Development of a Large-Scale (50 L) Apparatus for Ampholyte-free Isoelectric Focusing (Autofocusing) of Peptides in Enzymatic Hydrolysates of Food Proteins

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It has been demonstrated that peptides in enzymatic hydrolysates of proteins can be fractionated on the basis of the amphoteric nature of the sample peptides, by a laboratory-scale isoelectric focusing apparatus, without adding a chemically synthesized carrier ampholyte. This approach is referred to as autofocusing. In the present study, a large-scale (up to 50 L) autofocusing apparatus was developed and tested. A tank (125 cm \( \times \) 25 cm \( \times \) 20 cm) was divided into 12 compartments by 11 plates, each with a window covered in a thin agarose gel layer supported by a nylon screen (100 mesh). The compartments at both ends were filled with 0.1 N phosphoric acid (anode) and 0.1 N NaOH (cathode), respectively, functioning as electrode compartments. The remaining compartments were used for sample compartments. Autofocusing was carried out at constant voltage according to two different methods. In method 1, all sample compartments were filled with a 1% water solution of casein or milk whey protein hydrolysates. In method 2, two compartments located in the center of the tank were filled with 5% sample solution and the others were filled with deionized water. Compositional and sequence analyses of the autofocusing fractions revealed that peptides in the two hydrolysates can be fractionated within 24 h by the present apparatus. Better fractionation was obtained by method 2, whereas enrichment of some peptides occurred by using method 1.

KEYWORDS: Autofocusing; peptide fractionation; preparative isoelectric focusing; electrophoresis; functional foods; peptide; food

INTRODUCTION

Numerous beneficial biological properties regarding human health, such as the enhancement of intestinal adsorption of calcium, growth of lactic acid bacteria in the gut, and moderation of hypertension and hypercholesterolaemia, have been found in peptides derived from food proteins (1–8). The emerging market for nutraceuticals and functional foods has stimulated the production of enzymatic hydrolysates of food proteins as potential sources for active peptides.

Apparent biological activities of peptides in food protein hydrolysates have been frequently detected by in vitro assays using cell culture systems, enzymatic reaction mixtures, and similar methods. Some of the peptides with in vitro activity, however, might be degraded in the digestive system and consequently lose their in vitro activity. Alternatively, some of the inactive peptides detected by the in vitro assay might be converted to an active form by limited digestion in digestive tracts, blood, and so on. Therefore, the activity of peptides shown by the in vitro assays cannot be directly linked to beneficial activity for humans when orally ingested. Thus, the potential activity of the peptides must be evaluated through feeding experiments.

Liquid chromatography, especially reversed-phase (RP) mode, has been extensively used for the fractionation of peptides on a laboratory scale (9). This system allows a high resolution of peptides in a short time. In some cases, liquid chromatography has been used for industrial fractionation of peptides to prepare functional food ingredients (7, 10, 11). However, liquid chromatography is a relatively expensive system for the large-scale preparation of peptides to be used in feeding experiments, and some solvents used in liquid chromatography are harmful to animals and humans. Therefore, crude enzymatic hydrolysates of protein prepared by marginally selective procedures, such as selective precipitation and filtration techniques, have been frequently used in feeding experiments. It has been difficult to identify peptides with in vivo activity. To remedy this situation, a large-scale, low-cost, and biocompatible procedure for peptide fractionation has been demanded.

We have demonstrated that peptides can be fractionated on the basis of the amphoteric nature of the sample peptides dissolved in water using a laboratory-scale preparative isoelectric focusing apparatus (12, 13). This approach is referred to as autofocusing, which has advantages in cost and biocompatibility.

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over liquid chromatography because it does not require chemically synthesized ampholytes and organic solvents.

The objective of the present study is to develop a large-scale autofocusing apparatus that can process up to 50 L at one time, to demonstrate that autofocusing can be used for the fractionation of peptides required for animal experiments and small-scale human trials.

MATERIALS AND METHODS

Materials. An enzymatic hydrolysate of casein (CE90GMM) was commercially obtained from DMV Japan (Tokyo, Japan). This preparation consists of peptides with an average molecular weight of 640 Da and free amino acids up to 15%. A milk whey protein hydrolysate was a generous gift from Meiji Milk Products (Tokyo, Japan).

Acetonitrile (HPLC grade) and trifluoroacetic acid (TFA) were purchased from Wako Pure Chemicals (Osaka, Japan). Agarose powder (analytical grade) was purchased from Nacalai Tesque (Kyoto, Japan).

