Novel Approach for Large-Scale, Biocompatible, and Low-Cost Fractionation of Peptides in Proteolytic Digest of Food Protein Based on the Amphoteric Nature of Peptides

Asuka Akahoshi,† Kenji Sato,* Yuko Nawa, Yasushi Nakamura, and Kozo Ohtsuki

Department of Food Sciences and Nutritional Health, Kyoto Prefectural University, Shimogamo, Kyoto 606-8522, Japan

A large-scale, biocompatible, and low-cost procedure for peptide fractionation based on the amphoteric nature of peptide is developed. A sample cell (120 × 100 × 50 mm) with four joint tubes (17 mm i.d. and 20 mm in length) on the front and back was prepared. On the end of the joint tubes, a nylon screen (100 mesh)-supported agarose gel layer was formed. Five or nine of the sample cells were connected. A tryptic digest of casein (2.0−3.6 L) was applied to the sample cells. At each end of the sample cell apparatus, an additional cell filled with 0.1 M H_3PO_4 or NaOH was connected and used as anode and cathode compartments, respectively. Reproducible fractionation of peptide could be achieved by collecting fractions with specific pH values when voltage reached a plateau by applying direct current at constant power. Running time necessary for fractionation of peptide was inversely proportional to electric power and directly proportional to sample volume.

Keywords: *Peptide fractionation; preparative isoelectric focusing; autofocusing; electrophoresis; functional foods*

INTRODUCTION

Over many years, numerous beneficial biological activities for human health, such as moderation of hypertension, bacteriolysis, antioxidation of fat, and enhancements of calcium adsorption in digestive tract, have been found in peptides derived from food proteins [reviewed by Mills et al. (1992), Arai (1996), and Meisel (1997)]. It has been anticipated that oral intake of such peptides could be effective in preventing some diseases (Arai, 1996; Stephen, 1998). However, these peptides may be further degraded in the digestive tract and consequently lose their potential activity before reaching the target organ. Therefore, the potential activity of the peptides must be evaluated by feeding experiments.

In some cases, liquid chromatography has been used for large-scale preparation of an active peptide fraction for feeding experiments (Osajima et al., 1993). However, the high cost of liquid chromatography systems is a stumbling block to the preparation of large amounts of peptide despite its high selectivity. Crude peptide preparations obtained by procedures having low selectivity such as selective precipitation and filtration techniques, which usually just remove the undigested protein and proteolytic enzyme from the peptides, have been frequently used for feeding experiments and also for food ingredients. However, these preparations may contain the active peptide only in small levels. On the other hand, proteolytic digests of protein frequently show bitter or odd taste (Minamimura et al., 1972; Habibi-Najafi and Lee, 1996) and may contain peptides with unfavorable functions. In large doses, these preparations may cause undesirable side effects. Therefore, isolation or concentration of the beneficial peptide from the crude preparation is a critical step in the development of peptide-based functional foods.

In a previous paper, we demonstrated that peptides can be fractionated on the basis of their amphoteric nature using a laboratory-scale preparative isoelectric focusing apparatus (Yata et al., 1996). This approach is biocompatible and inexpensive and referred to as "autofocusing" to distinguish it from common isoelectric focusing using added carrier ampholytes. In the present study, we develop a large-scale focusing apparatus that can process a few liters of peptide sample dissolved in water.

MATERIALS AND METHODS

Materials. Hammarsten casein was purchased from Merck (Darmstadt, Germany). Trypsin (type II) was obtained from Sigma (St. Louis, MO). Acetonitrile (HPLC grade) and trifluoroacetic acid (TFA) were purchased from Nacalai Tesque (Kyoto, Japan). Dialysis membrane (size 36) was purchased from Wako Pure Chemicals (Osaka, Japan). Agarose powder (electrophoresis grade) was obtained from Funakoshi (Tokyo, Japan).

Preparation of Tryptic Digest of Casein. Casein was suspended in distilled water to 1% (w/v); 1 M NaOH was added to give pH 7.5, and the sample was digested with trypsin at an enzyme-to-substrate ratio of 1:100 (w/w) for 3 h at 37 °C.

