Separation of Catheptic Enzymes of Bovine Spleen by Isoelectric Focusing

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Catheptic proteases, which are potentially useful for meat processing, have been resolved and identified in bovine spleen extract by free solution zone convection isoelectric focusing. The isozymes of cathepsins A, B, D, L, and H have been separated, and their isoelectric points have been determined. The method is a very convenient and useful technique for analytical and preparative applications. Advantages over other methods of isoelectric focusing include simplicity of operation, ease of recovery and analysis of separated components, and no interference from proteins precipitating at their isoelectric points.

Recent studies (Robbins and Cohen, 1976; Robbins et al., 1979; Cohen et al., 1979) have demonstrated the potential of spleen extract as an exogenous tenderizer for mammalian muscle. The lysosomal acid proteases, cathepsins, present in these extracts attack myofibrillar proteins and connective tissue elements at post-mortem pH. These enzymes are relatively selective in their action on muscle proteins, and the advantages for their use in meat processing when compared to the less discriminating plant proteases, such as papain and bromelain, have been demonstrated and discussed previously (Robbins et al., 1979). Because bovine spleen is a readily available cheap source of catheptic enzymes for meat processing, we have investigated the types and quantities of various catheptic endopeptidases and exopeptidases present in spleen extract.

The catheptic enzymes of interest to us were the endopeptidase cathepsin D which has been shown to play a major role in the degradation of myofibrillar proteins (Robbins et al., 1979; Schwartz and Bird, 1977), the exopeptidase cathepsin A, which apparently acts synergistically with cathepsin D in degrading proteins (Iodice et al., 1966), and the sulfhydryl endopeptidases, cathepsins B, L, and H (Barrett, 1980; Kirschke et al., 1977). Cathepsins B and L have activity on collagen (Etherington, 1976; Barrett, 1980). Previous work on the catheptic enzymes has shown that each type of enzyme is generally present in a tissue in multiple forms as isozymes differing in their isoelectrical points. For this reason we decided to examine the spleen extract by the method of isoelectric focusing. This paper describes the separation of spleen extract by the method of zone convection isoelectric focusing (Valmet, 1969). This procedure gave a profile of the multiple forms of the catheptic enzymes of interest to us. The results of this work will have value in developing effective methods for obtaining quantities of the purified individual enzymes so that their activities against various muscle protein fractions can be evaluated and possible synergistic effects can be revealed.

EXPERIMENTAL SECTION

Enzyme Assays. Cathepsin D was assayed by the method of Keilova and Tomasek (1976) using acid denatured hemoglobin as the substrate. One unit of enzyme activity represents an increase of 1.00 absorbance unit at 280 nm in 1 min. Cathepsin B was assayed by using benzoylarginine-*p*-nitroanilide (BAPA) as the substrate by the method of Barrett (1972). One unit of enzyme activity represents 1.0 μmol of substrate hydrolyzed in 1 min. endopeptidase activity with azocasein as the substrate was carried out at pH 6.0 by using the conditions described by Kirschke et al. (1977) but modified by using 0.5% substrate rather than 2.5%. One unit of enzyme activity represents an increase of 1.00 absorbance unit at 366 nm in 1 min. Isozymes of cathepsins L and H were delineated from cathepsin B isozymes, which also degrade azocasein, by their higher specific activity on this substrate, relative to their BAPA hydrolase activity (Kirschke et al., 1977). Cathepsin A was assayed by using the conditions described by Logunov and Orekhovich (1972) with (carbobenzoxy)-glutamylphenylalanine as the substrate; the hydrolysis of the substrate to produce phenylalanine was followed by reaction with picrylsulfonic acid, as described by Snyder and Sobocinski (1975). One unit of enzyme activity represents the production of 1.0 μmol of phenylalanine in 1 h.

Gel Filtration. Freeze-dried spleen extract, prepared as previously described (Robbins and Cohen, 1976), was dissolved in 0.05 M acetate–0.15 M sodium chloride, pH 5.5 buffer and fractionated on a column of Sephadex G-100 (2.5 × 90 cm) equilibrated with the same buffer. Fractions containing the proteolytic activities were combined and dialyzed against pH 5.5, 0.005 M acetate buffer prior to isoelectric focusing.