Assembly of the Autofocusing Apparatus. A tank (20 cm inner width × 25 cm in height × 120 cm in length) was made from polycrlyl plate of 5 mm thickness. Eleven rows of slots (0.5 cm in width × 1 cm in depth) were made in the inner surface of the tank every 10 cm as illustrated in Figure 1. To divide the tank into 12 compartments, 11 polycrlyl plates (25 cm × 20 cm), each with a window (10 cm × 10 cm), were prepared. As illustrated in Figure 2, nylon screen (100 mesh) was mounted on the window and fixed by Scotch tape and a frame with four screws. The screen was wetted with 1% hot agarose solution and allowed to stand for a few minutes to form a thin agarose gel layer on the screen. On the agarose gel layer, additional agarose solution was loaded to 1–2 mm in thickness and also allowed to stand for a few minutes. Consequently, an agarose gel layer of 1–2 mm in thickness was prepared on the screen. The polycrlyl plate with the agarose gel layer is hereafter referred to as a separator. A separator was inserted into each slot of the tank. The compartment at either end of the tank was filled with 0.1 N phosphoric acid (anode) or 0.1 N NaOH (cathode) and used as, respectively, electrode compartments. The sample compartments were numbered from the anode side (no. 1) to the cathode side (no. 10). A titanium plate coated with platinum (6.5 cm × 3.5 cm) was purchased from Tanaka Kikinzoku Kogyo (Tokyo, Japan) and used as an electrode. To cool the sample during focusing, water jackets were attached to both sides of the tank. The water jackets were filled with 80% ethylene glycol cooled to −20 °C. In addition, a silicon tube, in which cold 80% ethylene glycol was circulated, was put into the sample compartments through the lid on the tank. Four coolers (977.5, 345, 345, and 150 W) were used to cool 80% polyethylene glycol in the water jackets. The polyethylene glycol solute in the silicon tube was cooled and circulated by an Eyela Coolace CCA-1100 (Tokyo Rikakikai, Tokyo, Japan). This assembly of the tank, separators, water jackets, and lid with silicon tube is referred to as a “focusing apparatus”. Autofocusing of peptides was performed at constant voltage according to two methods. In method 1, all sample compartments were filled with 1% sample solution; in method 2, sample compartments 4 and 5 were filled with 5% sample and the others were filled with deionized water (1–5 µL/cm). In both cases, direct electric current at a constant voltage of 500 or 600 V was applied to the electrodes. During focusing, all solutions in the compartments were stirred with magnetic stirrers.

Analytical Procedures. To monitor peptide fractionation, peptides in aliquots (20 µL) fractions were resolved by reversed-phase (RP) HPLC using a Cosmosil 5C18-MS (120 Å, 4.6 mm i.d. × 250 mm, Nacalai Tesque). Elution was performed with a linear gradient from 0 to 30% of acetonitrile in water in the presence of 0.1% TFA over 50 min at 1 mL/min. Absorbance at 214 nm of the column effluent was monitored. The column was maintained at 40 °C. In some cases, peptides in the autofocusing fraction were first fractionated by size-exclusion chromatography (SEC) before RP-HPLC. Aliquots (200 µL) from the autofocusing fractions were injected to a Superdex Peptide 10/30HR (Amersham Biosciences, Piscataway, NJ) equilibrated with 30% (v/v) acetonitrile containing 0.1% TFA at 0.5 mL/min.

To monitor fractionation of peptide by autofocusing, amino acid analysis of the autofocusing fractions was performed in triplicate according to the method of Bidlingmeyer et al. (14), with a slight modification (15). The amino acid sequence of peptide was determined with an automated pulse-limited liquid peptide sequencer based on Edman degradation (model PPSQ-21, Shimadzu, Kyoto, Japan). All operations were carried out according to instructions from the supplier.

RESULTS

The casein hydrolysate was fractionated according to method 1 at 500 V constant voltage; pH gradients from approximately pH 2 to 12 were formed after 12 and 24 h (Figure 3). Peptides were migrated to acidic (pH 2–6), neutral (pH 6–8), and basic (pH > 8) fractions. Approximately 50% of total peptides were distributed in the acidic fractions after 12 h; after an additional 12 h, >70% of the peptides were focused into the acidic fractions. On the other hand, peptide content in the basic fractions decreased with increased focusing time. These facts indicate that focusing of peptides was not completed after 12 h and that peptides continued to migrate during the next 12 h. As shown in Figure 4, panels A and B, the acidic (Fr 1–3) and basic fractions (Fr 9–10) were significantly richer in acidic (including Asn and Gln) and basic amino acids, respectively, indicating that fractionation of peptides occurs due to the amphoteric nature of the sample peptide without using added carrier ampholytes, such as ampholine. The acidic (Fr 1–4) and basic fractions (Fr 9–10) obtained after 24 h consisted of significantly higher proportions of acidic and basic amino acids.
respectively, than those obtained after 12 h. These facts also indicate that better fractionation of peptides was obtained by focusing prolonged beyond 12 h. No significant improvement in peptide fractionation was observed by focusing prolonged beyond 24 h. Therefore, in the following experiments, autofocusing by using the present apparatus was carried out for 24 h.