Assembly of Focusing Cells. An open-top sample cell with four joint tubes on the front and back as illustrated in Figure 1 was prepared. The cell was made from a polyacrylic plate and tube of 3 mm thickness. On the end of the joint tubes, an agarose gel layer was formed as illustrated in Figure 2. Nylon screen (100 mesh) was mounted on the end of the joint tubes of the first cell and fixed by inserting silicon tubes (Figure 2A,B). Then, the nylon screen was wetted with 1.5% of a hot agarose solution (Figure 2C). After gelation of the agarose on the screen (Figure 2D), an additional agarose solution was

* Author to whom correspondence should be addressed (telephone and fax +81 75 723-3503; e-mail k_sato@kpu.ac.jp).

† Present address: Faculty of Environment and Symbiotic Science, Prefectural University of Kumamoto, Kumamoto 862-8502, Japan.

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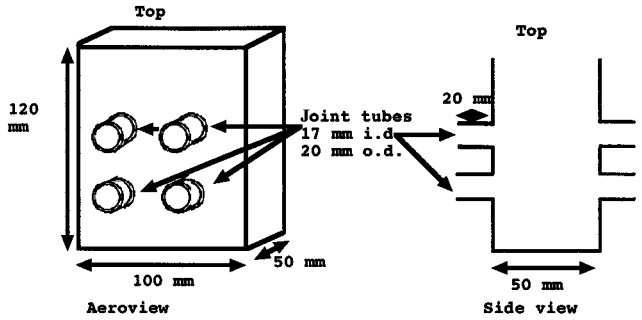

Figure 1. Schematic drawing of the sample cell.

pipetted on the thin agarose layer supported by nylon screen to 1−2 mm in thickness (Figure 2E). After gelation of the agarose over the screen of the first cell, the joint tubes of the next cell were inserted into the silicon tubes. Cell assembly was completed by repeating the same procedure as described above (Figure 3). An additional cell was connected at each end of the sample cell apparatus; these were used as electrode compartments. A platinum electrode was placed in each electrode cell. The joint tubes of the electrode and sample cell assembly were partitioned with dialysis membrane without the agarose gel layer.

Autofocusing of Tryptic Digest of Casein by the Large-Scale Apparatus. Five or nine sample cells were assembled as described above. For the electrode solutions, 0.1 M H_3PO_4 (anode) and 0.1 M NaOH (cathode) were used (Figure 3). Sample solution was put into all of the sample cells up to 400 mL. As shown in Figure 3, the sample cell was numbered from anode (acid) to cathode (base) side. To cool sample below 5 °C, a silicon tube coil (5 mm i.d.), in which −20 °C methanol was circulated, was put into the sample cells. The cell assembly was placed in a polyacrylic box (20 cm × 120 cm × 10 cm) with a lid during run time for safety. Direct current (50, 100, or 150 W) was applied to the electrodes. During focusing, the sample solution in each cell was stirred with a magnetic stirrer.

The tryptic digest was also fractionated in the autofocusing mode using a Rotofor (Bio-Rad), a commercially available preparative isoelectric focusing apparatus, as described previously (Yata et al., 1996).

Analytical Procedures. To monitor peptide fractionation, an aliquot (100 μL) from each cell compartment after autofocusing was analyzed by 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% of acetonitrile in water in the presence of 0.1% TFA over 30 min at 1 mL/min. Absorbance at 214 nm of the column effluent was monitored. The column was maintained at 40 °C.

Recovery of peptide was evaluated with amino acid analysis according to the method of Bidlingmeyer et al. (1984) with a slight modification (Sato et al., 1992).

RESULTS

Development of Large-Scale Autofocusing Apparatus. In the preliminary experiments, a sample cell with joint tubes of smaller inner diameters (5 or 10 mm) was also prepared. When these sample cells with narrow tubes were used for autofocusing, the electric current was frequently disturbed by bubbles formed in the narrow joint tubes. On the other hand, a constant power of up to 200 W could be applied to the sample cell with joint tubes of 17 mm (i.d.) without trouble, and this design was used in the following experiments. Originally, the sample cells were assembled without the agarose gel layer. Reversed-phase HPLC analysis, however, revealed that no significant fractionation of peptides occurred by using the apparatus without the agarose gel layer even after prolonged focusing (data not shown). We supposed that peptides were diffused through the nylon screen, which decreased the resolution of the peptides. To improve the resolution, a simple modification, the agarose gel layer was introduced on the screen, was made, which enabled fractionation of the peptide as described below.

