Acta Chemica Scandinavica B 28 (1974) 1003—1012

Studies on the Heterogeneity of Commercial Pronase-P Based on a Preparative Separation Method Using Sephadex G-75 Superfine

BO LÖFQVIST AND JAN-ERIK KLEVHAG

Biochemistry 1, Chemical Center, University of Lund, P.O.B. 740, S-220 07 Lund 7, Sweden

A highly improved separation method for Pronase-P was developed by gel chromatography on a specially selected more uniform fraction of the commercial superfine Sephadex G-75 beads under suitable packing and running conditions. A 10 × 90 cm bed capable of separating 1300 mg of Pronase was packed for preparative purposes and found to have better resolving efficiency than smaller columns. The enzymatically active material in Pronase was by this chromatography method separated into seven fractions with molecular weights ranging from 15 000 to 50 000 dalton. Thus Pronase components are far more heterogeneous in molecular size than had earlier been appreciated. Complete resolution was obtained between components of an estimated difference in molecular weight as small as 2000 – 3000 dalton. The possibility of intrinsic proteolysis causing specific digestion of some of the Pronase components was also demonstrated using the gel chromatography method. The results confirmed the extensive heterogeneity of Pronase earlier found using polyacrylamide gel electrophoresis, thus indicating the presence of at least 14 different enzymatically active components.

Pronase was first isolated by Nomoto and Narahashi [1] from the culture broth of *Streptomyces griseus* strain K1, and characterized as a homogeneous proteolytic enzyme with a broad substrate specificity. [2,3] Further characterization [4,5] revealed the presence of several proteolytically active components in Pronase. The most extensive analysis of the heterogeneity of Pronase to date was performed by Löfqvist and Sjöberg [6] using polyacrylamide gel electrophoresis. By this technique 14 different bands were resolved, of which 13 showed enzymatic activity using casein or LNA* as substrates. On the other hand Jurášek *et al.* [7] were able to separate only 6 different components using CM-Sephadex at pH 5 and a Ca^{2+} free medium. Since the electrophoretic separation method [6] permitted separation only on an analytical scale and further characterization of the heterogeneity of Pronase and the Pronase components required larger amounts of material, a preparative separation method had to be developed.

Already Nomoto *et al.*, [3] however, observed that the presence of Ca^{2+} ions was crucial to stabilize Pronase against autodigestion. In proceeding investigations [8] it has also been shown that different buffer-anions as well as pH have a marked influence on the stability of the Pronase components. Because of these restrictions in composition of the medium the number of suitable preparative separation techniques available was limited. In preliminary investigations the use of preparative polyacrylamide gel electrophoresis [9] was examined, but the resolution obtained was inferior to the separation of Pronase on analytical gels under similar conditions.

Since the resolution of gel chromatography is independent of the composition of the elution buffer, this technique was also considered suitable. The separation medium could thus be selected due to the stability demands of the Pronase components. Earlier reports [10,11] indi-

* *Non-standard abbreviations.* *N*-Benzoyl-L-arginine methyl ester.HCl, BAME; L-leucine-β-naphthylamide.HCl, LNA; Pronase-P lot No. 511324, Pronase I: Pronase-P lot No. 592045, Pronase IV.

cated that the molecular weights of four of the Pronase components were between 15 000 and 27 000 dalton. A preparative separation, using Sephadex G-75, therefore seemed possible. The present report describes the optimization of such a method based on a specially prepared Sephadex G-75 Superfine, and the application of this method to the examination of the heterogeneity and stability of Pronase-P.

MATERIALS AND METHODS

Pronase-P was purchased from Kaken Chemical Company, Tokyo, (lot No. 511324 and 592045, abbreviated Pronase I and IV). In the present investigation Pronase IV was used, while Pronase I was used only as a reference to the work of Löfqvist and Sjöberg.[6]

Sephadex G-75 (particle diameter in the dry state 40 – 120 μ), and Sephadex G-50, G-75, and G-100 Superfine (particle diameter in the dry state 10 – 40 μ) were obtained from Pharmacia Fine Chemicals. Several lots of G-75 Superfine, differing in resolving capacity, were used.

For the analysis of enzymatic activity were used L-leucine-β-naphthylamide.HCl, *N*-benzoyl-L-arginine methyl ester.HCl, ammonium sulfonate, N(1-naphthyl)-ethylenediamine dihydrochloride purchased from Sigma Chem. Co., and casein of Hammarsten grade from Merck AG. Acrylamide, *N,N'*-methylene-bisacrylamide and *N,N,N',N'*-tetramethylene diamine were obtained from Eastman Organic Chem. The staining of analytical gels was performed with Coomassie Blue (Sigma Chem. Co.).