The same sample was also fractionated according to method 2 at 600 V for 2 h and at 500 V constant voltage from 2 to 24 h. A pH gradient from pH 2 to 12 with shallow slope regions in the acidic and basic regions was formed (Figure 5). More than 85% of peptides were distributed in Fr 3–7. Only small amounts of peptides were distributed in Fr 1, 2, and 8–10, where the slope of the pH gradient became shallow. Approximately 65% of total peptides were focused into the acidic Fr 1–5. As shown in Figure 6, the peptide fractions obtained according to method 2 had compositional features similar to corresponding fractions obtained by method 1. However, ratios of basic amino acids in the acidic Fr 1–4 generated according to method 2 were significantly smaller than any of the fractions produced according to method 1. Similarly, ratios of basic amino acids in Fr 7–10 produced according to method 2 were higher than those in method 1.

Aliquots of the autofocusing fractions of the casein hydrolysate from both methods 1 and 2 were analyzed by RP-HPLC; elution profiles are shown in the Figure 7. Three large peaks in the chromatograms were identified as tyrosine (Tyr), phenylalanine (Phe), and tryptophan (Trp); the other peaks can be considered peptide peaks. In both cases, the elution profiles of peptides differed among the autofocusing fractions having different pH values, further evidence that peptide fractionation occurs by use of the present apparatus. In method 1, amino acid and peptide peaks with the same retention time were distributed in wider fractions and pH ranges than those in method 2. For example, Fr 1 and 2 achieved according to method 1 yielded
peptide profiles similar to those of Fr 3 and 4, whereas Fr 1 and 2 achieved according to method 2, which had pH values very similar to the corresponding fractions in method 1, yielded only small peptide peaks. Taken together with the results shown in Figures 4–7, it can be concluded that better fractionation of peptides, especially quantitatively minor acidic and basic peptides, can be obtained with method 2. On the other hand, some peptide peaks in the autofocusing fractions obtained according to method 1 are larger than those in the start material, whereas such enrichment is not observed in the results from method 2. Sequence analysis was carried out for some peptides isolated by the RP-HPLC from the fractions obtained according to method 2. Identified primary structure, origin, and estimated pI of peptides, on the basis of their primary structure, are summarized in Figure 8. Most peptides in Fr 4 had acidic pI lower than 4. Caseinphosphopeptides (peaks 3, 6, and 7) that may have lower pI than that estimated by their amino acid sequence were focused into this fraction. In Fr 6, most peptides had a pI value approximately between 4 and 6. A peptide potentially modified by a sugar chain (peak 11) was recovered in this fraction. It is worth noting that peptides in peaks 5 and 6 in Fr 4 had almost the same retention times as peptides in peaks 12 and 13 in fraction 6; however, these peaks consisted of different peptides. Therefore, some of the peptides, which cannot be separated by the RP-HPLC, can be fractionated by the present apparatus. In Fr 7, most peptides had pI values > 5.5. The peptides having intermediate pI values between Fr 6 (pH 6.5) and 7 (pH 9.4) were distributed.
Large-Scale Autofocusing of Peptides

**Figure 9.** Amino acid composition of autofocusing fractions. Whey protein hydrolysate was fractionated according to method 2 for 24 h.

<table>
<thead>
<tr>
<th>Fraction number</th>
<th>Peak number</th>
<th>Amino acid</th>
<th>Isoelectric point</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 (pH 3.5)</td>
<td>1</td>
<td>PDOEKFET</td>
<td>3.98</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>VEDIFPE</td>
<td>3.79</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>LIDDKLD</td>
<td>3.32</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>LGPDFTI</td>
<td>6.22</td>
<td>13</td>
</tr>
<tr>
<td>5 (pH 4.4)</td>
<td>5</td>
<td>EKLTPDE</td>
<td>6.22</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>VDDSPV</td>
<td>3.79</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>LDDKLD</td>
<td>3.32</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>VDDSPQD</td>
<td>3.79</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>LGPDFTI</td>
<td>6.22</td>
<td>13</td>
</tr>
<tr>
<td>8 (pH 11.0)</td>
<td>10</td>
<td>KYTPGA</td>
<td>10.00</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>LGPDFTI</td>
<td>6.22</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>EKDTPF</td>
<td>6.49</td>
<td>13</td>
</tr>
</tbody>
</table>

**Figure 10.** Summary of characteristics of isolated peptides from autofocusing of whey protein hydrolysate by method 2. Peptides were isolated by a series of SEC and RP-HPLC. a This value was estimated without considering possible modification with phosphate. b Potential residue for phosphorylation. c Potential residue for modification with o-link sugar chain.

in both fractions (peaks 9 and 16 and peaks 12 and 17). These data also confirm that peptides are fractionated on the basis of the isoelectric point of the sample peptides by the present apparatus.

