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Development of Continuous Type Apparatus for Ampholyte-Free Isoelectric Focusing (Autofocusing) of Peptides in Protein Hydrolysates

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An apparatus for continuous fractionation of peptides on the basis of amphoteric nature of sample peptides was developed. A tank (66.5 cm × 8 cm × 8 cm, L × W × H) was divided into 12 compartments by a thin agarose gel layer. A drain tube (5.5 cm in length and 0.7 cm in i.d.) was fixed through the bottom of each compartment to give a height of 4 cm from the bottom. The tank with 12 compartments and electrodes was referred to as an autofocusing unit. The peptide solution or water was delivered to the sample compartments of the first unit. The solutions drained from the first unit were successively delivered to the second and third units. To the electrodes of three units, a direct electric current was applied. By using the present apparatus, peptides in casein hydrolysate can be continuously fractionated at least for 5 h. Better resolution was obtained in the second and third units.

KEYWORDS: Autofocusing; peptide; peptide fractionation; continuous; isoelectric focusing; electrophoresis; functional foods

INTRODUCTION

For past decades, it has been demonstrated that oral ingestion of some enzymatic hydrolysates of food proteins can promote human health (*1*−*8*). In most cases, crude enzymatic hydrolysates of food proteins are used as functional food ingredients. As interest in the biological activity of peptides in food protein hydrolysates has increased, demand for fractionation of peptides in an industrial scale has increased. In some cases, liquid chromatography (LC) has been used for industrial fractionation of peptides (*7*, *9*−*12*). The LC system is, however, relatively expensive for the preparation of food ingredients, while it generally shows a high resolution.

We have demonstrated by using a commercially available preparative isoelectric focusing apparatus (Rotofor, Bio-Rad Laboratories, Hercules, CA) that peptides in enzymatic hydrolysates of food proteins can be fractionated on the basis of the amphoteric nature of peptides dissolved in water (*13*). This approach has been referred to as autofocusing, which has potential for preparation of active peptide fractions for functional foods and nutraceuticals (*13*, *14*). Polyester membranes were used in the Rotofor to avoid diffusion of the sample. However, such types of membranes could not prevent the diffusion of sample, when the sample compartment volume became larger than 500 mL (*15*). To solve this problem, thin agarose gel layers supported with nylon screens were inserted between the sample compartments (*15*, *16*). By using this technique, peptide samples up to 50 L could be fractionated in the previous batch type apparatus (*16*). As the fractionation of peptides occurs in the agarose layer, the focusing time by using this system is considerably shorter than that by using large-scale free zone electrophoresis (*17*−*24*). For industrial applications of this approach, much larger amounts of sample should be processed. It might be difficult to simply scale-up the inner volume of the apparatus due to difficulty in preparation of a large agarose gel layer. Then, an alternative approach for scale-up may be effective. For this purpose, an apparatus, to which sample solution was continuously applied, was developed in the present study.

MATERIALS AND METHODS

Materials. An enzymatic hydrolysate of casein (CE90GMM) was commercially obtained from DMV Japan (Tokyo, Japan). This preparation consisted of peptides with an average molecular mass of 640 Da and free amino acids up to 15% (w/w).

Agarose powder (analytical grade) was purchased from Nacalai Tesque (Kyoto, Japan). Other reagents were of analytical grade or better. Scotch tape (sealing tape in replacement accessories for Rotofor) was obtained from Bio-Rad Laboratories. All reagents were analytical grade or better.

Assembly of the Autofocusing Apparatus. Three tanks (66.5 cm × 8 cm × 8 cm, L × W × H) were made from polyacryl plates of 0.5 cm thickness. As illustrated in **Figure 1A**, 11 rows of slots (0.5 cm in width × 0.5 cm in depth) were made in the inner surface of each tank every 5.5 cm. Between the slots, a tube (5.5 cm in length × 0.7 cm i.d.) was fixed through the bottom to give a height of 4 cm from the

