

Isoelectric Focusing of Acidic Proteins

Studies on Pepsin

O. VESTERBERG

Chemical Division, Occupational Health Department, The National Board of Occupational Safety and Health, S-100 26 Stockholm 34, Sweden

The development of acidic carrier ampholytes (Ampholine) for the pH range 2.5–4 will allow studies on isoelectric focusing of most acidic proteins. These carrier ampholytes are of the aliphatic polyamino-polycarboxylic acid type. Isoelectric focusing in density gradient and polyacrylamide gel have been used for studies on multiple molecular forms of pepsin of hog and human origin. Detection of the enzymatic activity after focusing in gel using a modified zymogram procedure is described. Samples of human gastric juices show different patterns at a high degree of resolution, that might be promising for further studies on possible relationships between specific patterns and ulcers and cancer.

Isoelectric focusing has been used for separation and characterising many different proteins (for review articles see Refs. 1–4). Most of the proteins studied up to now have been isoelectric in the pH range 3.5–10. The principal reason why very few studies have been made outside this pH range is that buffer substances, carrier ampholytes, have not been commercially available to cover very acidic and basic pH regions. Recently, it was possible to extend the useful pH range on the alkaline side up to about pH 11.⁵ During the last years the problem with the acidic pH region has been tackled. This has resulted in the development of a suitable system of aliphatic polyaminopolycarboxylic acids for the acidic pH, analogous to the carrier ampholytes for other pH ranges.^{6,7} (Vesterberg, to be published in more detail elsewhere.)

There are many interesting acidic proteins with isoelectric point (pI) < 4, *e.g.* glycoproteins in serum, from micro organisms, and cell membranes. One of the best known acidic proteins is pepsin. A correlation between increased concentration of pepsin and the incidence of duodenal ulcers⁸ and cancer in the stomach⁹ has been found. This protein has been shown to occur in multiple molecular forms,^{10,11} one of which has been shown to be more prominent in some patients with peptic ulcers.¹² Because isoelectric focusing has been shown to give a resolution comparable to or surpassing carefully conducted electrophoresis in gels, it was of interest to apply the method on pepsin.

MATERIALS, METHODS, AND RESULTS

Pepsin was purchased from Merck, Germany, types Crude DABG, and pepsin krist. lyophilisiert 100 mU/mg, and from Sigma Chem. Co., Mo., USA, type 2 × Crystallized and Lyophilized, 2700 U/mg. The samples of gastric juice were obtained by aspiration from different patients with complaints indicating gastritis and in some cases ulcers, courtesy of Professor H. Lagerlöf at Karolinska sjukhuset, Stockholm. Some samples were obtained after stimulation with gastrin. All samples had been stored at -20° for about one month. Isoelectric focusing in density gradient was performed essentially as described earlier.⁴ A gradient mixer was used. A column of 110 ml capacity (Type 8101 LKB-Produkter AB, S-161 25 Bromma, Sweden) was used with circulating cooling water at $+4^{\circ}\text{C}$. The dense solution contained 24 g of sucrose, 2 ml of carrier ampholytes 20 % (w/v) pH 2.4–4 and distilled water to make a final volume of 49 ml; the less dense solution contained 0.5 ml of carrier ampholytes 20 % (w/v) pH 2.5–4 in a final volume of 49 ml. 15 mg of pepsin was dissolved in this solution. The anode solution for the central tube and the bottom of the column comprised 20 ml of a sucrose solution 55 % (w/v) to which 1.0 ml 1 M H_3PO_4 was added. After filling the column 10 ml of water containing 0.25 ml of Ampholine 20 % (w/v) pH 2.5–4 was added to provide the cathode solution. A potential of 500 V was applied for 2 h and was then increased to 700 V for 46 h. Fractions of 2.0 ml were collected. The pH of the fractions was measured at $+4^{\circ}$. Pepsin was assayed on hemoglobin as substrate essentially as described earlier.¹⁰

The result of focusing a purified preparation of pepsin in a density gradient column is shown in Fig. 1. The total yield of pepsin after focusing was often close to 90 %. Two

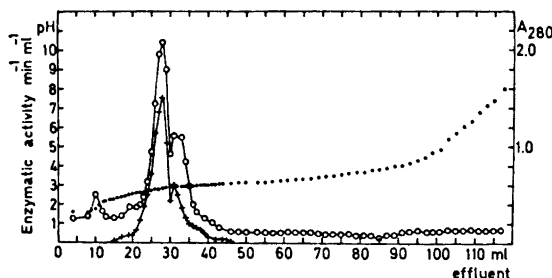


Fig. 1. The result of isoelectric focusing at $+4^{\circ}$ of 15 ml of pepsin (Sigma). Symbols: pH at $+4^{\circ}$...; absorbance, A_{280} O; enzymatic activity measured as increase in A_{280} min^{-1} , +.