Highly purified, crystallized samples of bovine serum albumin, soybean trypsin inhibitor, and lysozyme from Sigma Chem. Co., ovalbumin, β-lactoglobulin, and chymotrypsinogen from Calbiochem AG, and whale myoglobin from Schwartz were used for the estimation of the molecular weights of the Pronase components. All other reagents were of analytical grade.

Column chromatography. The columns used were, if nothing else is indicated, of the hydrodynamic precision type K 25/45 (bed size 2.5 × 40 cm), K 25/100 (bed size 2.5 × 90 cm), and K 100/100 (bed size 10 × 90 cm). (Pharmacia Fine Chemicals). UV-monitor and fraction collector for the eluate was from LKB-Products.

Gel swelling. The Sephadex beads were swelled for 24 h in standard buffer (30 mM boric acid – NaOH, pH 7.5; 30 mM $CaCl_2$; and 3 mM NaN_3). The same buffer was used for the separations, which were performed at 25 ± 0.5 °C.

Gel sieving. To obtain more defined beads the swelled gels were sieved on nylon nets allowing particles of maximum 40 μ to pass. During this procedure the small beads were washed through the net by buffer. The distribution in particle size in the obtained fractions was examined in a light microscope. As illustrated in Fig. 1, the sieving procedure gave a sharp cut in particle size between the beads, which stayed on the net, and those, which were washed through. In one case the beads that passed the 40 μ-net were also sieved on a 28 μ-net. The diameter of the beads, which were removed through the 40 μ- and 28 μ-net corresponded to 20 and 14 μ, respectively, in dry state.

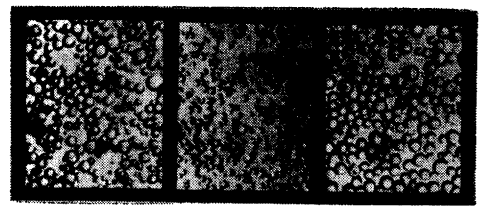

Fig. 1. The distribution in particle size of: (a) the beads of commercial Sephadex G-75 Superfine: (b) the beads removed by wet sieving on a 40 μ nylon net: (c) the residual beads after sieving.

Gel packing and sample application. In order to pack the Sephadex Superfine beads into a separating bed with good flow characteristics a special packing procedure had to be developed. A cylindrical perspex extention tube with the same inner diameter as the column was mounted on top of the column. The column was filled with buffer to a height of 5 cm above the bottom net (28 μ). The swelled gel was diluted to 1.5 – 1.6 bed volumes and filled into the center of the column along a glass rod. The lower end of the glass rod was kept below the liquid surface in the column. A 40 cm hydrostatic pressure was maintained during the packing procedure. Two or three bed volumes (V_t) of buffer were passed through the column before its flow properties were tested. The column K 100/100 had to be packed by a special procedure which will be reported separately as well as further details concerning optimal conditions for gel packing.

A solution (2 mg/ml) of Blue Dextran 2000 (Pharmacia Fine Chemicals) was used for control of the flow characteristics of the packed bed, and for determination of the void volume (V_0).

Pronase-P was dissolved in standard buffer at a concentration of 80 mg/ml. Samples were applied to columns within 2 min in order to reduce the intrinsic proteolysis of the Pronase components. The sample load was usually kept at 16 mg per cm^2 gel area. For all columns, except for K 100/100, the sample was applied by means of a syringe beneath the buffer surface on the top of the column.

Elution. The operating pressure was kept at

about 40 cm and regulated by a Mariotte flask to get a constant flow rate. The obtained flow rates for most beds were from 2 to 3 ml/cm² h. The smallest beads (diameter 14 – 20 μ in the dry state), and the K 100/100 column produced flow rates of 1.6 – 2.0 ml/cm² h. The collected fractions were stored at +4 °C.

Enzyme assays. Caseinolytic activity at pH 7.5 and 10 was measured according to the method of Kunitz, [12] activity towards BAME was determined using the method of Schwert *et al.* [13] and leucine aminopeptidase activity was measured using LNA by the method of Goldbarg and Ruthenburg, [14] all modified according to Löfqvist and Sjöberg.[6]

Analytical polyacrylamide gel electrophoresis was performed [6] in order to explore the heterogeneity of the gel chromatography peaks. Samples of either 20 or 50 μl of the column eluates were applied to the gels without prior concentration. The buffer used was 0.38 M Boric acid – 0.01 M CaO, pH 6.8.