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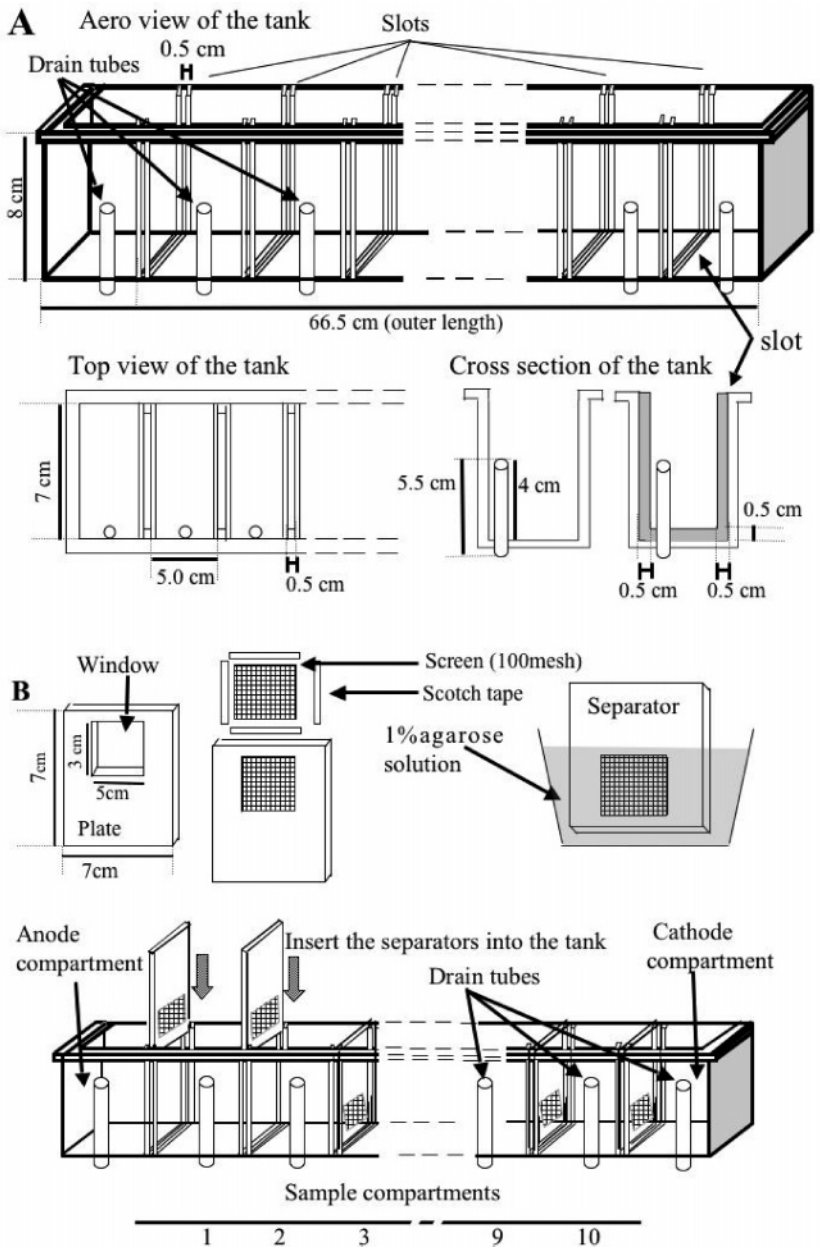

Figure 1. Schematic drawing of an assembly of an autofocusing unit. (**A**) Tank with slots and drain tubes. (**B**) Preparation of separator to make compartments.

bottom, which was named as a drain tube. As shown in **Figure 1B**, polyacryl plates (7 cm × 7 cm), each with a window (5 cm × 3 cm), were prepared. A nylon screen (100 mesh) was mounted on the window and fixed with the Scotch tape. On the screen of the plate, an agarose gel layer was made as described previously (*16*). The polyacryl plate with the agarose gel layer was named as a separator. To divide the tank into 12 compartments, the separators were inserted into the slots of the tank. The compartments at either end of the tank were filled with 0.1 N phosphoric acid (anode) or 0.1 N NaOH (cathode) and used as electrode compartments. Titanium plates coated with platinum (6.5 cm × 3.5 cm) were purchased from Tanaka Kikinzoku Kogyo (Tokyo, Japan) and used as electrodes. As shown in **Figure 1B**, the sample compartments were numbered from the anode side (no. 1) to the cathode side (no. 10). Before delivery of the sample solution, the sample compartments were filled with water (1−5 μS/cm). As illustrated in **Figure 2**, the tank was covered with a lid with 12 holes, through which sample and electrode solutions were delivered into the compartments. The tank with separators, electrodes, and lid was referred to as an autofocusing unit. To the electrode compartments of the first unit, the electrode solutions were delivered at 8 mL/min. To the sample compartments, water or casein hydrolysate solution (1 or 5%) was

