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Isoelectric Spectra of Native and Base Denatured Crystallized Swine Pepsin

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Twice crystallized commercial swine pepsin (EC. 3.4.4.1.) has been investigated with respect to heterogeneity and isoelectric point by the method of isoelectric focusing. The enzyme was resolved into three active components, the major component isoelectric at pH 2.20 and two minor components isoelectric at pH 2.82 and 3.06. From the commercial sample, base-denatured pepsin has been prepared and investigated. In this case the protein was resolved into two components, one major component isoelectric at pH 3.47 and a minor component isoelectric at pH 3.85.

The isoelectric pH of the major component of the base-denatured pepsin corresponds to the isoionic pH, calculated on the basis of the number of ionizable groups in the protein and their intrinsic dissociation constants (pK_{int}). It is concluded that the lower isoelectric pH found for the native enzyme is partially due to the presence of some basic groups buried in the interior of the protein molecule, and partially due to combination of the protein with anions which were present in the electrolyte medium where the electrofocusing of the native form of the protein was carried out.

No reliable determination of the isoelectric point of pepsin has been reported. Various authors [1–3] have indicated that the isoelectric point is below pH 3. The work of Tiselius *et al.*[4] showed that the protein migrated as an anion from pH 4.6 down to pH 1. Herriott *et al.*[5] found purified pepsin to be an anion in 0.1 M HCl. From these results, it has been concluded that the isoelectric point of pepsin is less than 1.

Commercial, crystalline swine pepsin preparations often appear heterogeneous. Rajagopalan *et al.*[6] chromatographed several such samples and found all of them to be heterogeneous. Two clearly separated zones of active material and a more diffuse active zone between them were obtained, as well as evidence of heterogeneity by end group analysis.

MATERIALS AND METHODS

Proteins. The pepsin used was twice crystallized swine pepsin (PM 8 JB from Worthington Biochemical Corporation, Freehold, New Jersey, U.S.A.). The native enzyme was dialyzed against distilled water prior to the isoelectric focusing experiments.

For base-denaturation, 10 mg of pepsin was dissolved in 5 ml of 0.1 M phosphate buffer of pH 8.0. A few drops of 1 M NaOH were added to bring the solution to pH 8.6, whereupon the mixture was maintained at 55° for 2 h. The enzyme showed no activity after this procedure. Prior to the isoelectric focusing experiment, the denatured pepsin was dialyzed against a 2 % water solution of carrier ampholytes of pH 4. Dialysis against this solution gave no precipitate, while dialysis against distilled water produced a light precipitate.

Chemicals. To create pH gradients above pH 3, Ampholine carrier ampholytes, 40 % water solutions, were purchased from LKB-Produkter, Box 76, Stockholm Bromma 1, Sweden. In the pH region between 1 and 3, for which commercial carrier ampholytes are not available, the gradients were created by using sulphuric, phosphoric, acetic, citric and formic acid (all of analytical reagent grade), and the ampholytes aspartic acid, glutamic acid, and histidine (*puriss.*). Sucrose analytical reagent grade, from Mallinckrodt, St. Louis, U.S.A., and sorbitol, extra pure for bacteriology, from E. Merck, Darmstadt, Germany, were used for the density gradients.

Electrolysis column. An electrolysis column of 110 ml volume (LKB 8101) from LKB-Produkter was used.

Preparation of the column and isoelectric focusing by electrolysis. When working with natural, stable pH gradients above pH 3, this was essentially done as described earlier.[7] In an earlier work,[8] pH gradients in the region between 1 and 3 have been used and preparation methods have been described by Pettersson.[9]

The protein sample was added to both the dense and the less dense solution and, consequently, the protein was from the start of the experiment evenly distributed throughout the whole column.

The column was thermostatted to +4°C during operation. In order to get pI values at +25°C, the electrolysis was continued, after the steady state was reached, for 4 to 6 h with thermostatted water of +25°C circulating through the cooling mantle. The time required for the focusing was about 60 h for Ampholine carrier ampholyte gradients at about 1.5 W. With acid gradients the focusing is slow. These electrolysis runs were allowed to proceed for about 150 h with about 1.5 W applied.

pH and spectroscopic measurements. UV absorption (280 nm) and pH (+25°C) were measured continuously on the column effluent by means of flow-through cells.[10] For subsequent enzyme activity measurements, fractions were collected manually.