Isoelectric Focusing. Zone convection isoelectric focusing was carried out using an apparatus similar to that described by Talbot and Caie (1975). The apparatus is shown in Figures 1 and 2. For details of its construction, the original paper by Talbot and Caie should be consulted. The apparatus which is constructed of Lucite differs from that of Talbot and Caie in several features; the width of the separation cell was doubled to increase sample capacity from 30 to 60 mL, and it was constructed so that coolant could be circulated through it for controlling temperature; the electrodes were sealed into filter frit so that they do not directly contact the sample.

Separations are carried out with the apparatus set at a 45° angle (Figure 1). In this position, solution in the 36 separation chambers forms a continuous channel between the electrodes. After completion of the separation, the sample is autofractionated into 36 fractions by lowering the apparatus to a horizontal position, where the solutions in the chambers are no longer in contact (Figure 2). pH measurements to determine the pH gradient are made directly in the separation chambers. Focusing was carried out at 4 °C using an LKB Model 2103 power supply (LKB Instruments, Inc., Rockville, MD). Sample size was 60 mL and the samples contained 1% Ampholine (LKB, Rockville, MD). Phosphoric acid (0.3 M) was used at the anode and 0.3 M ethanolamine at the cathode. The initial current was set at 2.5–4 mA. Initial voltages varied from 300 to 550 V. The limiting voltage was set at 1800 V.

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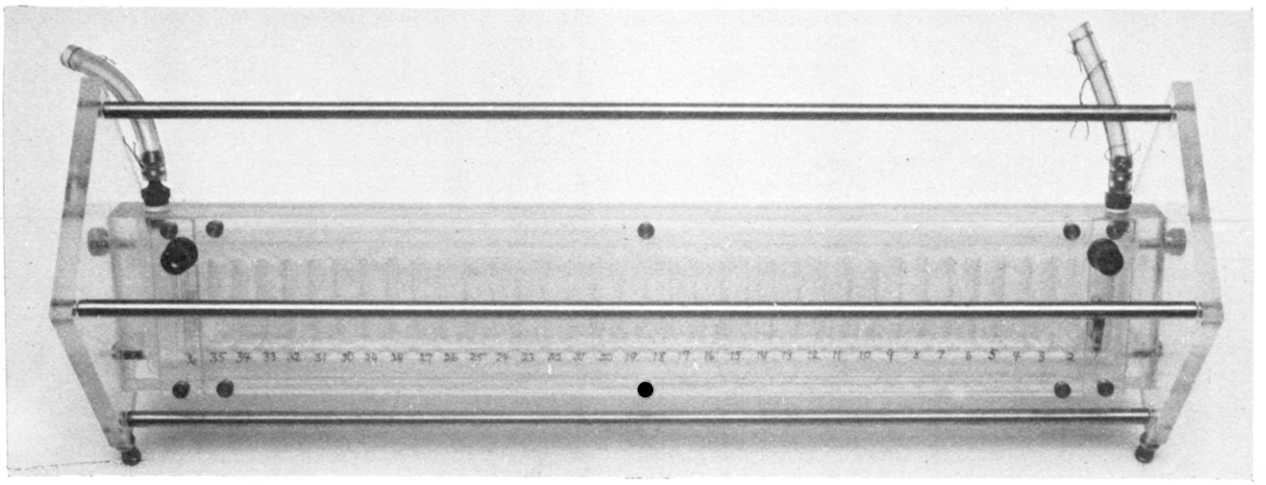

Figure 1. Photograph of the assembled zone convection isoelectric focusing apparatus. The separation cell is set at 45° with a movable metal positioning pin for carrying out the electrophoretic run (see the text).