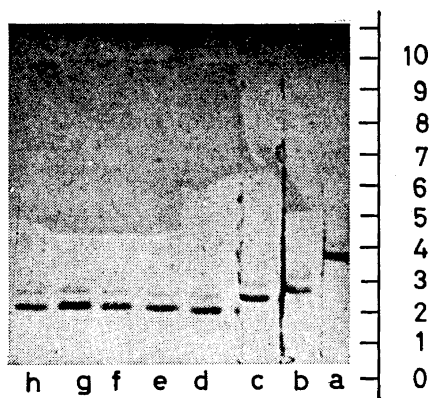
components with enzymatic activity were resolved, characterised by isoelectric points (pI's) at 2.86 and 2.94, respectively. The protein concentration as estimated by A_{280} correlated well with the enzymatic activity in the fractions showing significant activity. The elevated A_{280} values in the very acidic fractions were probably caused by impurities in the sucrose, or products formed at the anode. Thus the results indicated that the pepsin preparation used was probably not contaminated by protein impurities to any high extent.

Isoelectric focusing in thin layer polyacrylamide gels (IFPAG) was done in principle as described elsewhere.¹³ The gels contained 1 % (w/v) of Ampholine (LKB) pH 2.5–4 and were polymerized with riboflavin as catalyst. The anode was soaked in 1 M H_3PO_4 and the cathode was soaked in 0.1 % of Ampholine pH 2.5–4. The distance between the electrodes was 10.5 cm. Protein samples were usually applied after being soaked into small rectangular pieces of filter paper, e.g. Whatman 3 MM, and usually placed close to the cathode. A potential of 800 V was used for 4 h.* In some cases protein was applied close to both electrodes in the same strip of the gel. After focusing for 2, 3, and 3.5 h,

* Note added in proof. For further details and recommendations see Vesterberg, O. *Science Tools* 20 (1973) probably in No. 2.

Fig. 2. The result obtained after staining of the protein zones of pepsin preparations after isoelectric focusing in polyacrylamide gel. The distance from the anode is shown to the right (cm scale).

a–d, Sample application: 25 μ l containing 50 μ g of pepsin (Sigma) was soaked into each of two pieces of filter paper measuring 5 \times 0.5 cm, which were placed close to each electrode. Focusing time in hours: (a) 2, (b) 3, (c) 3.5, and (d) 4. e–h, Sample application: Protein solutions were soaked into rectangular pieces of filter paper measuring 1.0 \times 1.0 cm, and placed close to the cathode. e, 25 μ l containing 50 μ g of pepsin (Sigma). f, Same as e. g, 100 μ l containing 50 μ g of pepsin (Merck "krist"). h, 50 μ l of the same solution as in g.



narrow strips were cut out. During this operation the current was switched off. The strips were stained for protein as described earlier.¹³ By this procedure it was possible to see when protein zones migrating from either electrode had coalesced and focused sharply (Fig. 2). With the voltage used, this occurred within 2 h although the proteins continued to migrate slowly towards the anode. Nevertheless a high degree of resolution could be obtained (Fig. 2). A main component was always obtained. When sufficient protein was applied a faint band was also seen on the cathodic side of the main component. This component was more pronounced in impure preparations. By using a zymogram procedure (*vide infra*) it was possible to show that both zones contained enzymatic activity.

After switching off the current, pH was measured on the surface with a surface pH electrode¹³ (Fig. 3). Fastening the electrode with a clamp fixed to a stand, which can be moved a little for each pH measurement, aids the process. When these pH values were compared to the corresponding positions of the stained protein zones, the main component possessed a pI of 2.8 and the minor one a pI of 2.9.

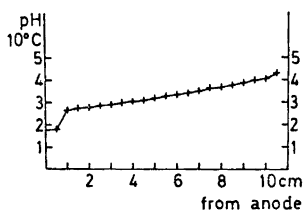


Fig. 3. Record of pH measurements at +10° from anode to cathode of the experiment shown in Fig. 2(e–h).