Enzyme activity measurements. Substrate for assay of proteolytic activity was acid-denatured casein. The substrate solution was adjusted to pH 1.9 with HCl solution. The concentration of casein was then 1 %. From each fraction of the column, 200 µl was taken and mixed with 3 ml of this solution. The samples were allowed to stand at +25°C for 10 min. The reaction was terminated by addition of 4 ml of 0.3 M trichloroacetic acid (TCA). The absorbance of the filtrate was determined at 280 nm. Samples where the TCA solution was added before the solution from the column were used as blanks. The increase in absorbancy, using 1 cm cells in the photometer, was used as a measure of pepsin activity.[11]

RESULTS

In a run on a gradient ranging from pH 3 to 5 using Ampholine carrier ampholytes, the native enzyme migrated to a pH below 3. pH gradients between 0 and 3 were used in the following experiments. Fig. 1 shows the enzyme activity, UV absorption, and pH, as functions of the level in the column, in a representative run. Fifteen mg of enzyme was used in this experiment. Three fully resolved active components can be seen, one major component isoelectric at pH 2.20 and two minor components isoelectric at

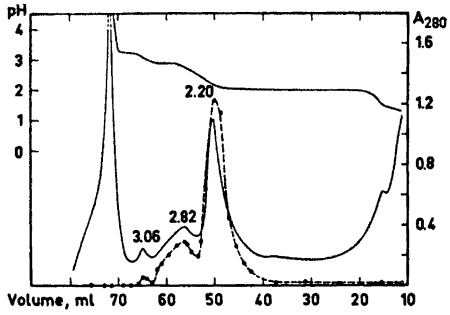

Fig. 1. pH (+25°C) course (upper solid curve), UV absorption (280 nm) course (lower solid curve), and enzyme activity course (- - O - -) of the column effluent after isoelectric separation of twice crystallized commercial swine pepsin. The figures above the UV absorption peaks give the pI values of the active enzyme components. The large peak to the left which shows no enzyme activity is mostly due to an impurity in the material used for creating the pH gradient.

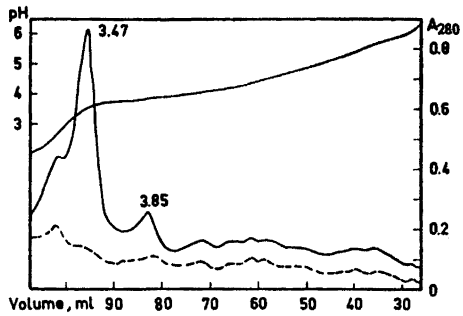

Fig. 2. pH (+25°C) course (upper solid curve), UV absorption (280 nm) course (lower solid curve) of the column effluent after isoelectric separation of base-denatured commercial swine pepsin. The figures above the UV absorption peaks give the pI values of the protein components. The broken curve shows the UV background absorbancy (280 nm) due to the carrier ampholytes. This was obtained by running a blank of the same carrier amphlytes as used in the separation experiment. To make the figure clearer, this curve has been displaced about 0.05 absorbancy units below its real value.

pH 2.82 and 3.06. The large UV absorption peak to the left, which showed no enzyme activity, was also present in an electrolysis experiment without pepsin. The peak is thus due to an impurity in the material used for creating the pH gradient or the density gradient.

As distinguished from the native pepsin, the base-denatured form was focused in an Ampholine gradient ranging between pH 3 and 5. The two solid curves in Fig. 2 show the UV absorption and pH distribution in the column. The broken curve shows the UV background absorbancy due to the carrier ampholytes. This was obtained by running a blank with the same carrier ampholytes as used in the separation experiment. To make the figure clearer, this curve has been displaced 0.05 absorbancy units below its real value. A comparison between the two absorption curves shows that the base-denatured pepsin was resolved into two components, one major component isoelectric at pH 3.47 and a minor component isoelectric at pH 3.85.

