



## Multidigestion in continuous flow tandem protease-immobilized microreactors for proteomic analysis

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### ABSTRACT

Proteolysis by sequence-specific proteases is the key step for positive sequencing in proteomic studies integrated with mass spectrometry (MS). The conventional method of in-solution digestion of protein is a time-consuming procedure and has limited sensitivity. In this study, we report a simple and rapid system for the analysis of protein sequence and protein posttranslational modification by multienzymatic reaction in a continuous flow using the enzyme (trypsin, chymotrypsin, or alkaline phosphatase)-immobilized microreactor. The feasibility and performance of the single microreactor and tandem microreactors that were connected by the different microreactors were determined by the digestion of nonphosphoprotein (cytochrome *c*) and phosphoproteins ( $\beta$ -casein and pepsin A). The single microreactor showed rapid digestion compared with that of in-solution digestions. Multiple digestion by the tandem microreactors showed higher sequence coverage compared with that by in-solution or the single microreactor. Moreover, the tandem microreactor that was made by using the combination of protease-immobilized microreactor and phosphatase-immobilized microreactor showed the capability for phosphorylation site analysis in phosphoproteins without the use of any enrichment strategies or radioisotope labeling techniques. This approach provides a strategy that can be applied to various types of linking microreactor-based multienzymatic reaction systems for proteomic analysis.

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The identification of the protein sequence is a first and important step in proteome analysis. In addition to the primary structure of protein, posttranslational modification such as phosphorylation is also important information in understanding the role of target protein in the regulation of fundamental cellular processes. Because dysregulations of mechanisms of modifications are implicated in various diseases, including cancer [1], the characterization of protein sequence is useful for biological and clinical research.

Peptide mass mapping and tandem mass spectrometry (MS/MS)<sup>1</sup>-based peptide sequencing are key methods in current protein

identification for proteomic studies. Obtaining reliable peptide maps and meaningful sequence data by mass spectrometry (MS) analysis needs not only the separation of the peptides but also the proteolysis condition [2,3]. The conventional method of in-solution digestion by sequence-specific proteases is a time-consuming procedure due to a low digestion rate that is especially observed with diluted substrate (low protease-to-substrate ratio). In addition, the ionization efficiency of the digested fragments, including a modified peptide such as a phosphopeptide, is dependent on peptide size or peptide sequence, which directly correlates with sequence coverage of the target protein by MS analysis. Therefore, the multidigestion by several proteases [4–6], demodification by appropriate enzymes such as phosphatase for phosphorylation or modification-specific enrichment strategies [7,8] are often carried out to enhance the sensitivity for MS-based analysis. However, these procedures need multiple steps and a long time in addition to the requirement for purification of digested peptides from the reaction systems.

Microreactors are powerful tools for handling small-volume (nanoliter to microliter) samples in microchannels to perform enzymatic reactions [9–11]. Enzyme-immobilized microreactors have been used for proteolysis in proteome analysis due to several advantages [9]. A high concentration of protease is immobilized on

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<sup>1</sup> Abbreviations used: MS/MS, tandem mass spectrometry; MS, mass spectrometry; PTFE, poly(tetrafluoroethylene); TY, trypsin; CT,  $\alpha$ -chymotrypsin; TY-microreactor, trypsin-immobilized microreactor; CT-microreactor, chymotrypsin-immobilized microreactor; Cyt-C, cytochrome *c*; CHCA,  $\alpha$ -cyano-4-hydroxycinnamic acid; GPNA, *N*-glutaryl-L-phenylalanine *p*-nitroanilide; AP, alkaline phosphatase; BAPA, benzoyl-L-arginine *p*-nitroanilide; AP-microreactor, alkaline phosphatase-immobilized microreactor; PB, phosphate buffer; HPLC, high-performance liquid chromatography; ESI-TOF, electrospray ionization time-of-flight; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; TFA, trifluoroacetic acid; pS, phosphoserine.

the microchannel, thereby resulting in a high enzyme-to-substrate ratio and consequently allowing the protease-immobilized microreactor to perform rapid digestion. Furthermore, the immobilized protease can be easily isolated and removed from the protein digests prior to MS, thereby eliminating the influence of fragments from the enzyme on MS results. Another advantage is the possibility of multiple uses (reusability) due to low autodigestion and high thermal or chemical stability [12–14]. Several methods for protease immobilization have been reported where the enzyme was covalently bound, trapped, or physically adsorbed onto different supports based on either silica and polymer particles or monolithic materials [9–11,15]. We previously reported the procedure for immobilizing enzyme on the internal surface of the poly(tetrafluoroethylene) (PTFE) tube by forming an enzyme polymeric membrane through a cross-linking reaction between Lys residues on the protein surface [16]. Using this system, we reported an efficient digestion method by the protease (trypsin [TY] or chymotrypsin [CT])–immobilized microreactor (TY–microreactor and CT–microreactor, respectively) [14]. Although this microreactor showed rapid digestion (5 min at 30 °C) compared with the conventional in-solution digestion (18 h at 37 °C) [14], it needs to improve the sequence coverage against the substrate protein that has a resistance to proteolysis. The improved sequence coverage is important to enhance the probability of identification and increase the likelihood of detection of structural variants generated by processes such as alternative splicing and posttranslational modifications.