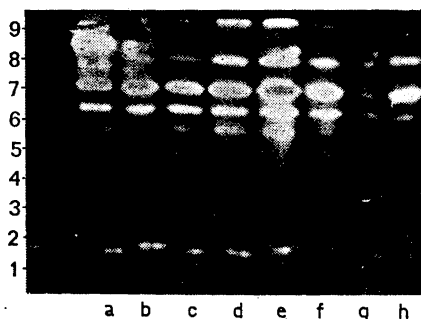


Fig. 4. Zymogram detection of proteolysis after isoelectric focusing. The samples of gastric juice (10 μ l) were applied to pieces of filter paper measuring 1.0 \times 0.3 cm and placed close to the cathode. The zones with enzymatic activity can be seen as white unstained zones against the stained background, which represents undigested albumin. Distance from the cathode (in cm) is shown to the left.

As was mentioned earlier, proteins were usually detected by staining. However, in some experiments protein samples were applied to the gel to permit slicing into two identical halves. One of the pieces was then used for detection of enzymatic activity by a modified zymogram procedure.¹⁴ It was only necessary to incubate the gel in albumin solution for 30 min. This is a shorter time than recommended earlier.¹⁴ Sharper zones are obtained with the new procedure because diffusion is reduced. Staining and fixing were done as described earlier.¹³ The patterns obtained with samples of gastric juice from different persons often showed differences in the number of zones detected, and also in the relative activities of particular zones. Up to 6 zones of activity were seen (Fig. 4).

DISCUSSION

As can be seen from Fig. 1 the useful pH range with these new carrier ampholytes extends down to about pH 2.2, which should be low enough to allow studies of most acidic proteins. A very important property of the carrier ampholytes is a sufficient buffering capacity.^{6,15} Measurements have shown that the carrier ampholytes forming the acidic pH region show more than twice the buffering capacity of those used earlier for the neutral pH range when compared on a weight for weight basis. This is due to the abundance of carboxylic groups dissociating in the acidic region, and is the principal reason why half the usual concentration of carrier ampholytes can be used for focusing in the acidic pH region. Another reason is that it is important to try to avoid too high a current at the voltage necessary for focusing. A lower concentration of Ampholine results in a smaller current, and thus a reduced total electrical load, minimising the risk of excessive heating that could otherwise destroy the density gradient.

The degree of focusing, *i.e.* the width of a protein zone, is determined among other factors by the local field strength.¹⁵ Quite good distribution of the field strength can be obtained with these new acidic carrier ampholytes judging from the width of the zones obtained after focusing in the column as well as in the gel. However, very critical inspection might perhaps indicate that the width of the main zones obtained after focusing both in the column and in the gel are somewhat larger than could be expected for homogeneous proteins. Some of the results obtained indicate that minor components are present close to the major ones and make them look somewhat broader than they actually should be. As was mentioned earlier pepsin has been proven by independent methods, such as ion change chromatography and electrophoresis in agarose gels, to occur in many multiple molecular forms, formed by cleavage of the peptide chain at different points during activation, and perhaps also differing in the number of amide groups. (For a summary of the most usual explanations for multiple molecular forms of proteins see Ref. 16).

During the IFPAG of pepsin it has been found that this protein migrates slowly in the direction of the anode. This is probably caused by electroendosmosis. Therefore, no stable final focusing position in the acidic pH region of the gel should be awaited before stopping any particular experiment. A method of determining the duration of an experiment has already been indicated. Proteins substantially larger than pepsin may require a somewhat longer time for optimal resolution than pepsin due to a lower electrophoretic mobility in the gel.

The pH measurement with the surface electrode has certain limitations. However, it can be seen by comparison of Figs. 1 and 3, that fairly good agreement between the values obtained in column and gel was obtained.

The fact that pepsin occurs in different molecular forms makes it difficult to make an exact comparison of the pI obtained by isoelectric focusing with that from electrophoresis. However, a value of 2.7 with the latter method has been reported,¹⁷ which is in good agreement with the pI for the main component obtained here. Earlier electrophoretic data which estimate the pI in the vicinity of 1 are most probably false, and probably resulted from binding of anions to the protein. Moreover, such a low value is not in accord with the amino acid composition of the protein. Another interesting fact is that an isoionic point close to pH 3 has been reported for pepsin.¹⁸ This is also close to the pI obtained here for the main component and may be considered additional evidence for the earlier proposed statement that the pI obtained by isoelectric focusing is also close to the isoionic point of a protein.^{7,15}

The patterns obtained with the different samples of gastric juice (Fig. 4) show many different zones of activity in accord with earlier observations.^{11,12} The resolution obtained by isoelectric focusing is very high and seems very promising for continued studies, *e.g.* on the significance of different patterns and their possible correlation with ulcers and cancer.