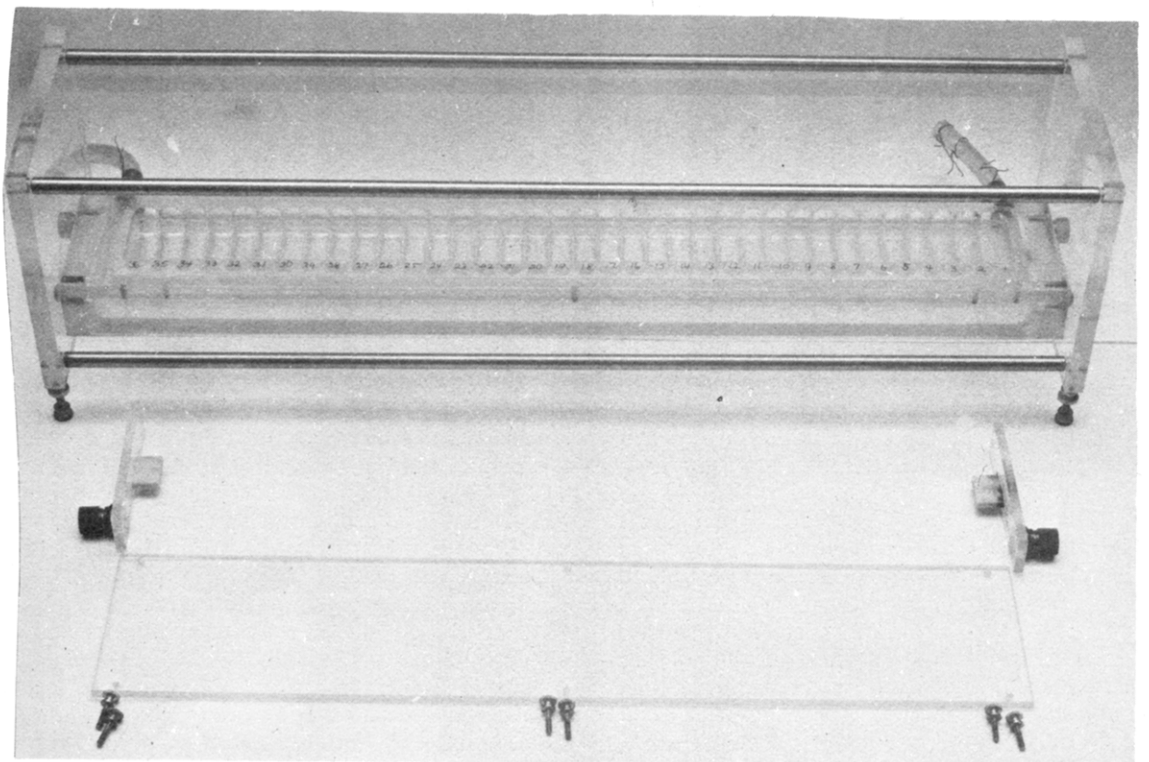

Figure 2. Photograph of the zone convection isoelectric focusing apparatus showing the separation cell in the horizontal position for autofractionation of the focused sample. The electrodes and cover have been removed from the separation cell.

Limiting power was 3 W. Separation times varied from 43 to 66 h.

pH Measurements. At the termination of the separation the sample was autofractionated, and the pH of each fraction was measured at 4 °C by using a Beckman 4500 digital pH meter with a Miramark, microcombination electrode, Markson Science, Inc., Del Mar, CA.

RESULTS AND DISCUSSION

A preliminary isoelectric focusing experiment was carried out with spleen extract using a wide range pH 3.5–10 ampholyte in order to assess the complexity of the catheptic enzyme mixture and to determine the range of their p*I* values.

The results show (Figure 3) the majority of cathepsin D activity focused in two closely spaced peaks having apparent p*I* values of 5.30 and 5.50. There are additional unresolved isozymes which give rise to a shoulder on the high pH side of the major component in the region pH 6–7. Additionally, there are minor components focusing below pH 5 and above pH 7.

Cathepsin A activity separates as a major peak having an apparent p*I* of 5.80. Additional isozymes present give rise to shoulders on the low pH side of the main component. Several minor peaks of cathepsin A activity are also present in the pH range 4–5 and above 6.

The bulk of the cathepsin B activity focuses as a large somewhat asymetric peak centered at pH 5.10. The broadness of the peak suggests the presence of several isozymes with p*I* values in the range 4–6.

Only minor amounts of BAPA hydrolase activity are observed above pH 6 in the pH range 6–9. This minor activity is apparently due to isozymes of cathepsins L and H, which focus in this range and have only weak BAPA hydrolase activity. These sulfydryl proteases have relatively high endopeptidase activity on protein substrates such as azocasein (Kirschke et al., 1977), however, and can be distinguished from cathepsin B isozymes by their higher