The conventional approach using multidigestion by different proteases for improved sequence coverage is based on parallel digestions of the same samples and analyzes overlapping peptides. However, this approach takes a long time and uses a multistep procedure. In this study, we prepared the tandem microreactor that was connected by different protease-immobilized microreactors using a Teflon connector. Connection was made easy because the current microreactors were made of a PTFE tube. The multienzymatic reaction by the tandem microreactors can be used for rapid analysis of protein sequence with high sequence coverage.

## Materials and methods

### Materials

*N*-Tosyl-L-phenylalanyl chloromethyl ketone-treated trypsin (TY) was purchased from Worthington (Lakewood, NJ, USA).  $\alpha$ -Chymotrypsin (CT), cytochrome *c* (Cyt-C), glutaraldehyde, and paraformaldehyde were obtained from Wako Pure Chemical (Osaka, Japan).  $\beta$ -Casein,  $\alpha$ -cyano-4-hydroxycinnamic acid (CHCA), *N*-glutaryl-L-phenylalanine *p*-nitroanilide (GPNA), pepsin A, and poly-L-Lys hydrobromides (MWs = 62,140 and 4200 Da) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Alkaline phosphatase (AP) was purchased from Biozyme Laboratories (South Wales, UK). Benzoyl-L-arginine *p*-nitroanilide (BAPA) was obtained from Peptide Institute (Osaka, Japan). *p*-Nitrophenylphosphate was obtained from Nacalai Tesque (Kyoto, Japan). All other chemicals were of analytical grade. PTFE tube was purchased from Flon Chemical (Osaka, Japan).

### Preparation of protease-immobilized microreactors

Protease-immobilized microreactors were prepared as reported previously [13,16]. Briefly, enzymes were reacted with bifunctional cross-linker agents, paraformaldehyde, and glutaraldehyde to facilitate enzyme–enzyme covalent binding on PTFE tube (500  $\mu$ m i.d. and 13 cm length). For the CT–microreactor and AP-immobilized microreactor (AP–microreactor), the enzyme (10 mg/ml) and

poly-Lys (MWs = 62,140 Da for CT and 4200 Da for AP, 10 mg/ml) were mixed at a 1:1 volume ratio in 50 mM phosphate buffer (PB, pH 8.0). The cross-linker in 50 mM PB (pH 8.0) (4% paraformaldehyde and 0.25% glutaraldehyde) and enzyme/poly-Lys solutions were supplied to PTFE tube using a T-shaped connector that creates a laminar flow [13]. Solution introduction was performed at different pumping rates (0.75  $\mu$ l/min for the cross-linker and 0.5  $\mu$ l/min for the enzyme/poly-Lys) by an EconoFlow syringe pump (Harvard Apparatus, Holliston, MA, USA). The polymerization reaction was performed for 15 h at 4 °C. Other procedures were the same as reported previously [13,16]. For the TY immobilization microreactor, poly-Lys was omitted and polymerization was performed as reported previously [14].

### Digestion by microreactor

Proteolysis was carried out in buffer A (10 mM ammonium acetate, pH 8.5) at 30 °C. Substrate proteins were dissolved to a concentration of 100  $\mu$ g/ml in buffer A. The protein solution was pumped through the microreactor at a flow rate of 1.2 to 15  $\mu$ l/min using a Pico Plus syringe pump. The peptide fragments were collected in 1.5-ml test tubes and analyzed by MS and high-performance liquid chromatography (HPLC) measurements.

To determine the kinetic parameters,  $K_m$  and  $V_{max}$ , the initial velocities ( $v$ ) were measured at various substrate concentrations ( $[S]$ ) in buffer B (50 mM Tris–HCl, pH 8.0) at 30 °C. The substrate solution was pumped through the microreactor at a flow rate of 5.0  $\mu$ l/min. Synthetic compounds (BAPA for TY–microreactor, GPNA for CT–microreactor, and *p*-nitrophenylphosphate for AP–microreactor) were used as substrates. The reaction was evaluated as the amount of released *p*-nitroaniline or *p*-nitrophenol calculated from absorbance at 405 nm using a spectrophotometer (Multiskan JX, Thermo Fisher Scientific, Waltham, MA, USA). The data were fitted to the Michaelis–Menten equation.

