Fractionation of Peptides in Protease Digests of Proteins by Preparative Isoelectric Focusing in the Absence of Added Ampholyte: A Biocompatible and Low-Cost Approach Referred to as Autofocusing

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Peptides in the protease digests of some acidic and basic proteins in the range of 0.1−1.0% (w/v) could be fractionated with high reproducibility by a preparative isoelectric focusing on the basis of the amphoteric nature of samples in the absence of added carrier ampholytes. Enrichment of peptides of up to 15-fold could be achieved by the present method. This technique, referred to as autofocusing, did not require or yield any toxic solvents or solutes; thus, the present approach can be a low-cost and biocompatible method for peptide fractionation.

Keywords: *Peptide; peptide fractionation; preparative isoelectric focusing; electrophoresis*

INTRODUCTION

Recently, peptides from partial enzymic and chemical hydrolysates of food proteins have been prepared and tried as food ingredients because of their high nutritional value and beneficial biological activities such as inhibitory activity against angiotensin converting enzyme, opioid activity, antibacterial activity, and enhancement of intestinal absorption of calcium [see reviews by Mills et al. (1992) and Ito (1994)]. For purification and characterization of the peptides in hydrolysates of proteins in laboratory scale, liquid chromatography (LC), especially high-performance liquid chromatography (HPLC), has been used extensively (Mant et al., 1992). However, the high cost of preparative LC apparatus and chemicals has been a stumbling block to the use of LC to prepare peptides in sufficient quantity to examine their values in vivo. In addition, acetonitrile, methanol, and trifluoroacetic acid, which have been extensively used for peptide preparation by LC systems, are undesirable solvents for food processing because of their toxicity. Therefore, a low-cost and biocompatible approach for peptide fractionation has been demanded. In the present paper, we demonstrate that peptides in the protease digests of some acidic and basic proteins can be fractionated by a preparative matrix-free isoelectric focusing based on the amphoteric nature of the sample itself in spite of the absence of added ampholyte. This approach will be referred to as autofocusing hereafter.

MATERIALS AND METHODS

Materials. Egg white lysozyme (salt free), porcine pepsin, and bovine trypsin (TPCK treated) were obtained from Sigma (St. Louis, MO). Ovalbumin (Nacalai tesque, Kyoto, Japan) was dialyzed against distilled water and then denatured by heating in a boiling water bath before tryptic digestion. Crude egg white lysozyme (chloride form) was obtained from Tokyokasei (Tokyo, Japan) and further purified with an anion exchange chromatography by using a column packed with DEAE-Toyopearl 650 (C) (Tosoh, Tokyo, Japan) to remove protease inhibitor, if present. The crude lysozyme chloride, which was dissolved in 0.02 M sodium phosphate buffer, pH 8.0, followed by dialysis against the same buffer, was applied to the column equilibrated with the same buffer. The unadsorbed fraction eluting with the buffer was collected and dialyzed against water and used for further experiments. Whey protein concentrate from cow's milk was a kind gift from Meiji Milk Products (Tokyo, Japan). Hammarsten casein and trifluoroacetic acid (TFA) were purchased from Merck (Darmstadt, Germany). Acetonitrile (HPLC grade) was purchased from Nacalai tesque.

Table 1. Voltage Values[a] Developed by Autofocusing of Protease Digests of Proteins

substrate protein	concn (%)	starting voltage (V)	terminal voltage (V)
lysozyme chloride	0.68	440	1116
	0.3	371	1540
	0.1	958	2795
lysozyme	1.0	741	2245
	0.5	1293	2632
ovalbumin	1.0	775	2337
	0.1	1469	1891
casein	1.0	515	1106
whey[b]	1.0	422 ± 96	1060 ± 69

[a] At constant power of 12 W. [b] $n = 8$.

Protease Digestion. Casein, lysozymes, and denatured ovalbumin were dissolved in distilled water; 1 M NaOH was added to give pH 8.0, and the proteins were digested overnight with trypsin at an enzyme to substrate ratio of 2−3:100 (w/w) at 37 °C. The protease digestion was terminated by heating the mixture in a boiling water bath for 15 min. Whey protein concentrate was dissolved in distilled water, adjusted to pH 3.0 by adding 1 M HCl, and digested overnight with pepsin at 37 °C at the same enzyme/substrate ratio as stated above. The reaction was terminated by heating, and then the digest was neutralized by adding 1 M NaOH before focusing. In some cases, the digests were clarified by centrifugation at 8000*g* for 30 min and/or by filtration through a cellulose acetate membrane (3.0 μm, Advantec, Tokyo, Japan).