DISCUSSION

As was pointed out in the introductory section, it has been suggested that the isoelectric pH of native pepsin is below 1. This is a remarkably low value. As will be shown below, neither this value nor the value, pH 2.20, obtained in this work (for the major component) are consistent with the amino acid composition of the protein.

The amino acid composition of pure pepsin has been determined by Rajagopalan *et al.*[6] From their data, the number of ionic groups can be calculated to 39 side-chain carboxyls, 1 α-carboxyl, and 5 basic groups. According to Perlmann,[12] pepsin contains in addition a phosphate group diester-linked to the protein. This gives a total of 41 acidic groups. Tanford [13] has given the following intrinsic pK values (pK_{int}) for these groups: protein-linked phosphate 1.3, α-carboxyl 3.8, and side-chain carboxyl 4.6. Since pepsin possesses many more acidic than basic groups, it is clear that the isoionic pH (the pH where the average net charge of the molecule is zero if no ions other than protons are bound to it) must be below the pK_{int} value of the side-chain carboxyls. A reasonable assumption is that the most acidic group, the phosphoryl group, is fully or almost fully ionized, and the α-carboxyl, next in acidity, is partially ionized at this pH. Since practically all the basic groups will be charged here, 3 to 4 of the 39 side-chain carboxyls must be dissociated. Since pK_{int} is the intrinsic pK_a when the average net charge of the molecule is zero, the isoionic pH may be calculated from the equation $pH = pK_{int} + \log \alpha/1-\alpha$ with $\alpha = 3/39$ or $4/39$ and $pK_{int} = 4.6$. This gives an isoionic pH between 3.5 and 3.6. Naturally, this kind of calculation is not very accurate, but it may suffice to fix the region of pH in which the isoionic point may be expected to fall.

In the calculation, it was assumed that all the ionic groups in the protein really are free to ionize. This assumption is reasonable for the base-denatured form. According to Clement,[14] the number of titratable carboxyl and basic groups for base-denatured pepsin agreed well with the number obtained from the amino acid composition as given by Rajagopalan *et al.* The pepsin used in the present electrofocusing experiments and that used by Clement were both purchased from Worthington. Clement chromatographed the pepsin and used the fractions of highest absorbance for the titration.

In the electrofocusing experiment where Ampholine carrier ampholytes were used, the isoelectric pH of the base-denatured pepsin (major component) was determined as 3.47. According to arguments presented by Vesterberg and Svensson,[15] an isoelectric pH obtained in such an experiment should be considered as the isoionic pH also. The experimental value thus shows good agreement with the calculated one.

Native pepsin did not focus in an Ampholine pH gradient. The protein collected near the anode at a pH below 3, which shows that it is isoionic below this value. According to Clement, the same number of carboxyls were titrated in native pepsin as in the denatured form. However, pepsin is denatured above pH 6 so it is not possible to determine by titration whether the basic groups are free to ionize in the native form. An isoionic pH lower than the calculated value can be explained if some of the basic groups are "buried". Indications that at least two of these groups are unreactive have been reported in the literature. According to Stark,[16] when pepsin was treated with ethoxyformic anhydride only one ethoxyformyl group was introduced. The site of modification was the α-amino group of isoleucine. However, he postulates that this reagent is expected to react with "exposed" histidine residues, but there was no reaction with the single histidine in pepsin. Perlmann [17] has reported on acetylation of pepsin with acetylimidazole. The type of groups

which may react with this reagent are lysyls, aromatic and aliphatic hydroxyls and histidyls. Perlmann concluded that only tyrosine residues and possibly the α-amino group of the isoleucine residue reacted with the reagent. This indicates that also the lysine residue of pepsin can be situated in the interior of the molecule.