The stability of reusable microreactors was determined using Cyt-C for TY and  $\beta$ -casein for CT in buffer A at 30 °C and was analyzed by MS. The concentration of each substrate was 100  $\mu$ g/ml. The flow rate of the substrate was 2.5  $\mu$ l/min. After each hydrolysis reaction, the protease-immobilized microreactors were washed with buffer B and stored at 4 °C.

The chemical stability of the protease-immobilized microreactors was tested using the synthetic compounds in buffer B with or without 4 M urea at 30 °C. The flow rate of the substrate was 5.0  $\mu$ l/min (reaction time = 5.2 min). The concentrations of BAPA for TY and GPNA for CT were 0.5 and 1 mM, respectively. For the in-solution experiment, the concentration of protease was 40  $\mu$ g/ml and assays were performed for 5.2 min.

### In-solution digestion

The in-solution digestion was performed by adding proteases into the substrate protein at a substrate-to-protease ratio of 50:1. The reaction solution was incubated in buffer A at 37 °C for 18 h. Acetic acid was added into the solution to stop the reaction.

### MS analysis

Electrospray ionization time-of-flight (ESI–TOF) MS measurements were performed using a Mariner mass spectrometer (Applied Biosystems, Foster City, CA, USA) with a scan range of  $m/z$  200 to 2500. The digested samples were dissolved in 50% aqueous acetonitrile and 1% formic acid at a concentration of 50 to 100  $\mu$ g/ml. Under 40  $\mu$ g/ml of the digested samples were difficult to detect the signals using Mariner instrumentation. The acceleration voltage was 4 kV. The electrospray signal was stabilized by a flow of

nitrogen curtain gas set (1 L/min) and nitrogen nebulizer gas set (0.3 L/min). Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS measurements were carried out using Bruker Autoflex (Bruker Daltonics, Billerica, MA, USA). A total of 1.0  $\mu$ l of sample solution and another 1.0  $\mu$ l of CHCA matrix solution (10 mg/ml in 50% [v/v] acetonitrile/H<sub>2</sub>O) were mixed on the plate. Reflection mode was used for detecting the digested peptides. Positive ion was detected, and 50 single-shot spectra were acquired from each sample spot. The acceleration voltage was 20 kV. Peptide fragments were assigned based on the Swiss-Prot database using PeptideMass of the ExPASy Proteomics Server (<http://expasy.org/cgi-bin/peptide-mass.pl>) with the following constraints: tryptic or chymotryptic cleavage and up to two missed cleavage sites. All matching spectra for the digests were manually identified.

#### HPLC analysis

The HPLC analysis of the digested samples was carried out by an Agilent 1100 HPLC system (Agilent Technologies, Santa Clara, CA, USA) using a Zorbax SB-C3 column (4.6  $\times$  250 mm) at room temperature. Elution was performed by using solution A (H<sub>2</sub>O/0.05% trifluoroacetic acid [TFA]) and solution B (acetonitrile/0.04% TFA). The linear gradient was 5% to 65% solution B for 30 min at a flow rate of 1.0 ml/min. The digested sample (10  $\mu$ g) was analyzed.

## Results and discussion

#### Preparation of enzyme-immobilized microreactors

We used two protease-immobilized microreactors in this study: TY- and CT-microreactors. TY hydrolyzes peptide bonds after Arg and Lys residues. Because these basic residues are usually located on the surface of protein, especially in soluble proteins, the digested peptides generally fit the range (<2 kDa) required for analysis by MS. However, if Pro residue is located at the C-terminal side of Arg or Lys, hydrolysis will not occur. In addition, it is possible that the conformational stability of the protein has a resistance for proteolysis. These possibilities will cause the digested peptides to become too large to be detected by MS. Therefore, aside from TY, other endoproteases can be used for MS-based analysis to cover the whole sequence of the target protein. CT hydrolyzes peptide bonds after aromatic residues (Phe, Trp, and Tyr) and after Leu and Met in a less specific way and is often used for proteolysis of hydrophobic proteins such as membrane proteins [5,17]. It is expected that the combination of MS results obtained with the tandem microreactor that carries out multidigestion may give significantly higher sequence coverage than that obtained with

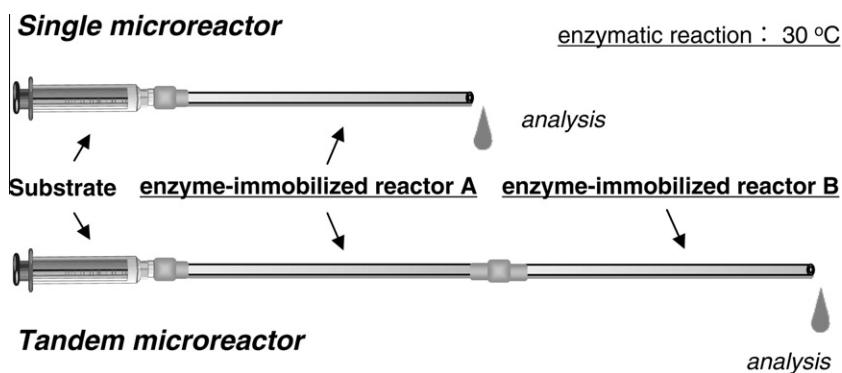
individual digestion by the single microreactor. Based on a similar idea, a reactor that was a bonded mixture of TY and CT to an epoxy monolithic silica column was reported [17]. In contrast to the mixture of TY- and CT-immobilized reactor [17], an interesting feature of our microreactor is the ease in linking each microreactor by using a connector (Fig. 1). In our system, we can easily change the order of each microreactor (e.g., CT-TY or TY-CT) according to our preference.