The milk whey protein hydrolysate was also fractionated according to method 2 for 24 h. As shown in **Figure 9**, amino acid analysis of each fraction revealed that the acidic and basic fractions were rich in acidic and basic amino acids as well as the casein hydrolysate. After 24 h of focusing, aliquots from fractions 2 (pH 3.5), 5 (pH 4.4), and 8 (pH 11.0) were then subjected to fractionation by the SEC mode. The SEC fractions eluted between 26.0 and 28.0 min were further fractionated by the RP-HPLC. As shown in **Figure 10**, elution profiles differed between fractions with different pH values. Sequence analysis confirms that peptides in the milk whey hydrolysate are also fractionated by their isoelectric points (**Figure 10**). This preparation contained some κ-casein-derived peptides. A casein-phosphopeptide (peak 1) was recovered in Fr 2.

**Figure 11** illustrates electric current and power developed by autofocusing of the casein hydrolysate via methods 1 and 2. In method 1, the current was high at the start of focusing and decreased with focusing time, until finally reaching a constant value. Approximately 6000 W h was consumed by autofocusing in method 1. In method 2, the current was low at the start and gradually increased until 12 h and then decreased; ~8500 W h was consumed by method 2.

**DISCUSSION**

Many varieties of matrix-free, preparative, isoelectric focusing apparatuses have been developed. By using those apparatuses, enzymes, antibodies, peptides, and so on have been isolated without the addition of carrier ampholytes (12, 16–24). This approach has been referred to as autofocusing. The main factor in decreasing the resolution of those apparatuses was diffusion of sample by convection current. In the previous study, we demonstrated that resolution was extensively improved by introducing a thin agarose gel layer between sample cells (13). As the fractionation of peptides occurs only in the agarose layer, this system can be considered an agarose gel preparative isoelectric focusing based on the amphoteric nature of the sample. The previous type of autofocusing apparatus consisted of 7–12 pieces of sample cells (500 mL) with four joint tubes (17 mm i.d.) on the front and back connecting the sample cells. A thin agarose gel layer, supported by a nylon screen, was placed between the adjusting joint tubes (13). This apparatus can process up to 5 L of the sample solution at one time. However, it requires considerable skill to connect the four joint tubes without damaging the agarose gel layer between the joint tubes. In addition, electric current was frequently disturbed by bubbles formed in the joint tubes of the previous apparatus. Therefore, we concluded that it might be difficult to make a larger scale version of this apparatus. In the present study, plates containing a window covered with an agarose gel layer are prepared and used instead of the joint tubes. By simply inserting the plates, referred to as separators, into the slots of the tank (50 L), the tank can be divided by the thin agarose gel layer into 10 sample and 2 electrode compartments. By using this apparatus, large-scale peptide fractionation (up to 50 L) occurred on the basis of the isoelectric points of the sample peptides without complications (**Figures 4, 5, and 8–10**).

In the previous studies (13), all sample cells were filled with the same peptide sample solution (method 1). In the present study, another type of sample application method was also used (method 2), in which the concentrated sample was applied to only compartments 5 and 6. Groleau et al. (24) reported that neutral peptides show a broader distribution than acidic and basic peptides when autofocused by the sample application corresponding to method 1, due to low electrophoretic mobility. The peptides with low electrophoretic mobility might remain near the compartments where the sample was applied. In method 1, these peptides with low mobility might show broad distribution;
in method 2, these peptides might remain near compartments 5 and 6, where the sample solution was applied, whereas acidic and basic peptides with high mobility might migrate from these compartments. Actually, method 2 gave better resolution of peptides than method 1, especially for acidic and basic peptides (Figures 2 and 4). On the other hand, an enrichment of some peptide constituents resulted from using method 1, whereas dilution occurred with method 2. Therefore, method 1 may be preferable when peptide concentration of the start material is relatively low.

In the present study, we have developed a batch system autofocusing apparatus, which can process up to 50 L at one time. A peptide sample of ~500 g or more can be fractionated within 24 h. Then, we can evaluate the biological activity of peptides, fractionated on the basis of isoelectric points, through feeding experiments, which would facilitate identification of the in vivo active peptides. However, effects of sample amount, the presence of salt in a sample, electric voltage, etc. on the resolution should be examined. Another aim of this project is to develop an autofocusing machine suitable for industrial applications. Now, further studies on the optimization of autofocusing conditions and the development of a larger autofocusing apparatus are in progress to demonstrate the potential of this approach for industrial applications.

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LITERATURE CITED