Autofocusing of Tryptic Digest of Casein by Large-Scale Focusing Apparatus. The tryptic digest was fractionated with a five-cell apparatus at 150 W constant electric power. Reversed-phase HPLC analysis revealed that each fraction obtained by focusing for 4 h showed significantly different peptide patterns, indicat-

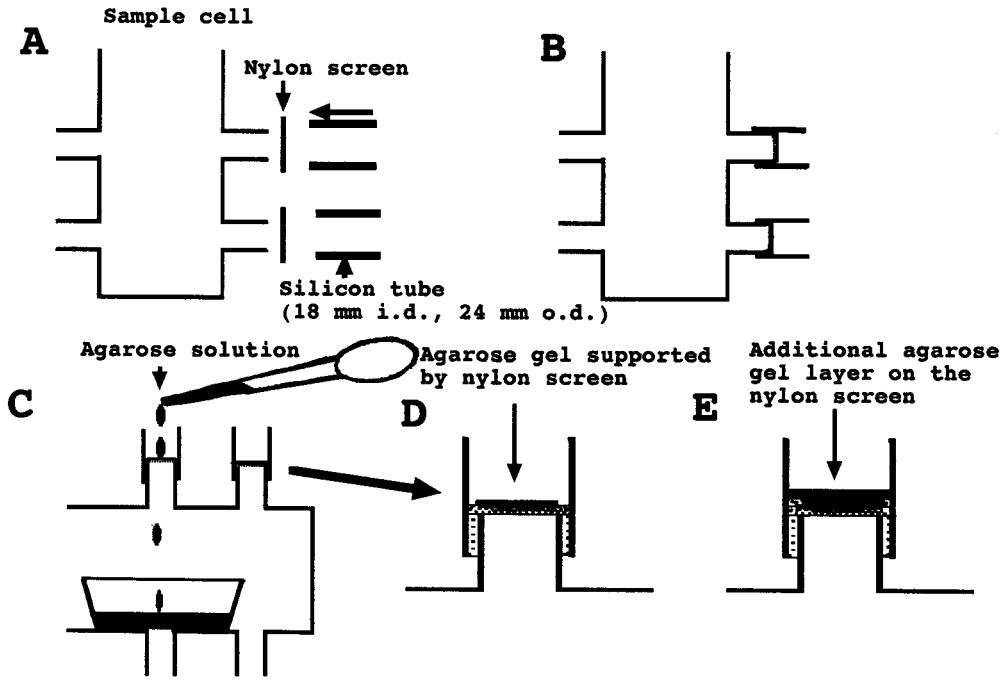

Figure 2. Formation of an agarose gel layer on the end of the joint tube of the sample cell. Refer to Materials and Methods for details.

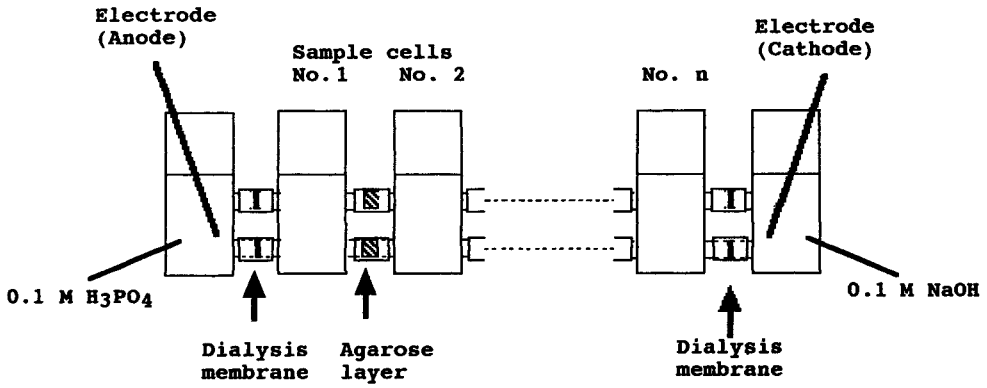

Figure 3. Schematic drawing of the cell assembly.