TY- and CT-microreactors were prepared as reported previously [14,16]. In addition to these protease-immobilized microreactors, we also prepared an AP-microreactor for analysis of protein phosphorylation. Because the pI value of AP is 5.9, an improved polymerization procedure [13] described as an immobilization method for acidic enzyme using poly-Lys as a booster was used for the AP-microreactor. When high molecular weight of poly-Lys (MW = 62 kDa) that was intended for the CT-microreactor in this study was used for the AP-microreactor, an aggregation of protein was readily observed, suggesting that high positively charged poly-Lys (MW = 62 kDa) was quickly interacted with the acidic AP protein by electrostatic interaction. Because our enzyme polymeric membrane is formed on the inner wall of the microchannel (500  $\mu$ m i.d.) through cross-linking polymerization in a laminar flow (see Materials and methods), the quickly aggregated enzyme and poly-Lys can be stuck on the microchannel during cross-linking polymerization. Therefore, it is suggested that the large-molecular-weight poly-Lys (MW = 62 kDa) is not appropriate for preparation of the AP-microreactor. To overcome this problem, a low-molecular-weight poly-Lys (MW = 4 kDa) was used for the polymerization of AP. As expected, with the use of poly-Lys (MW = 4 kDa), quick aggregation was suppressed and the AP-microreactor was successfully prepared.

Enzyme immobilization on PTFE tube was analyzed by the Bradford method to measure the total polymerized enzyme. For example, 50  $\mu$ g of CT was formed by polymerizing on a 1-cm-long PTFE tube. This concentration of immobilized protein was the same as the reported value [14]. Other enzymes were also immobilized on PTFE tubes with similar concentrations. Because these concentrations of the immobilized proteases are higher than those used in the experimental conditions of in-solution digestion, it may be suggested that our microreactors can perform rapid digestion compared with in-solution digestion.

#### Characterization of enzyme-immobilized microreactors

The individual enzymatic activity of the enzyme-immobilized microreactors was evaluated using synthetic small substrates at 30 °C in 50 mM Tris-HCl (pH 8.0): BAPA for the TY-microreactor



**Fig. 1.** Schematic representation of enzymatic reaction using enzyme-immobilized microreactors. The substrate was pumped through the enzyme-immobilized microreactors using a syringe pump. The reaction was carried out at 30 °C. The digests were collected in a sample tube and then analyzed by reverse-phase HPLC and ESI-TOF MS.