Preparative Isoelectric Focusing. Preparative isoelectric focusing of protease digests of proteins was performed in the autofocusing mode by using a Rotofor (Bio-Rad, Richmond,

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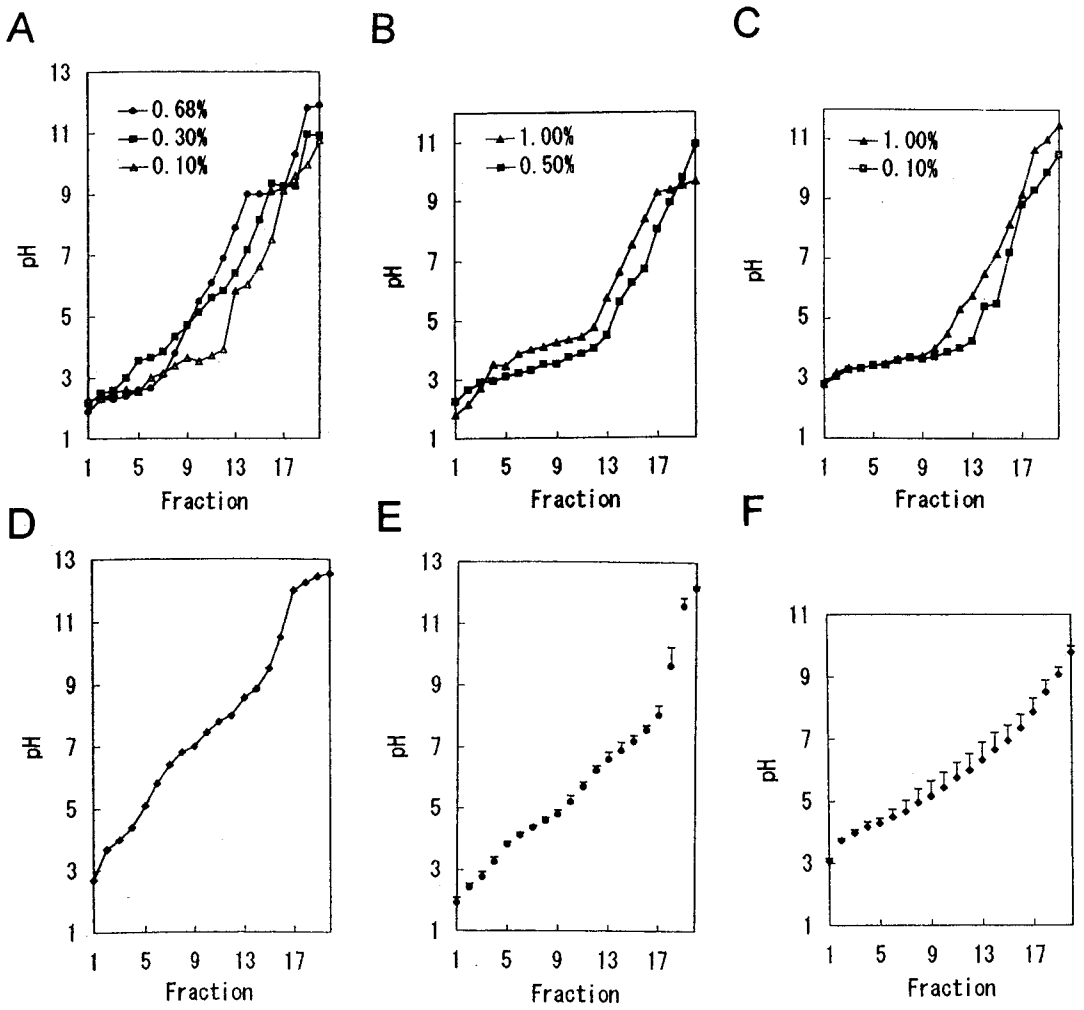

Figure 1. pH gradients formed by autofocusing of peptides in the protease digests and by normal isoelectric focusing with Bio-Lyte 3/10: (A) tryptic digest of lysozyme chloride; (B) tryptic digest of lysozyme; (C) tryptic digest of ovalbumin; (D) tryptic digest of 1.0% casein; (E) peptic digest of 1.0% whey protein concentrate; (F) 2.0% Bio-Lyte 3/10. Bars in E and F represent SD of the pH value ($n = 8$ and 6, respectively).