Naturally, it cannot be exluded that an isoionic pH below 3 can also be ascribed to some very acidic carboxyls. Kinetic studies have led Cornish-Bowden [18] to the conclusion that proteolysis by pepsin requires one carboxyl with an apparent pK_a of 1.0. But even if consideration is taken to such a remarkably strong carboxyl, this causes only a minute change in the calculated isoionic pH if all the five basic groups of the protein are free to ionize. If pH 2.20, the pH where the major component of the native enzyme was focused, should represent the isoionic pH, this demands that the side-chain carboxyls on the average have a pK_{int} of 3.2. In that case most of these groups must be unusually strong. It is known that there are proteins with abnormally acidic carboxyls. The number of abnormal groups, however, has been small compared to the total present. It may be significant that these are proteins which have an unusually high content of cationic groups. By electrostatic interaction, the latter might be expected to reduce the pK_{int} of the particular carboxyls to which they are closely proximate. Pepsin has few cationic groups so it is not possible to explain many abnormal carboxyls in this way. That the native enzyme has an isoionic pH below 3, lower than the value obtained for the base-denatured form, is probably mainly due to "buried" basic groups which are not free to ionize.

The conclusion which can be drawn from this work is that native pepsin is isoionic between pH 2.20 and 3. It is true that the enzyme focused at pH 2.20, but this does not necessarily mean that it is isoionic at this pH. The isoelectric and isoionic points coincide only in the absence of ion-binding. If anions are bound, the isoelectric pH will be lower than the isoionic pH. The pH gradient where the enzyme focused was created not by Ampholine carrier ampholytes, but by a system of acids. Thus, small anions were present in the electrolyte medium. It is likely that pepsin has an unusually strong tendency to combine with anions. An indication of this fact was reported by Tiselius *et al.*[4] They found that in the most acid solutions the negative charge of the protein became larger when the ionic strength was increased from 0.02 M to 0.1 M, contrary to the usually observed decrease in electrophoretic mobility with increasing salt concentration. That a decrease in the ionic strength increases the isoelectric point can partially explain why the isoelectric pH obtained in this work lies higher than that obtained by the moving boundary technique. In the moving boundary experiments described in the literature, the ionic strength of the electrolyte medium has been 0.02 or higher. This may be compared with the ionic strength at the point of focusing for the major component (pH 2.20) which was about 0.006.

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REFERENCES

1. Michaelis, L. *Biochem. Z.* **17** (1909) 232.
2. Ringer, W. E. *Hoppe-Seylers Z. physiol. Chem.* **95** (1915) 232.
3. Ågren, G. and Hammarsten, E. *Enzymologia* **4** (1937) 49.
4. Tiselius, A., Henschen, G. E. and Svensson, H. *Biochem. J.* **32** (1938) 1814.
5. Herriott, R. M., Desreux, V. and Northrop, J. H. *J. Gen. Physiol.* **23** (1940).
6. Rajagopalan, T. G., Moore, S. and Stein, W. H. *J. Biol. Chem.* **241** (1966) 4940.
7. Jonsson, M. and Pettersson, E. *Acta Chem. Scand.* **22** (1968) 712.
8. Jonsson, M. and Pettersson, E. *Science Tools* **15** (1968) 2.
9. Pettersson, E. *Acta Chem. Scand.* **23** (1969) 2631.
10. Jonsson, M., Pettersson, E. and Rilbe, H. *Acta Chem. Scand.* **23** (1969) 1553.
11. Anson, M. L. In Northrop, J. H., Kunitz, M. and Herriott, R. M., Eds., *Crystalline Enzymes*, 2nd Ed., Columbia University Press, New York 1948, p. 305.
12. Perlmann, G. E. *Advan. Protein Chem.* **10** (1955) 23.
13. Tanford, C. *Advan. Protein Chem.* **17** (1962) 69.
14. Clement, G. E. *Biochim. Biophys. Acta* **140** (1967) 368.
15. Vesterberg, O. and Svensson, H. *Acta Chem. Scand.* **20** (1966) 820.
16. Stark, G. R. *Advan. Protein Chem.* **24** (1969) 261.
17. Perlmann, G. E. *J. Biol. Chem.* **241** (1966) 153.
18. Cornish-Bowden, A. J. and Knowles, J. R. *Biochem. J.* **113** (1969) 353.

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