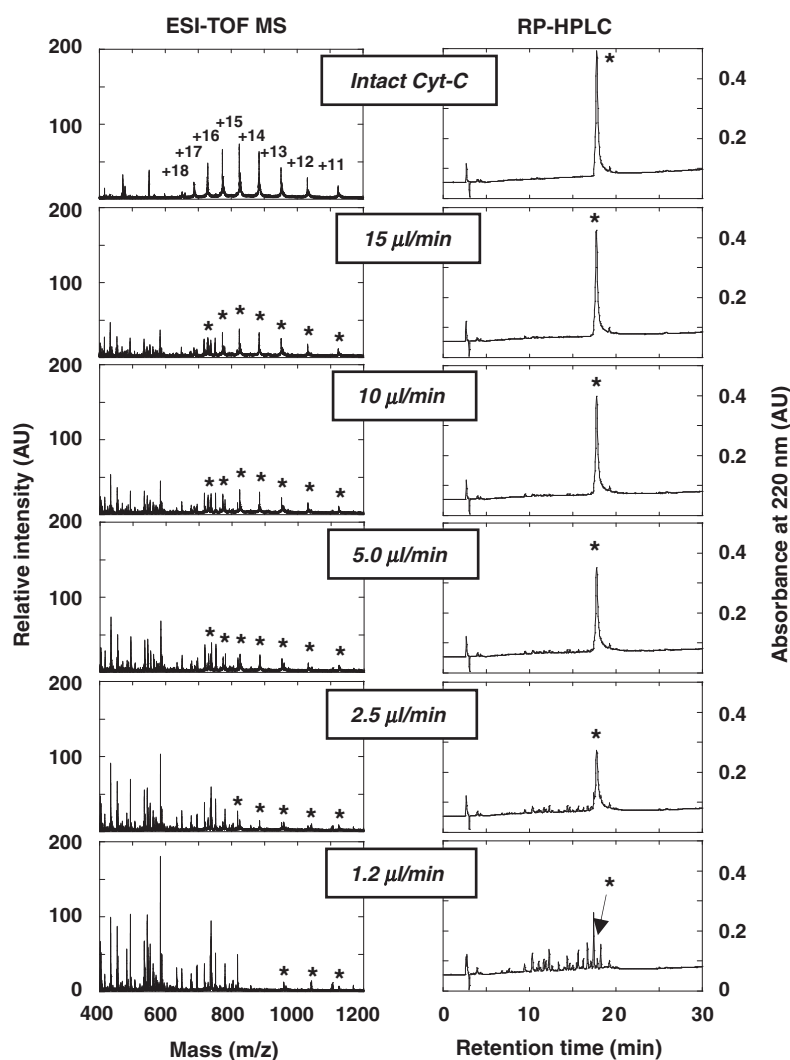
( $K_m = 4.2 \pm 0.7$  mM,  $V_{max} = 583 \pm 76$   $\mu$ M/min), GPNA for the CT-microreactor ( $K_m = 2.5 \pm 0.3$  mM,  $V_{max} = 493 \pm 46$   $\mu$ M/min), and *p*-nitrophenylphosphate for the AP-microreactor ( $K_m = 41 \pm 133$   $\mu$ M,  $V_{max} = 212 \pm 25$   $\mu$ M/min). The estimated kinetic parameters were similar to reported values [14,16], suggesting that immobilized enzymes in this study maintain their own hydrolysis activity after polymerization on PTFE surface.

Our previous proteolysis procedure using microreactors was carried out in 50 mM Tris–HCl (pH 8.0) solution [14]. In this buffer system, an additional purification step using reverse-phase micro-pipette tips prior to MS measurement could remove excessive amounts of buffer salt but could lead to sample losses, especially of hydrophobic peptides due to their inherent affinity to reverse-phase surfaces, leading to lower sequence coverage. In this study, proteolysis was carried out in a 10-mM ammonium acetate buffer (pH 8.5) that easily evaporated during ESI–TOF MS measurement without the need for any desalting procedure. The digested peptides were collected in a test tube and then directly analyzed by ESI–TOF MS and reverse-phase HPLC (Fig. 1). A syringe pump was used to deliver the substrates. A reaction time is correlated with the flow rate of the substrate. In the current single microreactor, proteolysis at a flow rate of 2.5  $\mu$ L/min yields a reaction time of 10.4 min (PTFE tube volume of 26  $\mu$ L). To study the effect of flow

rate on the digestion efficiency, we first investigated the digestion of Cyt-C by the TY-microreactor at several flow rates. Under our experimental conditions, with a flow rate increase from 1.2 to 15  $\mu$ L/min, the autodigestion peak of protease was not observed by MS and HPLC analyses (data not shown). In addition, MALDI–TOF MS analysis indicated that no free proteases or cross-linked aggregations came off from the PTFE tubes (spectrum not shown), demonstrating good mechanical stability.

ESI–TOF MS spectra and reverse-phase HPLC profiles of digests by microreactor at different flow rates are shown in Fig. 2. With a flow range of 2.5–15  $\mu$ L/min, the intact Cyt-C was observed while below 1.2  $\mu$ L/min over 90% of Cyt-C was digested by TY-microreactor. The results indicate that digestion at a lower flow rate (longer reaction time) is more efficient. Although intact Cyt-C remained in the samples at a flow rate of more than 2.5  $\mu$ L/min, the matched peptides covered 93% (97/104 amino acids) of the Cyt-C sequence. This value was the same as that of the digested sample at 1.2  $\mu$ L/min. The CT-microreactor showed similar characteristics in  $\beta$ -casein digestion (data not shown). Based on these results, the following experiments were carried out at a flow rate of 2.5  $\mu$ L/min.

The operational stability and reproducibility of the microreactors were tested based on the digestions of Cyt-C for the TY-microreactor and  $\beta$ -casein for the CT-microreactor. Between each



**Fig. 2.** ESI–TOF MS spectra (left) and reverse-phase HPLC (RP–HPLC) profiles (right) of the digests of Cyt-C by the TY-microreactor. Digestion of Cyt-C (100  $\mu$ g/ml) was carried out at different flow rates: 1.2, 2.5, 5.0, 10, and 15  $\mu$ L/min in 10 mM ammonium acetate buffer (pH 8.5) at 30 °C. The intact Cyt-C is marked with an asterisk (\*). For RP–HPLC analysis, the linear gradient was 5% to 65% acetonitrile in 30 min at a flow rate of 1.0 ml/min at room temperature.