CA) according to the instruction manual in the absence of chemically synthesized carrier ampholyte mixture. The run was done at 12 W constant power for 2−2.5 h. After the run, the sample was recovered into 20 tubes. The fraction in which precipitation occurred by the autofocusing was neutralized with 1 M NaOH or HCl to dissolve the precipitate. For comparison, a chemically synthesized ampholyte (Bio-Lyte 3/10, Bio-Rad) was used for development of pH gradient by the normal isoelectric focusing mode according to the instruction manual of the instrument.

Analytical HPLC. Aliquots of the autofocusing fractions were analyzed with reversed-phase HPLC using a Cosmosil AR 5C18-300 column (50 mm × 4.6 mm i.d., Nacalai tesque). Elution was performed with a linear gradient from 10 to 45% acetonitorile in water in the presence of 0.1% TFA over 30 min at 1 mL/min. Absorbance at 214 nm was monitored. The column was maintained at 43 °C. The HPLC apparatus consisted of a Shimadzu (Kyoto, Japan) LC-9A pump, an FCV-9AL gradient valve unit, a DGU-2 degasser unit, an SPD-6AV variable-wavelength detector, and a Tosoh AS-8010 autoinjector. The data were processed by an AI-450 work station (Dionex, Sunnyvale, CA).

Amino Acid Analysis. Recovery of peptide by autofocusing was evaluated with amino acid analysis. The samples were hydrolyzed with vapor HCl at 150 °C and derivatized with phenyl isothiocyanate according to the method of Bidlingmeyer et al. (1984) with a slight modification (Sato et al., 1992). Resultant phenylthiocarbamyl amino acids were separated by a Supersphare RP18 (e) column (Merck) at 0.8 mL/min according to the binary gradient elution as described previously (Sato et al., 1992).

RESULTS

Voltage Developed by Autofocusing of Protease Digests of Proteins. As shown in Table 1, terminal voltage values developed by the autofocusing of peptides ranged approximately from 1000 to 2800 V and depended on the species and concentration of substrate proteins. The following analyses with HPLC will reveal that the fractionation of peptides occurs for all samples examined.

Formation of pH Gradient. As shown in Figure 1, pH gradients approximately between pH 2.0 and 12.0 were formed by the autofocusing of tryptic digests of lysozymes, ovalbumin, and casein and peptic digests of whey protein concentrate, whereas isoelectric points of the substrate proteins were distributed between 4.5 (ovalbumin) and 10.7 (lysozyme). The reproducibility of pH gradient formation by the autofocusing of the peptic digest of 1.0% whey protein was comparable to that by the normal isoelectric focusing mode by using isoelectric focusing reagents, the example Bio-Lyte 3/10, in the criteria of SD values for pH of each fraction (E and F in Figure 1). However, the pH gradient patterns were somewhat varied with the concentration and origin of substrate proteins.

Fractionation of Peptides by Autofocusing. As shown in Figure 2, the elution patterns of peptides by the reversed-phase HPLC were different among the