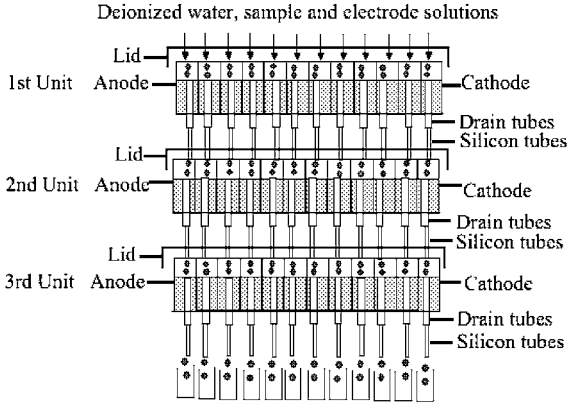

Figure 2. Schematic drawing of an assembly of a continuous type of autofocusing apparatus.

delivered at the same flow rate by two six-channel pumps (model 7553-80, Cole-Parmer, Barrington, IL). The second and third units were

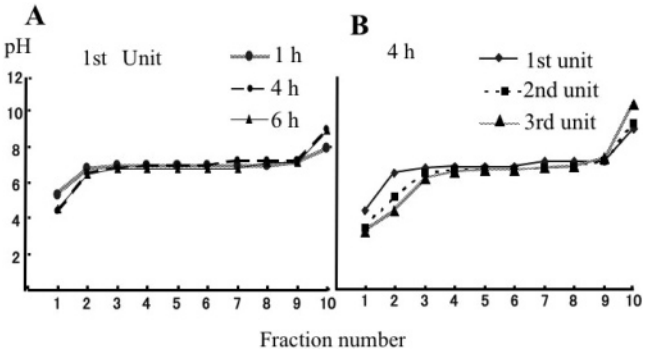

Figure 3. pH profiles in the autofocusing fractions in method 1 at 100 W. (**A**) pH profiles in the first unit. (**B**) pH profiles in the each unit after 4 h.

located under the first unit. When the level of solutions in the compartments of the first unit became higher than the drain tubes (4 cm), the solutions were poured into the drain tubes and delivered to the corresponding compartments of the second and third units through silicon tubes, which connected the drain tubes and the holes on the lid of the adjacent unit. To cool the sample during focusing, a silicon tube, in which 80% ethylene glycol cooled to −20 °C was circulated by an Eyela Coolace CCA-1100 (Tokyo Rikakikai, Tokyo, Japan), was put into the sample compartments. Autofocusing of peptides was performed at constant voltage or power modes in two sample application methods. In method 1, all sample compartments were delivered with 1% sample solution. In method 2, sample compartments 5 and 6 were delivered with 5% sample solution and the others were with the water.

The assembly of the three autofocusing units, cooler and pump units, and power supply (model HAR-1P 1200, Matssada Precision, Ohtsu, Japan) was referred to as a continuous type autofocusing apparatus. During focusing, all solutions in the compartments were stirred.

Analytical Procedures. 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. (*25*) with a slight modification (*26*). Peptide in a glass tube was hydrolyzed with 6 M vapor HCl at 150 °C for 1 h. Amino acids were derivatized with phenyl isothiocyanate. The derivatives were resolved by a Superspher RP-18(e) column (250 mm × 4 mm, Merck, Darmastadt, Germany). The elution condition was described in a previous paper (*26*).

Statistical Analyses. One-way analysis of variance and multiple comparison test of Fisher's PLSD were used to evaluate the difference in amino acid composition. Significant difference in the following sections means $P < 5\%$.

RESULTS

Resolution of peptides by using single unit was examined in the two sample application methods. When 1% casein hydrolysate solution was delivered to all sample compartments (method 1), most of the fractions showed a pH near 7 even after prolonged focusing except for fractions 1 and 10 as shown in **Figure 3A**. In addition, there were small differences in amino acid composition of peptides between fractions, which indicated that no significant fractionation of peptide occurred in method 1 by using a single unit at 100 W (**Figure 4**, upper row). When a 5% sample solution was delivered to sample compartments 5 and 6 (method 2), a higher voltage (1200 V) can be applied at 100 W due to high electric resistance by delivery of the water. As shown in **Figure 5A**, stable pH gradients approximately from 3.3 to 11.0 were formed between 1 and 6 h. More than 90% of peptide was distributed in fractions 5 and 6 (**Figure 5B**). However, only small but detectable amounts of peptide were migrated to other fractions. These acidic and basic fractions were rich in acidic and basic peptides, respectively (**Figure 5C**). These facts indicate that some acidic and basic peptides can be continuously separated by the present apparatus using a single unit at least from 1 to 6 h.