digestion, both microreactors were washed with buffer solution and stored at 4 °C for more than 60 days. The obtained 10 MS spectra (not shown) were identical to the similar sequence coverage of 93% (Cyt-C by TY-microreactor) and 57% ( $\beta$ -casein by CT-microreactor). In contrast, free proteases almost completely lost their activities at 25 °C within a couple of days, as reported previously [18]. These results indicate that the stability of proteases was increased by the prevention of autodigestion after an enzyme immobilization. In addition, the stability of the microreactors in urea solution was tested by the digestions of BAPA for the TY-microreactor and GPNA for the CT-microreactor. Similar to the previous report [16], both immobilized proteases were more stable at 4 M urea than were free proteases (Fig. S1 in Supplementary material). The proteolysis of Cyt-C by the protease-immobilized microreactor was also efficiently carried out with a high concentration of denaturant (3 M guanidinium chloride) [14]. These results suggest that the stability of immobilized proteases is superior to that of free proteases.

#### Protein digestion by protease-immobilized microreactors

Cyt-C (nonphosphoprotein),  $\beta$ -casein (phosphoprotein), and pepsin A (phosphoprotein) were used to test the performance of the tandem microreactors. The enzymatic reaction at a flow rate of 2.5  $\mu$ l/min yielded a reaction time of 10.4 min (single microreactor) or 20.8 min (tandem microreactor). The digestion efficiency by the microreactors was evaluated by analyzing the sequence coverage and the identified peptide.

Table 1 summarizes the ESI-TOF MS results of the protein digests using different digestion approaches. All identified peptides from the digestion of Cyt-C are listed in Tables S1–S5 in the Supplementary material. Some of the digested peptides by the microreactors were the expected peptides that have one or two missed cleavage sites. The *pI* value of Cyt-C is 9.6 (horse residues 2–104), suggesting that Arg or Lys residues locate the protein surface with high possibility. As expected, Cyt-C was digested by the TY-microreactor with higher sequence coverage (93%) at 30 °C. MALDI-TOF MS analysis also showed high sequence coverage (89% in Table S14). The value of sequence coverage was higher than the other TY-immobilized reactors reported [19,20] and the same as that performed by in-solution digestion (37 °C for 18 h), suggesting that the immobilized TY in this study showed rapid and efficient proteolysis. However, the CT-microreactor showed lower

sequence coverage (38%) than that by in-solution digestion (65%). A possible reason for the lower digestion ability could be the mass transfer limitation of folded Cyt-C in the cross-linked protease and poly-Lys complex matrix, as discussed previously [14].

The multidigestion of Cyt-C by the tandem microreactor that was connected by using the TY- and CT-microreactors was evaluated. The TY-CT tandem microreactor also showed rapid Cyt-C digestion with similar sequence coverage as that of the single TY-microreactor (Table 1). As shown in Table S3, multidigested peptides such as VQK, TGPNIHLGLF, and TGQAPGF were identified. Some digested peptides by the tandem microreactor were too small (<300 Da) to be identified by our MS analyses; therefore, based on comparison with the single TY-microreactor, the sequence coverage for Cyt-C by the tandem microreactor was found not to improve (88%, 91/104 amino acids) (Table 1). These results indicate that the peptide fragments by the TY-microreactor were also digested by the CT-microreactor, as expected.

Bovine  $\beta$ -casein (residues 16–224) is a phosphoprotein with well-characterized phosphorylated sites [6,21]. MS measurement of an intact protein revealed that  $\beta$ -casein in this study has five phosphorylated sites (data not shown). By measuring the decrease in the peak area at 220 nm of HPLC profiles of the digests, it could be estimated that more than 90% of  $\beta$ -casein was digested by the CT-microreactor for 10.4 min at 30 °C (Fig. 3A and B). ESI-TOF MS analysis revealed that 13 peptides containing 120 of the 209 possible amino acids of  $\beta$ -casein were obtained, producing a sequence coverage of 57% (Tables 1 and S7). This value was higher than that by in-solution digestion (45%, 95/209 amino acids). Moreover, the phosphopeptide containing 4 phosphoserine (pS) residues (pSpSpSEEpSITRINKKIEKF) was detected despite the low ionization efficiency of the phosphopeptide. To confirm this detection of the phosphopeptide by another MS system, MALDI-TOF MS analysis was performed. In addition to the pSpSpSEEpSITRINKKIEKF phosphopeptide, the QpSEEQQTDEL phosphopeptide was also identified by MALDI-TOF MS analysis (Table S15), thereby showing that all phosphorylation sites on  $\beta$ -casein in this study were detected from the digests by the CT-microreactor.