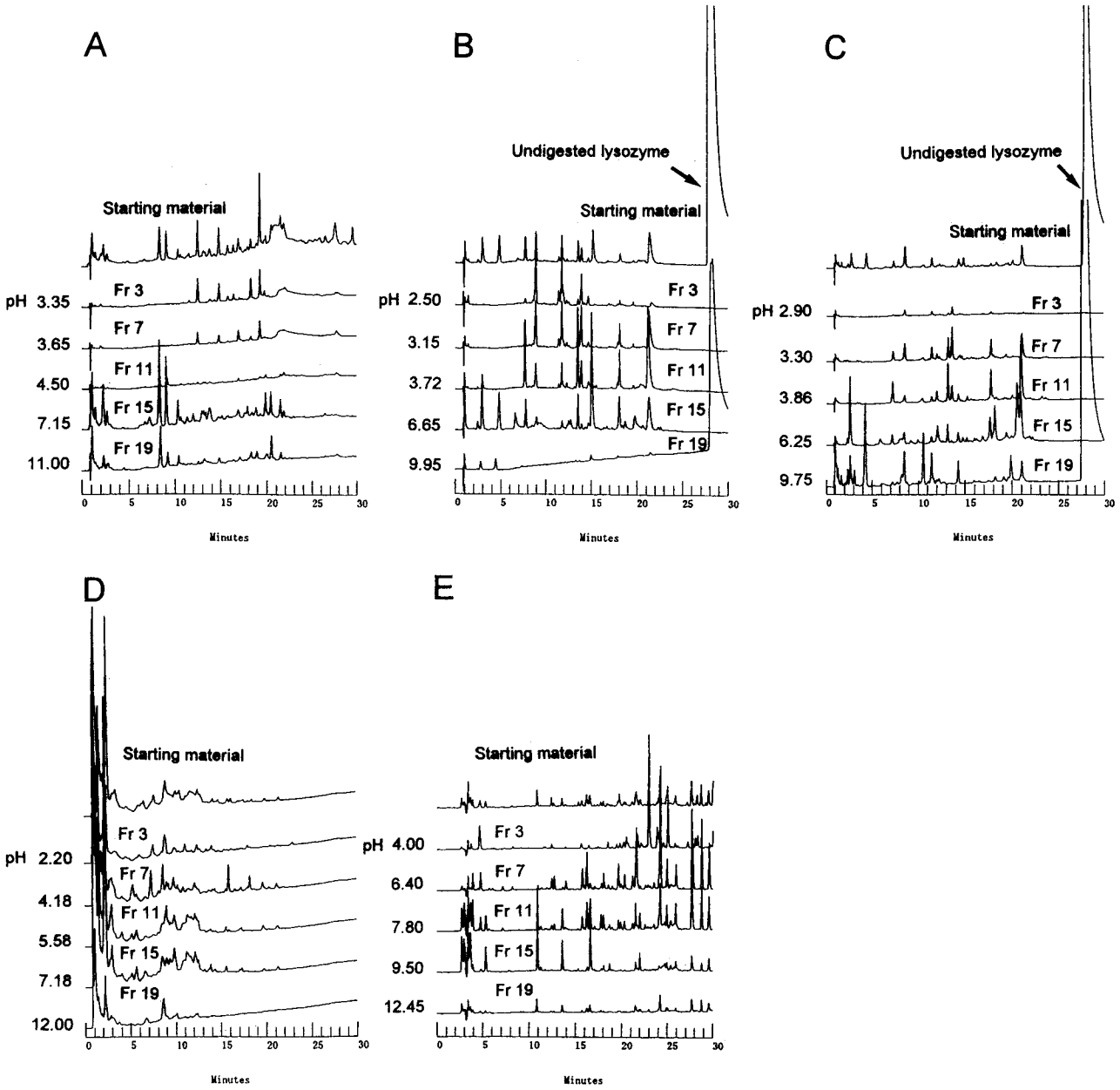

Figure 2. Analysis of the autofocusing fractions with reversed-phase HPLC: (A) tryptic digest of 1.0% ovalbumin; (B) tryptic digest of 0.1% lysozyme chloride; (C) tryptic digest of 0.5% lysozyme; (D) peptic digest of 1.0% whey protein concentrate; (E) tryptic digest of 1.0% casein. Undigested lysozymes in the chromatograms (B, C) were identified by SDS−PAGE according to the method of Laemmli (1970) (data not shown).

autofocusing fractions having different pH values, indicating that fractionation of peptides occurred. The HPLC analysis also demonstrated that an enrichment of peptide constituents occurred by the autofocusing on the basis of peak area of peptides up to 15-fold. As described above, the pH gradient patterns by the autofocusing were somewhat varried with the concentration of sample. As shown in Figure 3, the elution pattern of peptides in the autofocusing fraction was essentially the same as that of the fraction with the corresponding pH value from different concentrations of the same sample. Sufficiently reproducible fractionation of peptides can be achieved if similar pH fractions are collected.

During the autofocusing of the tryptic digest of ovalbumin, extensive precipitation was observed in most fractions (fractions 2−14). In this case, recovery of peptides, which was estimated on the basis of amino acid content in hydrolysates, was less than 40%. In the other cases, the recovery was greater than 70%.

The undigested lysozyme in the tryptic digest was focused into the fractions having pH values corresponding to the p*I* of lysozyme (10.7) and separated from most of its digest, indicating that proteins can also be fractionated with pH gradient formed by the autofocusing of peptides (Figure 2B,C).