To improve the resolution of peptide, three units were connected in tandem as shown in **Figure 2**. To all units, a direct electric current was applied in a constant voltage mode at 500 V. In method 1, similar pH and peptide profiles to the first unit were observed in the second and third units (**Figures 3B** and **4**, middle column). In method 2, the second and third units showed different pH and peptide profiles in comparison to the first unit. In the second and third units, pH gradients with shallow slope in the acidic and basic regions were formed (**Figure 6A**). In the first unit, most of the peptide remained in fractions 5 and 6, of which the pH was near 7. In the second and third units, higher amounts of peptides were migrated to acidic (fractions 1−5) and basic (fraction 7−10) fractions (**Figure 6B**). Resolution of peptide in the second and third units was significantly improved on the basis of amino acid composi-

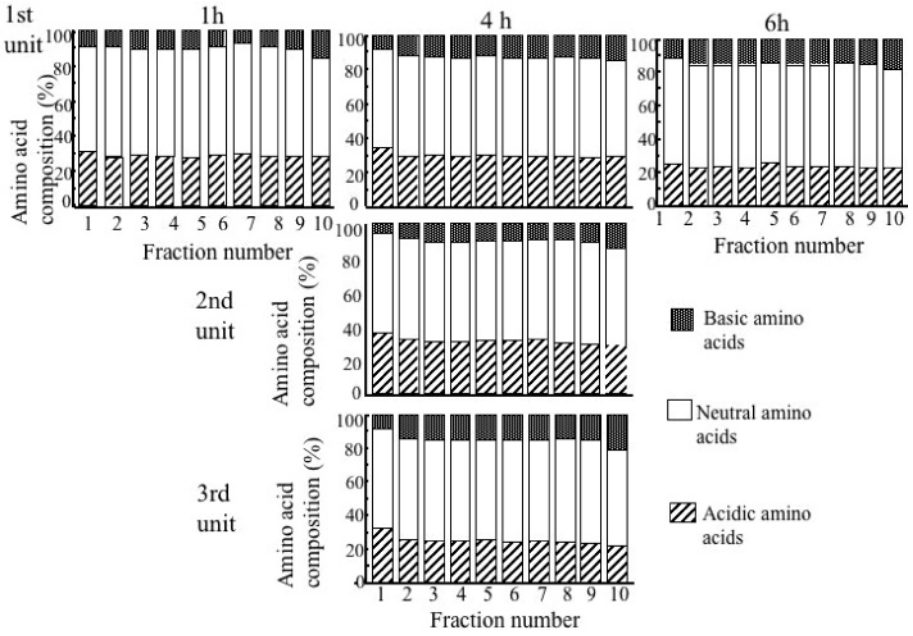

Figure 4. Amino acid composition of autofocusing fractions. Casein hydrolysate was fractionated in method 1 in constant electric power mode at 100 W.