In contrast, HPLC analysis of digested  $\beta$ -casein by the TY-microreactor (Fig. 3C) showed a broad profile and was different from that by the CT-microreactor (Fig. 3B). In addition to the *pI* value of  $\beta$ -casein (5.1), which was estimated from the primary structure and which did not take into account the number of phosphorylation sites, the total number of Phe, Trp, Tyr, Leu, and Met residues

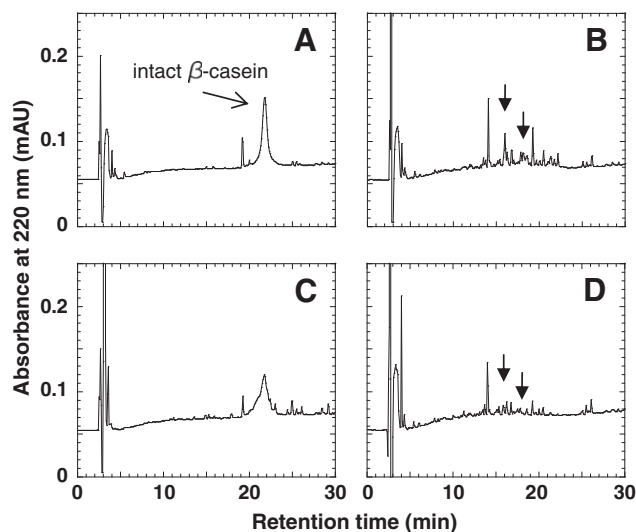
**Table 1**  
Summary of ESI-TOF MS results of digests of Cyt-C,  $\beta$ -casein, and pepsin A using different digestion approaches.

Digestion method <sup>a</sup>	Protein	Digestion time	Identified amino acids <sup>b</sup>	Sequence coverage (%)
TY-microreactor	Cyt-C	10.4 min	97	93
CT-microreactor	Cyt-C	10.4 min	40	38
TY-CT tandem microreactor	Cyt-C	20.8 min	91	88
TY (in-solution)	Cyt-C	18 h	99	95
CT (in-solution)	Cyt-C	18 h	68	65
TY-microreactor	$\beta$ -Casein	10.4 min	30	14
CT-microreactor	$\beta$ -Casein	10.4 min	120	57
CT-TY tandem microreactor	$\beta$ -Casein	20.8 min	146	70
TY (in-solution)	$\beta$ -Casein	18 h	44	21
CT (in-solution)	$\beta$ -Casein	18 h	95	45
TY-microreactor	Pepsin A	10.4 min	N.D.	N.D.
CT-microreactor	Pepsin A	10.4 min	179	55
TY (in-solution)	Pepsin A	18 h	19	6
CT (in-solution)	Pepsin A	18 h	196	60

Note. N.D., not determined due to undetectable hydrolysis activity.

<sup>a</sup> The reaction was carried out in 10 mM ammonium acetate buffer (pH 8.5) at 30 °C. The reaction time by the microreactor was 10.4 min (single microreactor) or 20.8 min (tandem microreactor) with a flow rate of 2.5  $\mu$ l/min. The in-solution digestion was carried out at 37 °C for 18 h. Concentrations of substrate and free protease were 100 and 2  $\mu$ g/ml, respectively.

<sup>b</sup> The detected peptides were identified by the MS-Fit tool of the Protein Prospector database. Identified peptides from the digests are listed in Tables S1–S13 in the supplementary material.



**Fig. 3.** HPLC profiles of  $\beta$ -casein digestion by different digestion approaches: (A) intact  $\beta$ -casein; (B) CT-microreactor; (C) TY-microreactor; (D) CT-AP tandem microreactor. For proteolysis, a total of 100  $\mu$ g/ml  $\beta$ -casein was injected through the microreactors at a flow rate of 2.5  $\mu$ l/min. The reaction was carried out in 10 mM ammonium acetate buffer (pH 8.5) at 30  $^{\circ}$ C for 10.2 min (single microreactor) or 20.4 min (tandem microreactor). The analytical conditions of HPLC are shown in Fig. 2. The peaks that disappeared after dephosphorylation reaction are marked with solid arrows.

(42) was larger than that of Arg and Lys residues (15). Therefore, it is suggested that the sequence coverage of  $\beta$ -casein by the TY-microreactor (14%) or by in-solution digestion by free TY (21%) is lower than that by the CT-microreactor (Tables 1, S6, and S9).