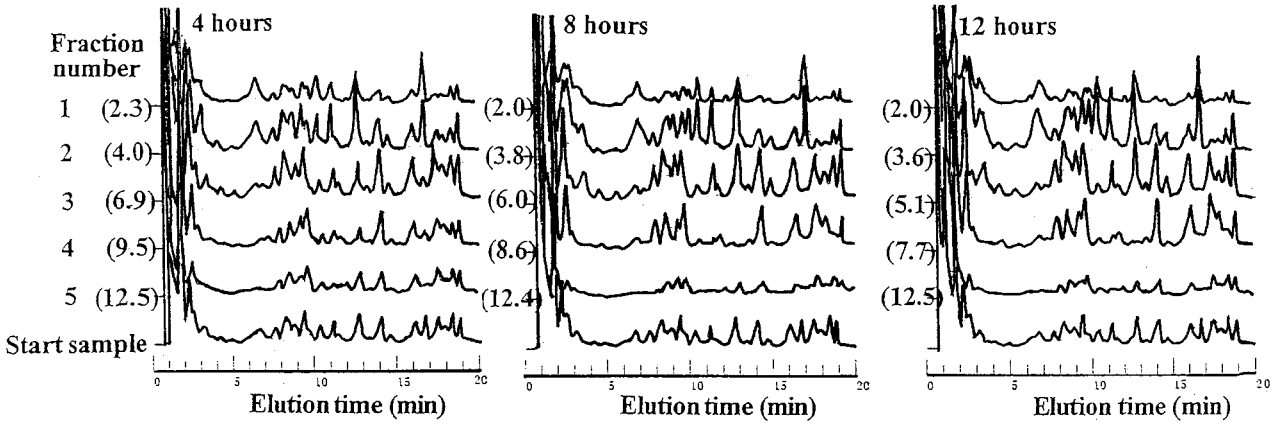

Figure 4. Analysis of the aliquot from each sample cell after autofocusing with reversed-phase HPLC; tryptic digest of 1% casein was fractionated by the five-cell apparatus at constant power of 150 W. pH values of each fraction are represented in parentheses.

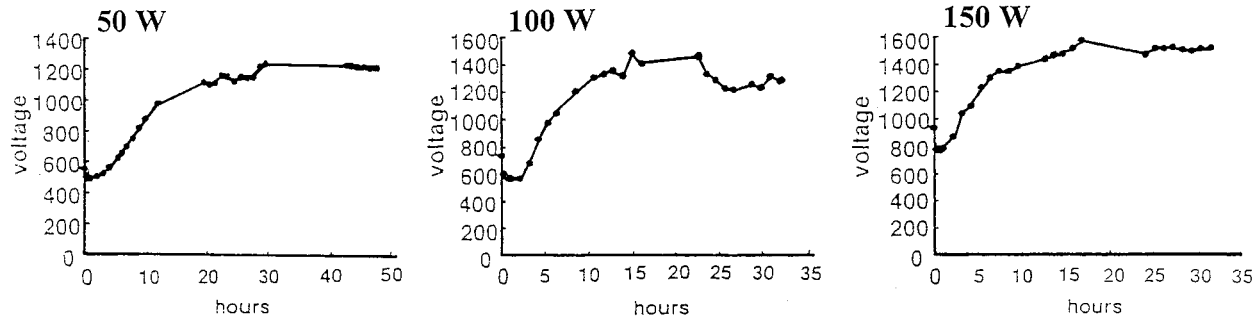

Figure 5. Voltage values developed by autofocusing using the five-cell apparatus at different electric powers.

ing that fractionation of the peptide occurred (Figure 4). On the basis of the peptide patterns, better fractionation was obtained after an additional 4 h. No significant improvement was, however, observed after that. Then, ~8 h was necessary for fractionation of the peptide in 2 L of the 1% tryptic digest of casein at 150 W. When peptide fractionation was completed, voltage reached a plateau (Figure 5). The same phenomenon was observed by using the laboratory-scale apparatus (Yata et al., 1996). The terminal point of fractionation of the peptide can be estimated by monitoring the voltage at constant power.

Fractionation of the peptide by the present apparatus was compared with that by using the laboratory-scale apparatus (Rotofor), which has 20 sample chambers and can fractionate samples of up to 50 mL. As shown in Figures 4 and 6, the peptide patterns in the fractions with similar pH values prepared by both apparatuses were comparable, whereas the Rotofor fractions contained fewer peptide peaks, indicating loss of some peptides. Actually, a significant amount of peptide in the tryptic digest of casein was precipitated during the autofocusing and remained in the core membrane of the Rotofor (Yata et al., 1996). On the other hand, recovery of peptide using the present apparatus was >80%, which was a significantly higher value than that (~40%) obtained by the Rotofor (Yata et al., 1996). The higher recovery by the present apparatus may be due to the significantly smaller volume of the agarose gel layer in comparison with the sample volume.