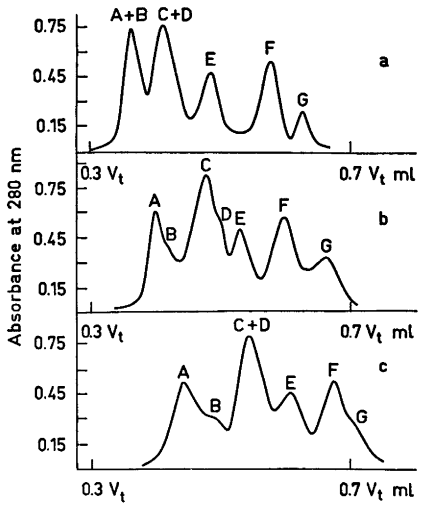

Fig. 3. The high molecular weight components of Pronase IV separated on a 2.5 × 40 cm bed of: (a) Sephadex G-50 Superfine: (b) Sephadex G-75 Superfine: (c) Sephadex G-100 Superfine. The flow rates were 2 – 3 ml/cm² h and the amount of sample applied was 16 mg/cm².

RESULTS

Conditions for preparative chromatography on Sephadex G-75 Superfine

The molecular weights determined for the Pronase components characterized so far [10,11] indicated that the pore size of Sephadex G-75 ought to be suitable for separation of Pronase. A 1.5 × 60 cm bed of Sephadex G-75 (particle diameter 40 – 120 μ in the dry state) was packed in a column of simple construction and examined for its resolving capacity on Pronase. A result similar to that of Narahashi *et al.*[15] was obtained (Fig. 2a). Two broad regions of A_{280}-absorbing material were eluted. The first region was poorly resolved into three fractions, which showed protease and peptidase activity using casein and LNA as substrates. The other region emerged close to V_t and contained components with a molecular weight below 5 000 dalton and without enzymatic activity. In order to examine whether a better resolution of the Pronase components could be obtained the column was repacked with Sephadex G-75 Superfine, particle size 10 – 40 μ in the dry state. (Usually these small beads are difficult to pack into separating beds with good flow characteristics and are therefore seldom used in column chromatography.) When Pronase was chromatographed on this bed (Fig. 2b) a better resolution than with G-75 was obtained of both the high and low molecular weight material. Five peaks could be distinguished in the high molecular weight region, and three peaks were indicated in the low molecular weight region. In order to further improve the separation results the packing procedure described in Materials and Methods was introduced along with the use of hydrodynamically more optimal columns (Pharmacia K 25/45). At the same time the separation on Sephadex G-50, G-75, and G-100 (all Superfine) were compared (Fig. 3) to establish the optimal pore size for

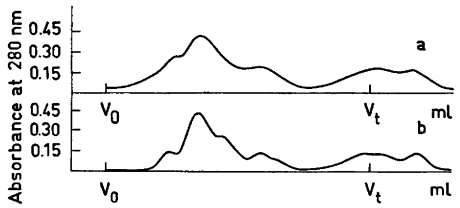

Fig. 2. The effect of bead size on the resolution of Pronase IV on a 1.5 × 60 cm gel chromatography bed of: (a) Sephadex G-75; (b) Sephadex G-75 Superfine. The rates of elution were 2.5 ml/cm² h and the amount of sample applied was 12 mg/cm².

the resolution of the Pronase components. Using Sephadex G-75 Superfine a remarkable improvement of the separation was noted on this bed compared to the results obtained on the earlier 1.5 × 60 cm bed (Fig. 2b). Thus seven different high molecular weight peaks (labelled A – G) were discerned, and five of these peaks were well separated from the other material. Using Sephadex G-50 Superfine a better resolution of F and G was achieved than with G-75 Superfine (Fig. 3a), while there was a poorer separation in the A – D region. As expected, the reverse was true, when Sephadex G-100 Superfine was used (Fig. 3c). With this material a better resolution of peaks A and B was obtained, while F and G separated poorly. Thus Sephadex G-75 Superfine was the most suitable for studies of the heterogeneity of Pronase, when an optimal separation of all the Pronase components was desired.