We next studied the feasibility of the enzyme-immobilized tandem microreactor. As expected, the multidigestion by the CT-TY tandem microreactor showed 20 digested peptides. The sequence coverage by the CT-TY tandem microreactor (70%) was higher compared with that of the single CT-microreactor (57% in Table 1). It is noteworthy that GVSK, VKEAMAPK, HKEMPFKP and YPVEFP peptides that were not identified by the single CT-microreactor (Tables S7 and S8) were identified by the tandem microreactor. The results indicate that an improvement of the sequence coverage in digestion by the tandem microreactor in comparison with the single microreactor and in-solution digestion can be expected. Moreover, the HPLC profile of the digested peptides by the CT-AP tandem microreactor revealed the disappearance of two peaks compared with that by the single CT-microreactor (Fig. 3B and D), suggesting that the phosphopeptides containing pSpSpSEEpSITRINKKIEKF were dephosphorylated by AP. MS analysis also revealed the dephosphorylation of the pSpSpSEEpSITRINKKIEKF phosphopeptide (spectrum not shown). The results indicate that the tandem microreactor that was made by using the protease-immobilized microreactor and the phosphatase-immobilized microreactor showed the feasibility of the identification of phosphorylation site in phosphoproteins without any enrichment strategies and radioisotope labeling.  $\beta$ -Casein has another well-known phosphorylated site (Ser35). Because the phosphopeptide containing Ser35 (QpSEEQQQTEDEL) was detected by our MALDI-TOF MS analysis (Table S15), it is possible that the one peak that disappeared in the HPLC profile (Fig. 3D) may be the phosphopeptide containing pS35.

Pepsin A (porcine residues 60–385) is an acidic protease ( $pI$  of 3.2) and a phosphoprotein that has one phosphoserine residue [7]. An optimal pH of pepsin A for its protease activity is approximately 2.0, suggesting that pepsin A does not have any activity under our digestion condition at pH 8.5. We confirmed that pepsin A

was inactive at pH 8.5 (data not shown); therefore, pepsin A was used as a substrate without any denaturation procedure. All identified peptides from the digests by ESI-TOF MS analysis are listed in Tables S11–S13. Similar to the digestion of  $\beta$ -casein, pepsin A was efficiently digested by the CT-microreactor but not by the TY-microreactor (Table 1), thereby explaining the difference between the total number of cleavage sites by CT (65 residues) and by TY (3 residues). The sequence coverage of 55% (179/326 amino acids) by the CT-microreactor was lower than that by in-solution digestion (60%, 196/326 amino acids). In addition, the phosphopeptide (EATpSQELSITY) was detected in the digestion by in-solution digestion but not by the CT-microreactor. When the HPLC profile of the digests by the single CT-microreactor was compared with the CT-AP tandem microreactor digest, it was found out that one peak disappeared after passing through the tandem microreactor (not shown). This suggests that the digests by the CT-microreactor also contain the phosphopeptide but that the size of the peptide was bigger than the EATpSQELSITY phosphopeptide from in-solution digestion. In addition, it is possible that our MS system was not able to detect the phosphopeptide from the CT-microreactor. A possible reason for the lower digestion ability of the CT-microreactor could be mass transfer limitation of folded pepsin A in the cross-linked CT and poly-Lys complex matrix. Similar lower digestion ability of the immobilized protease was observed in the digestion of Cyt-C by the CT-microreactor as described above.

## Conclusion

Several protease-immobilized microreactors have been developed for proteolysis [9,12,15,18,19]. Most of these studies have focused on rapid digestion and reduction of sample volume. So far, there is no study yet on a multienzymatic reaction system and analysis of posttranslational modification in protein. In the current study, we showed a simple and rapid analytical method for the identification of protein sequence by using tandem protease-immobilized microreactors. Proteolysis by the tandem microreactors showed higher sequence coverage, which is a remarkable result compared with that of the single microreactor or in-solution digestion (Table 1). In addition, the tandem microreactor composed of a protease-immobilized microreactor and a phosphatase-immobilized microreactor showed the capability to localize the phosphorylation site(s) in phosphoproteins. The current analytical method is much simpler than the other conventional methods [6–8]; for example, the phosphoprotein is just flowed through the microreactor, and it eliminates purification of digests from the reaction system without any enrichment strategies. These interesting features are superior advantages of our approach using the enzyme-immobilized microreactors over the conventional methods. In addition, it is known that in-solution digestion by trypsin can induce artificial modifications such as asparagine deamidation [22] and N-terminal glutamine cyclization [23,24] on target protein due to the elevated temperature and alkaline pH buffers used during digestion. Proteolysis by our protease-immobilized microreactors was achieved within a short period of time ( $\sim$ 10 min) at 30  $^{\circ}$ C, thereby suggesting these artificial modifications as a remote possibility.

In conclusion, the approach using a tandem enzyme-immobilized microreactor coupled with MS is a simple and rapid procedure for the analysis of protein sequence, including posttranslational modification. In addition, our enzyme immobilization method using poly-Lys as a booster can be applied to proteins with a wide range of  $pI$  values [13]; hence, the strategy based on multienzymatic reaction using the tandem microreactor provides a useful approach for other posttranslational modification analyses (e.g., acetylation, methylation, ubiquitination, glycosylation) or for

a consecutive enzymatic reaction system such as synthesis of organic compounds for clinical drugs.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.ab.2010.07.026](https://doi.org/10.1016/j.ab.2010.07.026).

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