When this manuscript was completed a paper appeared on isoelectric spectra of pepsin.¹⁹ In this investigation pH gradients below pH 3 were created by using mixtures of acids. Three components of pepsin were detected. It is not possible to make a strict comparison of the pI values of that study with those reported in this investigation, because different brands of pepsin have been used. However, M. Jonsson indicated that the pI of the most acidic component was too low, which could have been caused by binding of some of the anions used to obtain the pH gradient. He was also of the opinion that a pI obtained in the presence of carrier ampholytes was closer to the isoionic point than the pI obtained in the presence of acids. The use of ordinary acids to create a pH gradient has many drawbacks, some of which may be mentioned here:

- (1) the pI obtained is influenced by the type of acids and concentrations thereof used;
- (2) a very long time (about 150 h) may be required for separation of proteins;
- (3) the distribution of the field strength is unfavourable;
- (4) it is difficult to obtain a good shallow pH course which means among other things that the useful part of the column is very short;
- (5) no stable final pH gradient is obtained; instead there is a slow drift against the anode;
- (6) the resolution of proteins with a considerable difference in pI is not good.

The new carrier ampholytes for the acidic pH range circumvent all the mentioned drawbacks.

Acknowledgements. Thanks are due to Miss Vesna Gasparich for preparation of the carrier ampholytes, and also to Mrs. Gun Nise and Miss Birgitta Karlsson for skilful technical assistance in the experiments with pepsin and gastric juice. Dr. Cyril Smyth is acknowledged for valuable criticism and revision of the English text.

REFERENCES

1. Haglund, H. *Methods Biochem. Anal.* **19** (1971) 1.
2. Catsimpoolas, N. *Separ. Sci.* **5** (1970) 523.
3. Wrigley, C. W. In Niederwieser, A. and Pataki, G. *New Techniques in Amino Acid Peptide, and Protein Analysis*, Ann Arbor Science Publ. Inc. 1971, pp. 291 – 339.
4. Vesterberg, O. *Methods Enzymol.* **22** (1971) 389, 412.
5. Lundblad, G., Vesterberg, O., Zimmerman, R. and Lind, J. *Acta Chem. Scand.* **26** (1972) 1711.
6. Vesterberg, O. *Acta Chem. Scand.* **23** (1969) 2653.
7. Vesterberg, O. In Catsimpoolas, O. *Conference on Isoelectric Focusing and Isotachopheresis*, Ann. N.Y. Acad. Sci. **209** (1973) 23.
8. Cheret, A.-M. and Bonfils, S. *Pathol. Biol.* **18** (1970) 317.
9. Pastore, J. O., Kato, H. and Belsky, J. I. *N. Engl. J. Med.* **286** (1972) 279.
10. Rajagopalan, T. G., Moore, S. and Stein, W. H. *J. Biol. Chem.* **241** (1966) 4940.
11. Etherington, D. J. and Taylor, W. H. *Biochem. J.* **113** (1969) 663.
12. Taylor, W. H. *Nature* **227** (1970) 76.
13. Vesterberg, O. *Biochim. Biophys. Acta* **257** (1972) 11.
14. Hirsch-Marie, H., Burtin, P. and Conte, M. *Acta Gastro-Enterol. Belg.* **28** (1965) 373.
15. Vesterberg, O. and Svensson, H. *Acta Chem. Scand.* **20** (1966) 820.
16. Vesterberg, O. In Renoz, M. *Proc. VII Symp. Chromatogr. and Electrophoresis*, Presses Académiques Européennes, Brussels 1973.
17. Polonovski, M., Boulanger, P., Macheboef, M. and Roche, J. *Biochimie médicale*, 5° éd., Masson, Paris 1952, Vol. 1, p. 299.
18. Bovey, F. A., Yanarig, S. S. In *The Enzymes*, Academic 1960, Vol. 4, p. 63.
19. Jonsson, M. *Acta Chem. Scand.* **26** (1972) 3435.

Received February 28, 1973.