In order to improve the resolution of the Pronase components in peaks A – G a 2.5 × 90 cm bed was packed. For that purpose a new lot of Sephadex had to be used. The packing performance as well as the resolution of this lot of Sephadex G-75 Superfine was, however, not as good as expected. Therefore a number of different lots were tested. A considerable variation in the resolution power between the lots was found. The best lot was selected and packed in a K 25/100 column. The elution profile for Pronase fractions from this column is shown in Fig. 4a. The resolution was, however, still not complete between any of the peaks, and each peak eluted in a rather large volume. It was assumed that the variations in performance between different batches of Sephadex were due to inhomogeneities in particle size. Therefore, in an attempt to further improve the separation properties of the gel, the swelled Sephadex G-75 Superfine particles were sieved on a nylon net. About 10 % of the gel particles were in wet state smaller than 40 μ and passed through the net and were removed. The separation of Pronase-P on a bed (2.5 × 90 cm) packed with the sieved and more homogeneous gel material (Fig. 4b) resulted in an improved resolution compared to the unsieved gel (Fig. 4a). No new peaks were resolved but the elution volume for each peak was markedly reduced, clearly indicated by comparison of the elution volume of peak E in the two elution diagrams,

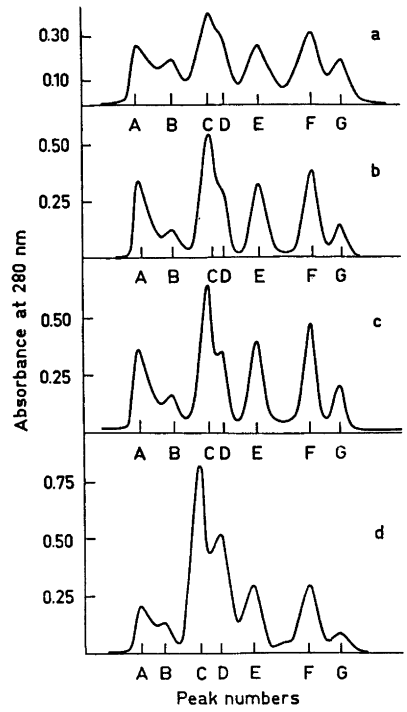

Fig. 4. The elution diagrams of the high molecular weight components in Pronase IV separated: (a) on a 2.5 × 90 cm bed of Sephadex G-75 Superfine (particle size 10 – 40 μ in the dry state): (b) on a 2.5 × 90 cm bed of sieved Sephadex G-75 Superfine (particle size 20 – 40 μ in the dry state): (c) on a 10 × 90 cm bed of sieved Sephadex G-75 Superfine (particle size 20 – 40 μ in the dry state). In (d) is shown the elution diagram of Pronase I on the same bed as used in (b). The flow rates were 1.6 – 3 ml/cm² h. The amount of sample applied was 16 mg/cm².

and a more complete separation of the peaks was thus obtained.

The same sieving procedure was also applied to batches with poor original resolving power in order to examine if their quality could be improved. This was, however, only possible to a limited extent, and it can for these batches be concluded that other factors than inhomogeneities in particle size are responsible for the poor separation characteristics. In accordance with the theory of gel chromatography [11] the smallest beads, which in wet state passed the 40 μ sieving net, should give a further improved resolution of the Pronase components. To facilitate the packing of this material it was

sieved further, and the smallest particles, which passed a 28 μ net, were removed. In spite of this fractionation it was not possible to pack the residual beads (particle diameter 14 – 20 μ in the dry state) into a separating bed with acceptable flow characteristics. Nevertheless a remarkably small zone spreading could be observed on the best packed bed, indicating that a further resolution could be achieved, provided a bed with good flow characteristics could be packed.

The applied amount of Pronase on the 2.5 × 90 cm bed (Fig. 4b) was 80 mg. With increased amounts of protein applied the resolution between the peaks declined considerably. Only a few mg of each component could therefore be obtained in each run. Consequently it was desirable to scale up the separation. A K 100/100 column was therefore packed, which with the same load per area would allow 16 times as much material to be separated. The separation obtained (Fig. 4c), where thus 1300 mg Pronase was chromatographed, was even better than that obtained when the K 25/100 column was used.

Investigation of Pronase heterogeneity

Qualitative and quantitative differences in the composition of different Pronase preparations. In all the separations presented so far Pronase IV was used. The elution diagram of Pronase I, the preparation used in earlier investigations,[6] is presented in Fig. 4d. A comparison of the K_{av} values for the peaks in Fig 4b and 4d (Table 2) indicated only small differences in the qualitative composition of the two Pronase preparations. Certain divergences were, however noted. Thus the K_{av} for peak A and D was substantially lower for Pronase IV than for Pronase I. The separation between peak D and E was also incomplete in the elution diagram of Pronase I. These differences were significant and reproducible, and indicate that the two analyzed Pronase preparations might contain different components.