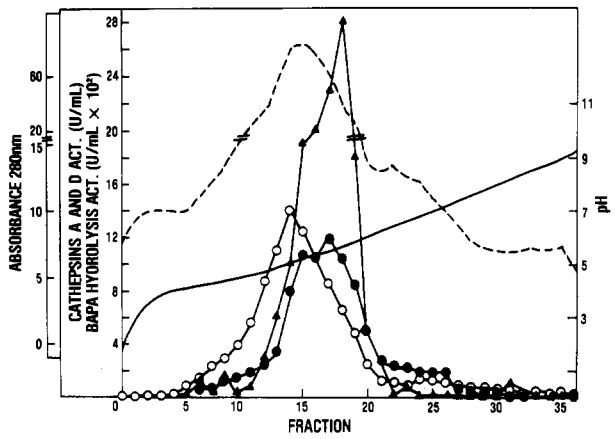

Figure 3. Isoelectric focusing of spleen extract in the pH 3.5–10 ampholyte. Sample contains 700 mg of protein. Separation conditions are as described in the text. Separation time was 43 h. Absorbance at 280 nm (---); cathepsin D activity (●); cathepsin A activity (▲); BAPA hydrolysis activity (○); pH gradient (—).

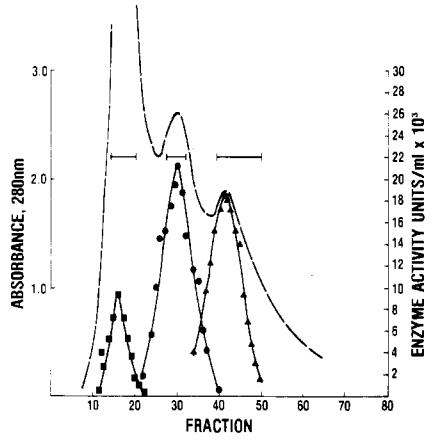

Figure 4. Gel filtration of spleen extract on a Sephadex G-100 column. A 12-mL sample containing 384 units of cathepsin A activity, 223 units of cathepsin D activity, and 1.8 units of BAPA hydrolysis activity; protein, 1.2 g. Separation conditions are as described in the text. Fraction volume, 4 mL. Absorbance at 280 nm (---); cathepsin A activity (■); cathepsin D activity (●); BAPA hydrolysis activity (▲). The bars indicate the fractions containing the various enzymatic activities which were combined for separation by isoelectric focusing.

ratio of endopeptidase activity to BAPA hydrolase activity (see below).

The results of the focusing experiment show that the majority of the catheptic enzymes of interest to us have isoelectric points spanning the pH range from 5 to 7 and suggest that optimum resolution of them could be obtained by focusing in a narrow pH gradient spanning this range.

Gel Filtration. Preliminary to isoelectric focusing in a narrow pH gradient, an initial separation of the catheptic enzymes into classes was carried out on Sephadex G-100 to take advantage of differences in their molecular weights (Figure 4). The elution volume of cathepsin A corresponds to a molecular weight of about 85 000. Cathepsin D elutes with a molecular weight of 45 000–50 000, and cathepsin B and other thiol proteases with BAPA hydrolase activity elute together with an elution volume corresponding to molecular weights in the range of 20 000–25 000. The fractions containing the desired enzymatic activity were combined as shown in Figure 4 and dialyzed against 0.005 M acetate, pH 5 buffer prior to separation by isoelectric focusing.

The combined fractions from the gel filtration step were each focused in a pH gradient prepared from pH 5 to pH 7 ampholyte, and the results of typical separations are described below. The p*I* values of major isozymes found for each catheptic enzyme are summarized in Table I.

Table I. p*I* Values of Catheptic Enzymes Separated from Bovine Spleen

enzyme	p*I*[a]
cathepsin D[a]	5.24-5.25, 5.40-5.50, 5.60-5.63, 5.75-5.80, 6.37-6.40, 6.55-6.65, 6.75-6.80
cathepsin A	5.16 ± 0.06, 5.34 ± 0.04, 5.68 ± 0.02, 5.90 ± 0.06
cathepsin B	4.90 ± 0.05, 5.11 ± 0.06, 5.23 ± 0.02, 5.34 ± 0.02
cathepsins L and H	6.09 ± 0.09, 6.26 ± 0.07, 6.44 ± 0.04, 6.75 ± 0.05, 8.60 ± 0.24

[a] Spread of isoelectric points for each isozyme from two separations (cathepsin D). Mean isoelectric points and standard error of isozymes of cathepsin A (three separations) and cathepsin B and cathepsins L and H (four separations).