DISCUSSION

Recently isoelectric focusing in free solution has been developed for fractionation and purification of proteins (Egen et al., 1988; Roman and Brown, 1994). This technique has been based on the formation of a pH gradient or window by the added carrier ampholytes. However, the commercially available carrier ampholytes have not been fully characterized chemically. Alternatively, acrylamide and its derivative have been used to immobilize the pH gradient (Righetti et al., 1989). The unpolymerized acrylamide and its derivative in the gel matrix are, however, potential toxic substances. These

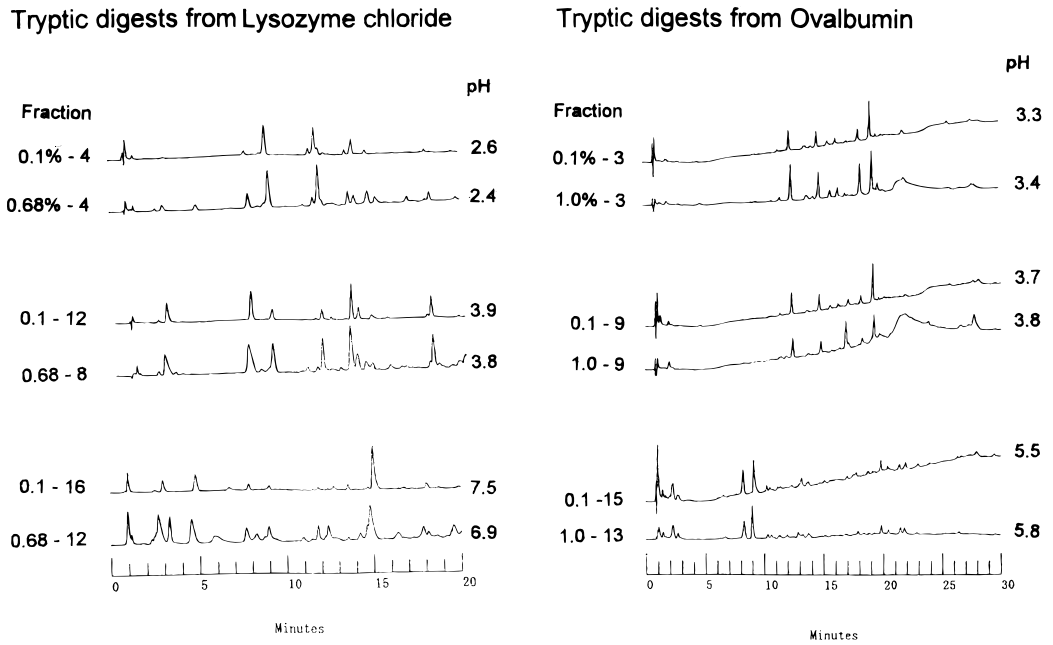

Figure 3. Comparison of elution patterns of peptides in the autofocusing fractions with corresponding pH from the same substrate protein of different concentrations. Tryptic digest of 0.1% ovalbumin was prepared by diluting tryptic digest of 1.0% ovalbumin. Tryptic digests of 0.1% and 0.68% lysozyme chloride were prepared by the independent digestion.

undesirable and expensive materials should (must) be removed completely from the final products when the products are to be used for biological assay and especially for food ingredients. The present study demonstrates that peptide mixtures can be fractionated on the basis of their amphoteric nature. The present approach, referred to as autofocusing, requires only 0.1 M NaOH and phosphoric acid in the electrode chambers and does not yield any toxic materials. Together with these facts, the present autofocusing is a low-cost and biocompatible approach for peptide fractionation. Its low running cost and biocompatibility are of advantage over HPLC system in some cases. Further study on the bioavailability of peptides prepared according to the present method for rat is now in progress.

Recently, a large-scale focusing cell (4−100 L) has been developed (Radola, 1993). If the autofocusing occurs in such a large cell, this technique would be useful for the preparation of peptides for food ingredients. In addition, we also expect that the present approach is useful for upstream fractionation of peptides for analytical purposes.

Reproducibility of pH gradient formation by the autofocusing of protease digest of proteins is comparable to that by commercially available carrier ampholytes, which has been used for the preparaton of protein. The protease digest of food protein could be used as a biocompatible carrier ampholyte for protein fractionation.

In some cases, the recovery of peptides by autofocusing was below 40%. Possibly some peptides, especially ones precipitated during focusing, remained in the membrane core which separates the focusing chambers of the Rotofor. Some instrumental improvement may be necessary to overcome this fault.

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