The quantitative differences between the two Pronase preparations were more pronounced judged from the amount of A_{280}-absorbing material found in different peaks (Table 1). Thus peak A is proportionally larger in Pronase IV, while in Pronase I peaks C and D are more predominant. The amount of enzymatically inactive low-molecular A_{280}-absorbing material is about 30 % in both the Pronase batches tested.

Molecular weights of the Pronase components. The bed used for chromatography in Fig. 4b and 4d was calibrated with a number of proteins of known molecular weights. When the logarithm of their molecular weights were plotted against the determined V_e/V_0 (Fig. 5) a straight line was obtained. The V_e/V_0 values for the peaks of Pronase IV are also indicated in Fig. 5. and show that the approximate molecular weights of the Pronase components vary between 15 000 and 50 000 dalton. Thus the Pronase components exhibit a much larger variation in molecular size than was suggested by the ultracentrifugation studies of Narahashi *et al.*,[15] where a single peak corresponding to a molecular weight of 20 000 dalton was obtained. The

Table 1. K_{av} and amount of A_{280}-absorbing material of the gel chromatography peaks A – G.

Peak	K_{av} of the gel chromatography) peaks		Amount of A_{280}-absorbing material, %		% Staining [c]
	Pronase IV[a]	Pronase I[b]	Pronase IV[a]	Pronase I[b]	
A	0.165	0.196	18	8.5	9
B	0.241	0.248	7	5	7
C	0.322	0.326	30.5	30	27
D	0.352	0.367		23	21
E	0.437	0.439	18	14	15
F	0.557	0.560	20	15	13
G	0.621	0.624	6.5	4.5	8

[a] Elution diagram 4b. [b] Elution diagram 4d. [c] Staining in the corresponding electrophoretic bands as measured by Löfqvist and Sjöberg in Pronase I.[6]

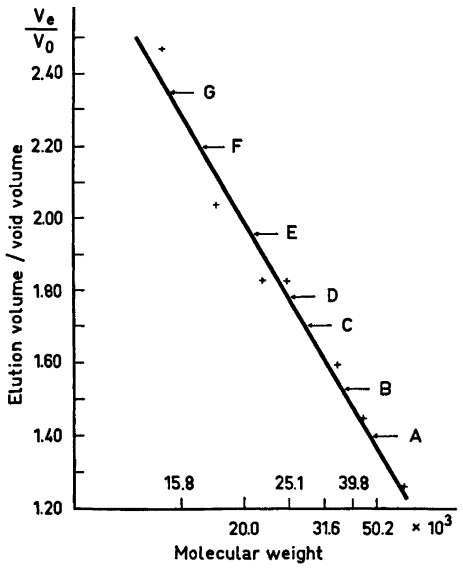

Fig. 5. The elution volume/void volume (V_e/V_0) of a series of standard proteins (see Materials and Methods) *versus* their molecular weights. V_e/V_0 values for the chromatographically separated peaks A – G are indicated by arrows.

molecular weight of peak G (15 000 dalton) corresponds well to the value (17 750 dalton) calculated by Bauer and Löfqvist [16] from the amino acid composition. On the other hand peak A with an apparent molecular weight of 50 000 dalton shows a molecular weight twice as high as previously reported for any Pronase component.[10,11]

Electrophoretic analysis of the different gel chromatography peaks. In order to identify the Pronase components present in the different peaks in terms of the electrophoretic bands identified by Löfqvist and Sjöberg [6] analytical polyacrylamide gel electrophoreses were run on each of the seven peaks in the elution diagrams presented in Fig. 4b and 4d. The content of electrophoretic bands in the two Pronase preparations are given in Table 2. All the major bands of Pronase I,[6] except band 11, which could not be examined in the buffer system used, were identified in the chromatography peaks. This further establishes that the heterogeneity of Pronase is actually as extensive as earlier shown [6] and not caused by intrinsic proteolysis during the separation. Most of the major electrophoretic bands of Pronase I were also identified in the corresponding peaks of Pronase IV (Table 2). A few interesting differences were, however, observed. Thus Pronase IV contained two components (one electrophoretic band 9 in peak A and one electrophoretic band 10 in peak CD), which were earlier not found in Pronase I.[6] On the other hand the major bands 2 and 5 occurring in peak F and the tailing edge of peak D of Pronase I were hardly detected or lacking in Pronase IV.