The relationship of electric power and focusing time was also examined with the five-cell apparatus. As a lower electric power was applied, the voltage reached a plateau in a longer time (Figure 5). It took approximately 24 and 12 h to reach a plateau of voltage by applying 50 and 100 W, respectively. As shown in Figure 6, fractions 2 and 3 obtained at 50 W showed slightly different peptide patterns from the corresponding fractions at 100 and 150 W, which can be explained by the slightly lower pH value of these fractions compared with

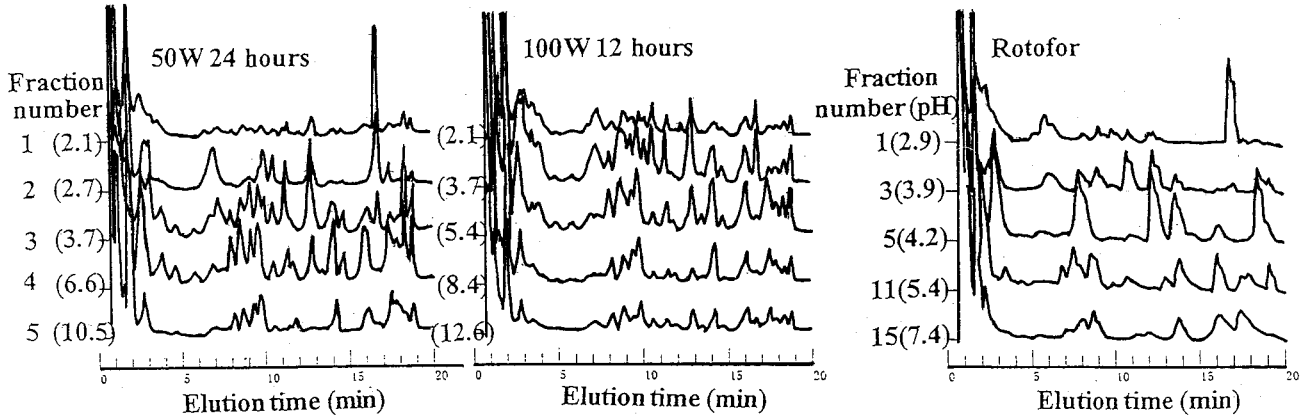

Figure 6. Peptide patterns of the autofocusing fractions by the five-cell apparatus (50 and 100 W) and the small-scale commercial apparatus (Rotofor).

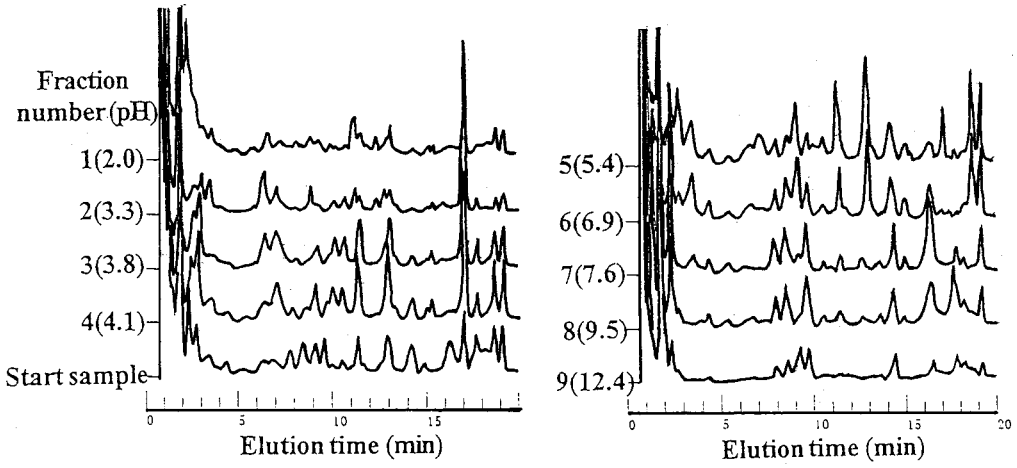

Figure 7. Peptide patterns of autofocusing fractions by the nine-cell apparatus.

their counterparts. Essentially the same peptide patterns were observed between the fractions with similar pH values, that is, fraction 3 at 50 W versus fraction 2 at 100 and 150 W (Figures 4 and 6).

