] sensitive to EDTA treatment. Band 9, however, likewise found in peak I, is not sensitive to EDTA treatment. Besides, it shows protease activity at an alkaline pH. Since this new component is only a minor part of peak I, it may have been difficult to detect in former investigations.

Peak II gives only one main band on the analytical gels. This band closely corresponds to band position 8. Its enzymatic activity and sensivity to EDTA treatment are also the same as that of band 8 in peak I. The properties of both bands are in agreement with the properties of peak II, as reported by Nara-

* This separation, kindly performed by Dr. S. Wählby, Institute of Medical Chemistry, University of Uppsala, Sweden, was done on a Pronase-P preparation, purchased from Calbiochem (lot No. 74543). Details of the separation method are described in Ref. 10.

hashi and Yanagita.[7] Further studies are needed to prove if these two bands (both numbered 8 here) do represent two different enzymatic entities.

As recently reported by Wählby [12] there are two main components in peak III. Both react with DFP and show esterase activity, one against BAEE and the other against PNPA. On the polyacrylamide gel in Fig. 9, these two esterases can be identified as the two main bands 4 and 3, respectively, both hydrolysing casein at neutral pH. In addition, band 4 hydrolyses BAME, while band 3 hydrolyses casein at pH = 10. Narahashi *et al.*,[7,9] and Trop and Birk [8] found one carboxypeptidase and one protease in peak III. The protease was reported to have activity towards both BAME and casein at alkaline pH, and was obviously a mixture of bands 3 and 4.

Regarding the carboxypeptidase, also described by Narahashi and Yanagita,[7] using Cbz-gly-leu as a substrate, we have not been able to detect this particular activity in any of the peaks (I – IV) from lot No. 64543.

Peak IV, finally, has been described by Narahashi and Yanagita,[8] and Wählby *et al.*[12] as an alkaline protease not inhibited by EDTA. By electrophoretic analyses it is found to be made up by bands 2 and 1 plus contaminations from peak III. The properties of band 1, the main constituent, correspond well to the results of Wählby and Narahashi. Trop and Birk [8] have related elastolytic activity to this special peak.

In conclusion, this comparison (Fig. 9) shows that each peak consists of several bands, and that the electrophoretic method provides a much better resolution of Pronase-P. But at the same time, there is a good correlation between the different enzymatic entities separated by ion exchange chromatography and by gel electrophoresis, both regarding their elution or migration order and their enzymatic properties. This, of course, adds further evidence to the hypothesis that Pronase-P is a rather stable but very complex mixture of proteolytic enzymes.

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