In addition to the major components minor bands appeared in most of the peaks of the elution diagrams. They only occupied a small fraction (less than 10 %) of the material in each peak and have either been regarded as

Table 2. The heterogeneity of the chromatography peaks A – G in terms of components found by analytical polyacrylamide gel electrophoresis.

Peak	Major electrophoretic bands[a] present in Both Pronase I and IV	Pronase IV only	Pronase I only	Minor electrophoretic bands present in Pronase I or IV
A	8	9	13 – 14[b]	–
B	6, 7	–	13 – 14[b]	8
C	12, 13 – 14[b]	–	–	4, 10, 9
D	13 – 14b, 10, 9	10[c]	5	4, 7, 12
E	4	–	–	7
F	1	–	2	3, 2
G	3	–	–	1

[a] The numbering of the bands refer to the earlier results by Löfqvist and Sjöberg.[6] [b] Band 13 and 14 were not separated in the system used for the electrophoretic analysis. [c] Band 10 appeared as a double band in Pronase IV.

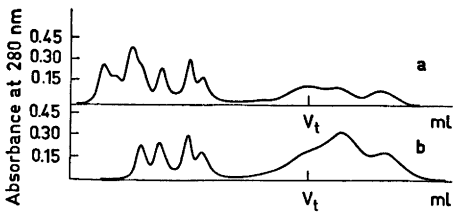

Fig. 6. The elution diagrams of Pronase IV in 30 mM ammonium acetate buffer, pH 4.8 (a) in the presence of 30 mM $CaCl_2$, (b) without $CaCl_2$ on a 2.5 × 90 cm bed of Sephadex G-75 Superfine. The flow rates were 5 ml/cm² h, and the sample loads were 16 mg/cm².

contaminants from a neighbouring peak or as impurities of less importance at this stage of investigation.

Stability of the Pronase components. The potential risk for intrinsic proteolysis of the Pronase components during the separation was considered as a critical factor in this investigation of the heterogeneity of Pronase. Since the resolving power of a Sephadex bed, however, is not influenced by the composition of the eluting medium used, the stability of the separated components in various media could be studied by comparing the elution profiles obtained in different buffers. This advantage of gel chromatography was used to investigate the stability of the Pronase components under the conditions used by Jurášek *et al.*[7] These investigators were able to identify 6 Pronase components after dialysis of Pronase against Ca^{2+} -free acetate buffer pH 5 and separation on a CM-Sephadex column. They thus concluded that "Pronase appears to be a less complex mixture of proteolytic enzymes than had previously been appreciated". When we separated Pronase on a 2.5 × 90 cm Sephadex G-75 Superfine bed equilibrated with a Ca^{2+}-free acetate buffer at pH 4.8 (Fig. 6b), peaks A, B and C were found to be completely digested into low molecular weight fragments. The protective effect of 30 mM Ca^{2+} under in other respects similar conditions is shown in Fig. 6a. The stability of peaks A, B, and C is remarkably increased but still not as good as that at pH 7.5 as judged by the amount of enzymatically inactive low molecular weight components produced. At pH 4.8 these occupy 40 % instead of the at pH 7.5 normally found 30 % of the A_{280}-absorbing material. This sign of increased intrinsic proteolysis was, however, not correlated with the disappearance of any particular peak in the elution diagram.

The stability of Pronase IV was further examined by analysis of the recovery of enzymatic activity after gel chromatography at pH 7.5 in the presence of Ca^{2+}. The residual enzymatic activities for a parallel sample of unfractionated Pronase IV stored beneath the Sephadex column (80 mg/ml) (Table 3) were also measured. A substantial loss in activity towards casein when analyzed at pH 7.5 and a small loss in activity towards BAME occurred both in the chromatographed Pronase sample and in the sample stored unfractionated. The activities towards casein at pH 10 and LNA were largely unaltered.

Table 3. Residual activity after separation of Pronase IV on Sephadex G-75 Superfine or preincubation at different concentrations in standard buffer.

Pronase IV dissolved in standard buffer at a concentration of	% Residual activity Casein at pH 7.5	Casein at pH 10	LNA	BAME
1.5 mg/ml	100	100	100	100
80 mg/ml, chromatographed and pooled before analysis	65	96	98	86
80 mg/ml, stored 36 h before analysis	61	90	97	87
80 mg/ml, diluted to 1.6 mg/ml after 2 min and analyzed	86	101	99	100

From Table 3 it can also be seen that the loss in activity is highly dependent on the concentration of Pronase in the solution. After 2 min of incubation a 15 % loss in enzymatic activity towards casein analyzed at pH 7.5 was observed at a Pronase concentration of 80 mg/ml. It thus appears that the presence of calcium ions was particularly important for the stability of the components that exhibited only neutral protease activity.