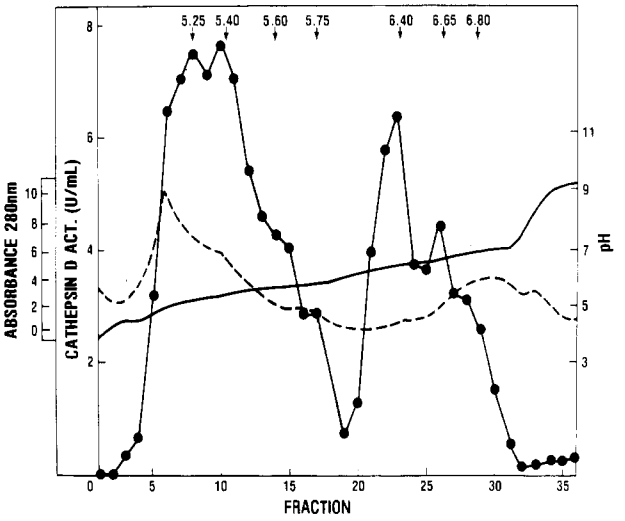

Figure 5. Isoelectric focusing of the Sephadex G-100, cathepsin D fraction in the pH 5–7 ampholyte. Sample: 140 mg of protein containing 169 units of cathepsin D activity. Separation conditions are described in the text. Separation time was 66 h. Absorbance at 280 nm (---); cathepsin D activity (●); pH gradient (—). The position of the cathepsin D isozymes and their p*I* values are indicated by the arrows.

Isoelectric Focusing of Cathepsin D. The separation pattern of cathepsin D is shown in Figure 5. There are at least seven isozymes of cathepsin D present with p*I* values spanning the range from 5.25 to 6.80. This observation is in keeping with the results of Press et al. (1960), who reported the presence of at least 10 isozymes of cathepsin D in bovine spleen. These p*I* values fall within the range reported by other investigators. Smith and Turk (1974) have isolated three isozymes from bovine spleen with p*I* values of 5.6, 5.9, and 6.4, and Ferguson et al. (1973) reported values of 6.1, 6.3, and 6.7. Huang et al. (1980) reported values of 6.49 and 6.04 for the bovine spleen isozymes.

Isoelectric Focusing of Cathepsin A. The separation pattern of cathepsin A isozymes shows (Figure 6) the majority of activity between pH 5 and pH 6 as four isozymes having p*I* values of 5.15, 5.30, 5.70, and 5.90. Smaller amounts of cathepsin A activity focus between pH 3–4 and pH 6.5–7.5. Longunov and Orekhin (1972) have reported the isolation of an isozyme of cathepsin A from bovine spleen with a p*I* value of 5–5.2.

Isoelectric Focusing of Cathepsin B. The isozymes of cathepsin B, identified by their BAPA hydrolase ac-

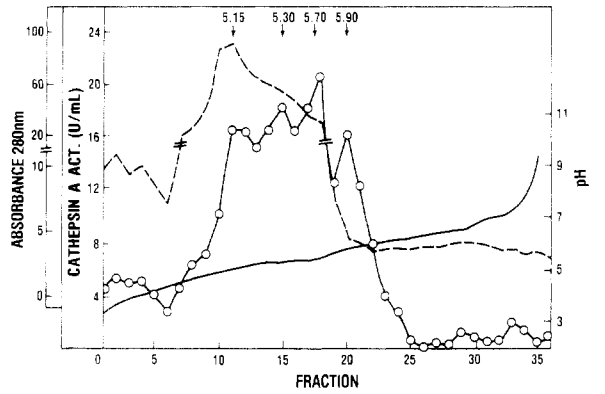

Figure 6. Isoelectric focusing of the Sephadex G-100, cathepsin A fraction in the pH 5–7 ampholyte. Sample: 220 mg of protein containing 200 units of cathepsin A activity. Separation conditions are described in the text. Separation time was 48 h. Absorbance at 280 nm (---); cathepsin A activity (O); pH gradient (—). The position of the cathepsin A isozymes and their p*I* values are indicated by the arrows.