When the number of sample cells was increased from five to nine, focusing time at 50 W increased from 24 to 48 h. Peptide patterns of the fractions with similar pH values were also comparable between the five- and nine-cell apparatuses (Figures 4, 6, and 7).

Together with above facts, reproducible fractionation of peptide can be obtained by collecting a fraction with a specific pH value when the voltage reaches a plateau. Therefore, >50-fold scale-up from the previous work can be achieved by the present means without decreases in the resolution and recovery.

Relationships of sample volume, electric power, and time necessary for fractionation of peptide can be summarized as shown Table 1. The running time necessary for the autofocusing of peptide is inversely proportional to electric power and directly proportional to sample volume even when different apparatuses are used. It can be anticipated that a larger volume of sample can be fractionated in a shorter time by the application of a higher electric power, if a powerful electric power supply and sample cooling systems are available.

Table 1. Summary of Fractionation of Tryptic Digest of Casein in Autofocusing Mode by the Present Large-Scale Apparatus in Comparison with a Commercially Available Apparatus (Rotofor)

focusing apparatus	sample vol (L), V	electric power (W), P	time for fractionation[a] (h), T	$P \times T/V$
five-cell apparatus	2	50	24	600
	2	100	12	600
	2	150	8	600
nine-cell apparatus	3.6	50	48	677
Rotofor	0.05	12	2.5	600

[a] Approximate time necessary for peptide fractionation.

DISCUSSION

The main factor that decreases the resolution of matrix-free isoelectric electrophoresis is diffusion of sample by convection current (Haglund, 1967). To minimize the effect of the convection current, gravity gradient with sucrose (Haglund, 1967; Rilbe, 1976; Acevedo, 1993), thin layer focusing cell (Bier and Long, 1992; Roman and Brown, 1994), sample cell partitioned with porous layer (Egen et al., 1988) or polyacrylamide gel (Righetti et al. 1989), and so on have been used. In the present study, our aim is to develop a method for the large-scale preparation of a peptide fraction adequate for feeding experiments. For this purpose, use of high-cost apparatuses and toxic chemicals should be avoided. Therefore, polyacrylamide gel cannot be used because of the potential toxicity of its monomer form. Addition of nontoxic chemicals such as sucrose also may interfere in the subsequent feeding experiment. Therefore, we developed a simple system, which consists of the sample cell connected by the joint tubes. Diffusion of sample through the joint tubes is prevented by the

biocompatible and thin agarose gel layer on the nylon screen between the joint tubes of the adjacent sample cells. As the fractionation of peptide occurs only in the thin agarose layer, this system can be considered as an agarose gel isoelectric focusing using peptide sample as ampholyte. Formation of a thicker (>3 mm) gel layer on the nylon screen should be avoided because the running time increased significantly.

Without doubt, liquid chromatography, especially reversed-phase HPLC, is a most effective tool for the separation of peptides [reviews by Huang and Guiochon (1989) and Mant et al. (1992)]. However, it is a relatively expensive system for large preparations, and most solvents used in the reversed-phase mode are harmful to humans. On the other hand, the sample cell in the present apparatus can be prepared for <$30 U.S., and no expensive apparatuses and materials are required. The low cost and biocompatibility of the present approach have an advantage over the conventional preparative liquid chromatography. By using the present apparatus, more than a gram order of peptide fraction can be prepared, which would enable the design of small-scale animal experiments to examine biological activity of peptide preparations with specific isoelectric points. In the previous study, we demonstrated that some indigestible pyroglutamyl peptides are induced during industrial peptide preparation (Sato et al., 1998). The indigestible pyroglutamyl peptide-enriched and -free fractions can be prepared by the present approach using the five-cell apparatus (manuscript in preparation). By using these preparations, we are examining the metabolic fate of the pyroglutamyl peptide by feeding trial with rat.

In addition, the present apparatus can be used for large-scale and biocompatible fractionation of protein because peptides in the proteolytic digest of food protein can be used as edible carrier ampholytes for protein fractionation (Haglund, 1967).

We demonstrate here that the peptide fractionation based on the autofocusing can be scaled up by the thin agarose layer in the focusing apparatus. By using this technique, we have started developing a process-scale machine for peptide preparation as food ingredients. Further studies on the optimization of the resolution of peptide and scale-up are in progress. We anticipate that the present approach has potential in accelerating the development of peptide-based functional foods at both research and industrial levels.

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