DISCUSSION

The resolution obtained by gel chromatography is highly dependent on the amount of sample applied. This fact is not always considered in practical separation work. Thus overloading especially in the case of complex samples often leads to a poor resolution. In the work of Narahashi *et al.* [15] and Awad *et al.*[10] 4 – 10 times as much Pronase was applied to Sephadex G-75 or G-100 columns as was used in the present report. Consequently this might be one reason, why these investigators were only able to partially separate two fractions and get weak indications of one additional component.

There are, however, several other parameters that have to be regarded in order to achieve good separation results on gel chromatography. The theory of gel chromatography gives the relation of these parameters to each other as well as their contribution to the separation efficiency.[17] The applicability of the theory in practical separational work is, however, limited by restrictions like packing performance of the gel, uniformity of the beads and obtainable flow properties of a packed bed, and these factors have to be examined and optimized in experimental work.

The effect of packing procedure but also to some extent column design on the separation result is illustrated by comparison of Figs. 2b and 3b. The superfine beads were found to be more difficult to pack and thus the in theory predicted advantage of using small beads was limited by the poorer packability of these beads. By modification of the packing procedure it was, however, possible to obtain a separating bed giving account for the larger resolving capacity expected from superfine beads. Further improvement of the separation results was also achieved, when a more uniform preparation of superfine beads (particle diameter in the dry state 20 – 40 μ instead of the normal 10 – 40 μ) was used. This was most likely due to improved packing performance of the gel with the more uniform particle distribution. The differences in resolving efficiency from one batch of Sephadex G-75 Superfine to another also indicated the presence of other hitherto not described variations in the uniformity of the Sephadex beads. Investigations in progress show that the separation efficiency can be further improved by partial elimination of such variations. The improved gel chromatography method on Sephadex G-75 Superfine columns, as described in the present paper showed, however, that the Pronase components were far more heterogeneous in molecular size than had earlier been appreciated. Thus Pronase was separated into seven peaks with molecular weights ranging from 15 000 to 50 000 dalton and with complete resolution between components of an estimated difference in molecular weight as small as 2000 – 3000 dalton.

Through analysis of each of the gel chromatography peaks by polyacrylamide gel electrophoresis the qualitative nature of the Pronase heterogeneity was investigated. From the result thereby obtained and the enzymatic activities observed for the different electrophoretic bands [6] it can be concluded that chromatography peaks A – D contain neutral proteases as well as leucine aminopeptidases. In the absence of Ca^{2+} ions at pH 4.8 these components are found to be highly susceptible to autodigestion and peaks A, B, and C are completely digested already during the course of separation. These results explain the findings of Jurášek *et al.* [7] that "Pronase appears to be a less complex mixture of proteolytic enzymes than had previously been appreciated", and that it does not contain any neutral proteases. The conditions used by Jurášek *et al.* [7] simply caused a total digestion of several of the Pronase components, and these investigators were only able to detect the most stable ones. A certain loss in neutral protease activity also occurred under the conditions used for the separations presented in this paper. This was probably caused by intrinsic proteolysis. The nature of the resulting products, however, has not been investigated, and the possibility that any of

the components originally present in Pronase are completely digested also under the conditions applied to this investigation cannot be excluded. However, this seems unlikely and is not supported by any results obtained by analysis of Pronase in various media on either gel chromatography or gel electrophoresis,[6] and we thus believe that the found heterogeneity gives a complete picture of the heterogeneity present in the investigated Pronase preparations.

Since the most pronounced heterogeneity is found in the region (A–D), where the neutral proteases are eluted and since both qualitative and quantitative variations (Tables 1 and 2) in the heterogeneity between different Pronase preparations also are found in this region, it can be questioned to which extent the found heterogeneity represents enzymes originally produced by the microorganism and to which extent the heterogeneity is caused by intrinsic proteolysis of the originally produced enzymes occurring during the fermentation and isolation processes. Further attention to the question of the originality of the identified Pronase components will be given in a separate paper.[18] At this stage, however, the fate of two of the Pronase components in the A–D region will be discussed in some detail.