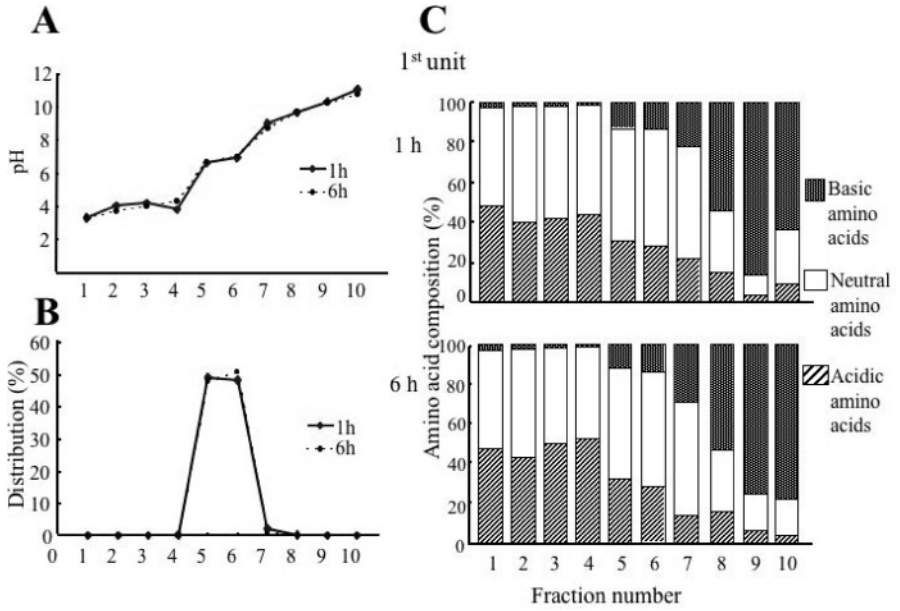

Figure 5. pH and peptide profiles in the autofocusing fraction from the first unit in the method 2 at 100 W. (**A**) pH profiles. (**B**) Distribution of peptides. (**C**) Amino acid composition.

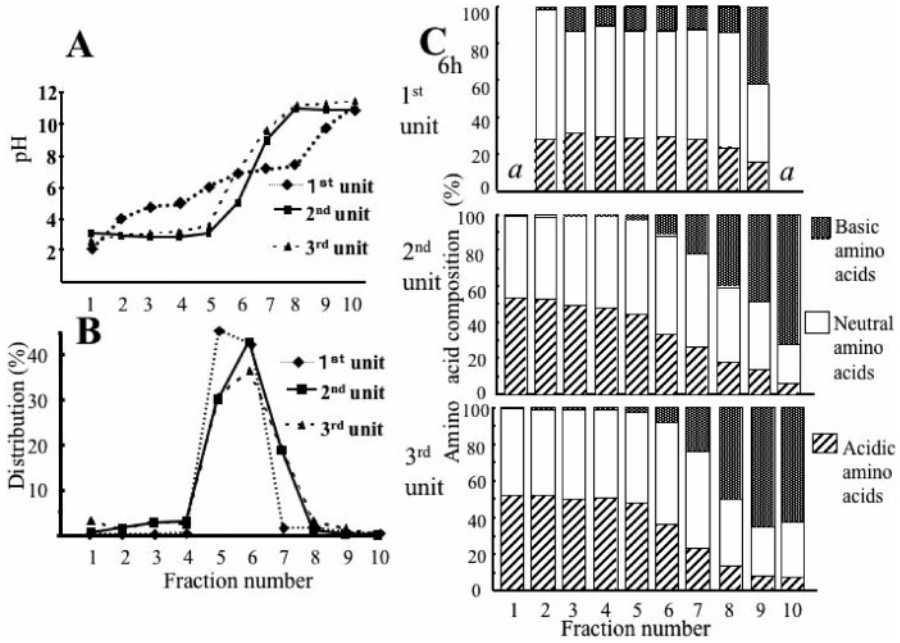

Figure 6. pH and peptide profiles in the autofocusing fraction from the first, second, and third units in method 2 at 500 V. [a]No significant amounts of peptide were observed in fractions 1 and 10. See the legend for **Figure 5** for marks **A**–**C**.

tion of each fraction (**Figure 6C**). Essentially, the same resolution of peptide was observed from 1 h of focusing (data not shown). The recovery of peptides was estimated to be approximately 90% on the basis of the peptide contents in the sample solution and the effluents from the third unit.

After prolonged focusing more than 8 h, the slope of pH gradient became shallow (**Figures 6A** and **7A**). A wider distribution of peptide was also observed (**Figures 6B** and **7B**). Differences in amino acid composition between fractions became smaller after 8 h (**Figure 7C**). In addition, portions of peptides penetrated into the anode compartment after 12 h (**Figure 8**).

DISCUSSION

In the present study, a prototype of continuous autofocusing apparatus was developed. No significant fractionation occurred in the sample application method 1 by using the present continuous one, while significant fractionation of peptide occurred in the previous batch type apparatus in method 1 after a prolonged focusing time (*16*). In the previous batch type apparatus, pH values of the fractions 1 and 10 became approximately 2 and 12, respectively, after 12 h. In the continuous apparatus, pH values of the corresponding sample compartments remained at 4 and 8 in method 1. It could be assumed that these pH values would not change after the prolonged focusing time due to continuous application of peptide sample with buffer action. Most of the peptides in the casein hydrolysate have pI values between 4 and 6 (*16*), which are not distant from pH values of fractions 1 and 10. In method 2, fractions 1 and 10 in the continuous type apparatus showed approximately pH 3 and 11, respectively. Thus, peptides in