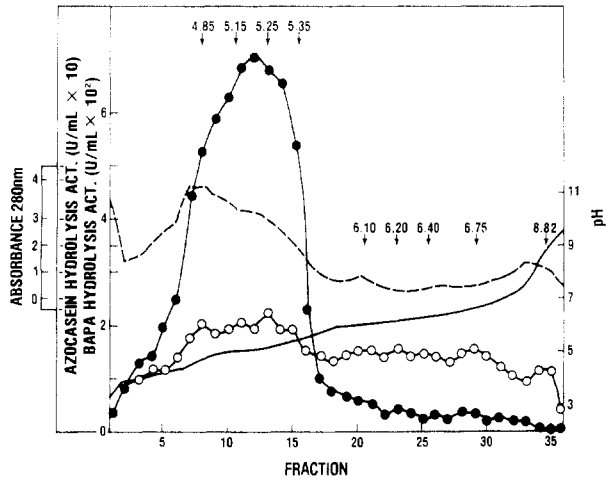

Figure 7. Isoelectric focusing of the Sephadex G-100, BAPA hydrolysis fraction in the pH 5–7 ampholyte. Sample: 125 mg of protein containing 1.2 units of BAPA hydrolysis activity. Separation conditions are as described in the text. Separation time was 45 h. Absorbance at 280 nm (---); BAPA hydrolysis activity (●); endopeptidase activity on azocasein (see the text) (O); pH gradient (—). The position of the cathepsin B isozymes' p*I* range (4.85–5.35) and the cathepsin L and H isozymes' p*I* range (6.10–8.82) is indicated by the arrows.

tivity, focus in the pH range 4.9–5.3 as a broad peak with shoulders on each side (Figure 7). The azocasein activity curve indicates the presence of at least four isozymes of cathepsin B. Etherington (1976) has also reported the presence of a major isozyme of the bovine spleen enzyme with a p*I* value of 4.9; a second isozyme with a p*I* value apparently higher than 4.9 (exact value not given) was also identified.

Isoelectric Focusing of Cathepsins L and H. The sulfhydryl endopeptidases with a high ratio of endopeptidase to BAPA hydrolase activity are of the cathepsin L and H type (Kirschke et al., 1977); in this study no attempt was made to differentiate between the isozymes of these enzymes. There are at least five isozymes of this type present in the spleen extract with p*I* values spanning the range 6.10–8.82 (Figure 7). There are no previous reports of the p*I* values for the isozymes of cathepsin L or cathepsin H in bovine spleen, but the p*I* values found here are close to those reported for the isozymes of rat liver cathepsin L, 5.8–6.1 (Kirschke et al., 1977), and human liver cathepsin H, 6.0 and 6.4 (Schwartz and Barrett, 1980). Etherington (1976) and Ducastaing and Etherington (1978), however, have reported the isolation and purification of an acid sulfhydryl protease from bovine spleen with activity on collagen. This enzyme, formerly known as collagenolytic cathepsin (Etherington, 1976), and recently renamed cathepsin N (Ducastaing and Etherington, 1978), has a p*I* value of 6.55, and Barrett (1980) has suggested that this protease is identical with cathepsin L.

Recovery of Enzymatic Activity. The recovery of enzymatic activity after isoelectric focusing of each type of catheptic enzyme exceeded 100% based on the apparent activity present before focusing. In the case of cathepsin B and D, the recoveries were 104% and 113%, respectively; 218% of the cathepsin A activity was recovered. The recovery of cathepsin L plus cathepsin H activity was not determined. these results indicate that catheptic enzyme inhibitors reported to be present in spleen tissue (Smith and Turk, 1974; Lenney et al., 1979) are separated from the catheptic enzymes during isoelectric focusing, giving rise to an increase in enzymatic activity.

The data obtained from the present study have been useful in designing rational separation schemes for the various catheptic enzymes and their isozymes in bovine spleen based on ion exchange methods and preparative isoelectric focusing. These methods, coupled with gel filtration and affinity chromatography, can provide purified proteases for characterization work and for studies of their reactivity on purified muscle fractions.

This work has demonstrated that the method of free solution isoelectric focusing utilizing an apparatus of the type described by Talbot and Caie (1975) can provide important information on the distribution of proteins and enzymatic activities in relatively crude biological extracts. The apparatus serves simultaneously as an analytical and preparative tool capable of handling gram quantities of protein mixtures. The method has several advantages over other methods of isoelectric focusing which employ supporting media or density gradients, including simplicity of operation, ease of recovery and analysis of separated components, high recoveries of separated components, and no interference from proteins precipitating at their isoelectric points. In addition, prefocusing of pH gradients and insertion of samples at specific pH values in the gradient after focusing are facilitated.