Firstly peak D of Pronase I was found to contain electrophoretic band 5, not visible in Pronase IV. Peak D in Pronase I also showed a higher K_{av} than that of peak D in Pronase IV, causing a poor resolution between peak D and E. The component equivalent to electrophoretic band 5 thus seems to be eluted as the smallest component in peak D. In the investigation by Löfqvist and Sjöberg [6] band 5 was the only component, which was not active towards any of the substrates tested. Later studies have shown that this component is active towards *N*-carbobenzoxy-L-glycyl-L-leucine and thus corresponds to the carboxypeptidase in Pronase,[15] The molecular weight can be estimated at around 23 000 dalton.

Secondly Pronase IV was found to contain two components with the same electrophoretic mobility. Thus both peak A and D produced a major band in position 9. In Pronase I, however, band 9 was only found in peak D. In the earlier investigation [6] this band also showed activity towards casein at pH 10. The molecular weight of the components in peak D was found to be 25 000 dalton. Band 9 in peak D thus seems to correspond well with the subtilisin-like alkaline protease with a molecular weight of 28 000 dalton isolated by Gertler and Trop [11] and Awad *et al.*[10] The other band 9, which was only found in peak A of Pronase IV, seems to be responsible for the lower K_{av} obtained for that peak in Pronase IV and would thus have the largest molecular weight, 50 000 dalton, of the Pronase components.

The heterogeneity of the remaining peaks E, F, and G is less complex. In the case of Pronase IV only one main component is found in each peak. The K_{av} values as well as the amounts of A_{280}-absorbing material of the two Pronase preparations also agreed well, thus indicating a higher stability of these components. From the activities earlier observed for the electrophoretic bands [6] it can be concluded that peak E contains the trypsin-like enzyme in Pronase, further characterized by Jurášek and Smillie [19] and peak F the elastase-like enzyme III, described by Gertler and Trop. [11] Peak G finally contains an enzyme with broad substrate specificity, showing similarities to both elastase and chymotrypsin.[16,20]

Acknowledgements. We wish to express our gratitude to Professor Gösta Ehrensvärd for his continuous interest in our work, and to Miss Ylva Persson for her skilful technical assistance. We also like to thank Drs. B. Jergil and C.-A. Bauer for many valuable discussions.

REFERENCES

1. Nomoto, M. and Narahashi, Y. *J. Biochem. (Tokyo) 46* (1959) 653.
2. Nomoto, M., Narahashi, Y. and Murakami, M. *J. Biochem. (Tokyo) 48* (1960) 593.
3. Nomoto, M., Narahashi, Y. and Murakami, M. *J. Biochem. (Tokyo) 48* (1960) 906.
4. Narahashi, Y. *Methods Enzymol. 19* (1970) 651.
5. Wählby, S. *Biochim. Biophys. Acta 151* (1968) 394.
6. Löfqvist, B. and Sjöberg, L.-B. *Acta Chem. Scand. 25* (1971) 1663.
7. Jurášek, L., Johnson, P., Olafsen, R. W. and Smillie, L. B. *Can. J. Biochem. 49* (1971) 1195.
8. Narahashi, Y. and Yanagita, M. *J. Biochem. (Tokyo) 62* (1967) 633.
9. Hjertén, S., Jerstedt, S. and Tiselius, A. *Anal. Biochem. 27* (1969) 108.

10. Awad, W., Soto, A., Siegel, S., Skiba, W., Bernström, G. and Ochoa, M. *J. Biol. Chem. 247* (1972) 4144.
11. Gertler, A. and Trop, M. *Eur. J. Biochem. 19* (1971) 90.
12. Kunitz, M. *J. Gen. Physiol. 30* (1947) 291.
13. Schwert, G. and Takenaka, Y. *Biochim. Biophys. Acta 16* (1955) 570.
14. Goldbarg, J. A. and Ruthenburg, A. M. *Cancer (Philadelphia) 11* (1958) 283.
15. Narahashi, Y., Shibuya, K. and Yanagita, M. *J. Biochem. (Tokyo) 64* (1968) 427.
16. Bauer, C.-A. and Löfqvist, B. *Acta Chem. Scand. 27* (1973) 3147.
17. Wink, H. *J. Chromatogr. 52* (1970) 205.
18. Löfqvist, B. *Acta Chem. Scand. B 28* (1974) 1013.
19. Jurášek, L. and Smillie, L. B. *Can. J. Biochem. 51* (1973) 1077.
20. Bauer, C.-A., Löfqvist, B. and Pettersson, G. *Eur. J. Biochem. 41* (1974) 45.

Received April 30, 1974.