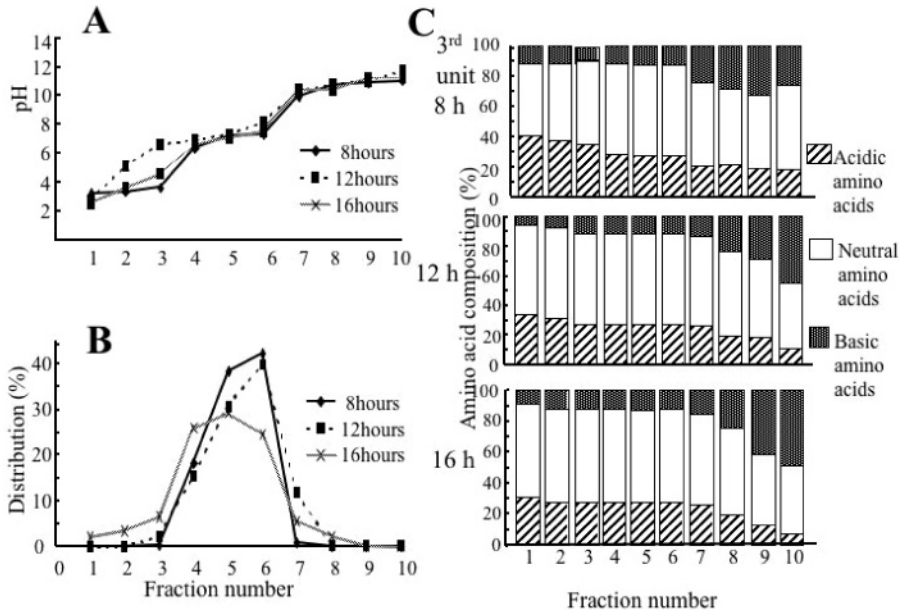

Figure 7. pH and peptide profiles in the autofocusing fraction from the third unit in method 2 at 500 V after prolonged focusing (8–16 h). See the legend for **Figure 5** for marks **A**–**C**.

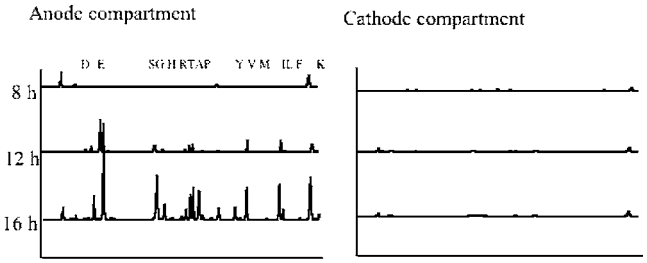

Figure 8. Presence of peptides in anode compartment after prolonged focusing. Aliquots from electrode compartments were hydrolyzed and subjected to amino acid analysis.

fractions 1 and 10 and adjacent fractions will have a weaker charge in method 1 in comparison to that in method 2 in the continuous apparatus. Together with these facts, the sample application method 2 is preferable for the continuous apparatus, while both methods can be used in the previous batch system. Resolution of peptide in method 2 was extensively improved by connecting the second and third units. Peptides in the casein hydrolysate can be continuously fractionated at least from 1 to 6 h with resolution corresponding to that obtained by the batch system apparatus after 24 h (*16*). After 8 h, the resolution of peptide, however, became poorer, although the same electrode and sample solutions were delivered in the same condition. These facts suggest that deterioration of the agarose gel layer might occur after the prolong focusing. The effect of prolonged focusing on resolution also might depend on sample peptide and sample loading conditions. The mechanism of this phenomenon remains to be solved. Further studies on optimization of sample loading and focusing conditions using a couple of sample peptides are in progress.

As summarized in **Table 1**, the present continuous type apparatus shows a higher performance by lower electric power in comparison to the 50 L batch type one, which demonstrates the advantage of the continuous type over the batch one in large-scale fractionation. If the large focusing units (e.g., 50 L) could be used in the continuous system, peptides might be processed in kg/h levels. Then, the present approach might have an advantage over liquid chromatography and membrane techniques in cost and selectivity, respectively, for the industrial application.

Table 1. Summary of Performance of the Previous Batch (50 L) and Present Continuous Types Autofocusing Apparatus

	batch type[a]	continuous type
time (h)	24	6
total processed sample (g)	400	288
performance (g/h)	16.6	57.6
consumed electric power (W h) for fractionate 1 g sample	20.4	1.3

[a] Ref *16.*

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