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Chemical Phosphorylation of Bovine β-Lactoglobulin

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Bovine β-lactoglobulin was phosphorylated with phosphorus oxychloride ($POCl_3$) at pH 8.5 to give a product that contained up to 14 mol of phosphorus/mol of protein. The acid lability of the phosphate residues and the ^{31}P NMR spectral data suggest that the protein lysine and histidine residues had been phosphorylated. Some dimerization of the protein also occurred. Circular dichroism showed that phosphorylation disrupted the native structure of the protein but did not denature it completely.

Whey, the principal byproduct of cheese making, is a fluid which contains 4–5% lactose, some mineral salts, and about 0.5% protein. The protein material can be separated from the whey and is a valuable source of essential amino acids such as lysine. It is soluble and stable between pH 2.5 and pH 10 and is able to form foams and gels under appropriate conditions (Marshall, 1979). It would be desirable, however, to extend the range of functional properties of whey protein, and this study was undertaken to explore the effects of phosphorylation on the major whey protein β-lactoglobulin as a possible prelude to the examination of phosphorylated whey protein.

Nonspecific protein phosphorylation can be accomplished with inexpensive reagents such as phosphoryl chloride ($POCl_3$) or phosphorus pentoxide dissolved in phosphoric acid, and several such artificial phosphoproteins have been studied (Heidelberger et al., 1941; Mayer and Heidelberger, 1946; Ferrel et al., 1948; Salák et al., 1965; Willmitzer and Wagner, 1975). The earlier studies indicated that the phosphate could become attached to amino nitrogen such as in lysine or to hydroxyl oxygen, as in serine, and that the properties of the phosphoprotein were not simply an extrapolation of those of the original protein to take account of the increased negative charge.

EXPERIMENTAL SECTION

Phosphorylation. Purified β-lactoglobulin (3× crystallized; Sigma Chemical Co., St. Louis, MO) was dissolved to give a 1% solution. Phosphoryl chloride ($POCl_3$) was added as small aliquots over various periods of time with vigorous stirring at 25 °C while maintaining the pH at 8.5 by the dropwise addition of 5 M NaOH. The $POCl_3$ was added in three different forms: (1) dissolved in light mineral oil, (2) dissolved in CCl_4, and (3) neat with no dispersing solvent. After the addition of all the $POCl_3$, the aqueous phase was separated from the organic solvent, where necessary, and dialyzed against 0.9% (0.15 M) NaCl and against two changes of deionized water before being freeze-dried. Samples of this freeze-dried material were further purified by dissolving them in water and chromatographing them on a 2.5 × 30 cm column of Sephadex G-25 in 0.10 M NaCl at a flow rate of 0.7 mL/min. The column effluent was fractionated, and the 280-nm absorbances and phosphate contents of selected fractions were determined. The fractions containing the protein peak were bulked, dialyzed and freeze-dried, and examined further or frozen. A control reaction involved adding 10% $POCl_3$ dissolved in CCl_4 into the aqueous system without protein for 4 h to achieve hydrolysis and/or polymerization of the $POCl_3$. The protein was then added, and the mixture was allowed to stand overnight. Subsequently, the control protein was purified as above for the phosphorylated protein. This control measured any electrostatic interactions between any polyphosphates that might be formed and the protein.

Poly-L-lysine (VII-B; 40 000 molecular weight; Sigma, St. Louis, MO) was phosphorylated and purified by the above procedure using the direct addition method at a $POCl_3$:substrate molar ratio of 4000:1.

Phosphoprotein Hydrolysis in Solution. Purified phosphoprotein samples were dissolved in water (5 mg in 0.5 mL), and the pH was adjusted to 1.5, 4.5, 7.5, or 10.5 with 1 M NaOH or HCl. Each of these solutions was held at 37 °C for 24 h, the pH was then adjusted to 7.5, and they were dialyzed twice against 1-L changes of 0.05 M Tris buffer at pH 7.5 in 24 h. The samples then had their protein and phosphate contents determined by absorbance at 280 nm ($\epsilon_{1\%}$ = 9.4; Tanford and Nozaki, 1959) and sample digestion in 60% $HClO_4$ followed by phosphate analysis (King, 1952), respectively.

A second and third set of experiments included an intestinal alkaline phosphatase, EC 3.1.3.1 (Type VII, Sigma,

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