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Pep-MS assay: Protease hydrolysis assay system using photo-cleavable peptide array and mass spectrometer

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Food processing technology such as protein hydrolysis using proteases has been receiving a lot of attention, and it is important to accurately understand the cleavage specificity of each protease for selecting a protease suited to aims. Although numerous methods have been reported to reveal the substrate specificity of proteases, there is no method to evaluate simply, quickly, reasonably, and accurately. This study set out to devise Pep-MS assay, a novel assay system that can be used to comprehensively clarify positions at which proteases cleave, by combining a mass spectrometer and a photo-cleavable peptide array. First, we evaluated peptide array corresponding to the primary sequences of α_{S1}-casein, α_{S2}-casein and β-casein with trypsin to verify the accuracy of the Pep-MS assay. The evaluation of cleavage positions by the trypsin protease reagent using the Pep-MS assay resulted in a matching rate of about 96.8% to rational cleavage positions. Next, we confirmed the cleavage positions in α_{S2}-casein or β-lactoglobulin by an industrial bacterial protease from *Bacillus subtilis* at some protease reaction temperatures or reaction times. The Pep-MS assay clarified the differences in the cleavage patterns due to the reaction temperature, and the change in the cleavage strength with the reaction time. Pep-MS assay is a promising method for evaluating the substrate specificity of proteases, which will be useful to find effective production conditions for functional peptide from foods and effective hydrolysis conditions for decreasing allergen of food proteins.

[**Keywords:** Peptide array; Screening system; Protease; Cleavage position; Mass spectrometer]

Various physiological activities have been identified for peptides derived from food proteins, and it is likely that food processing technology based on protein hydrolysis using proteases will become increasingly important in the future. However, although industrial proteases used for food processing exhibit a wide variety of enzymatic activities, the cleavage specificity for many of these proteases has not been clarified. To select an optimal protease for processing and to find effective production conditions for functional peptides, it is important to accurately understand the cleavage specificity of each protease.

A variety of methods have been reported to reveal the substrate specificity of the protease, such as phage display (1,2), position scanning (3–5), peptide arrays (6,7), fluorescence resonance energy transfer (FRET)-based peptide library assays (8–10), and liquid chromatography-tandem mass spectrometry (LC-MS/MS)-based peptide mapping assays (11,12). While each of these methods has provided valuable information about the substrate specificity of proteases and several of them remain popular choices for investigation, they each suffer from some drawbacks.

For example, substrate phage display techniques display a random substrate peptide added an antibody antigen sequence to the terminal. When the phage displaying the substrate is hydrolyzed with the protease and the phage is recovered by the antibody binding reaction, only the phage with the cleaved substrate remains in the solution. By reading the substrate sequence presented by the phage in this solution, it is possible to identify the selective substrates of the protease. It has the advantage of being able to generate and screen very large libraries and reveal individual substrate sequences for a protease, however it is not able to reveal the actual site of cleavage (1,2). FRET-based peptide library assay employs a fluorescent donor and quencher pair placed on the two termini of a resin-bound substrate peptide. Cleavage of the peptide by an endopeptidase removes the N-terminal quencher, and the bead becomes fluorescent (8–10). The FRET method has the advantages of being compatible with both natural and unnatural building blocks, which make it capable of handling large libraries, but it is not able to reveal the actual site of cleavage, the same issue as in the case of phage display.

To determine the site of cleavage, Edman sequencing of the partially-cleaved peptides on the positive bead can be used (10). This technique can provide information on individual sequences, but the main shortcoming is that the Edman sequencing is very time-consuming and expensive, making the method less ideal for high-throughput profiling of a large number of proteases. LC-MS/MS-based peptide mapping assays are often used for metabolomics analysis. In this method, each peptide fragment in a protein hydrolysate is comprehensively analyzed by LC-MS/MS and the

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amino acid sequence of each peptide is identified by comparing the analysis data with the database of a known protein. This method has the advantages of being able to confirm the cleavage specificity of a protease for proteins briefly and quickly, but has shortcomings of possibly leading to false positives and false negatives.

In the present study, to overcome the shortcomings of the known evaluation methods, we set out to develop a novel assay, Pep-MS assay, to identify protease cleavage positions by combining a photo-cleavable peptide array with a mass spectrometer, and aimed to reasonably and accurately evaluate the substrate specificity of proteases. We synthesized a substrate peptide on a cellulose membrane to prepare a peptide array, and hydrolyzed the peptide array using a protease. After peptide fragments on the peptide array are liberated by UV irradiation and collected, the molecular weight of the peptide fragments was determined with a mass spectrometer to identify the site cleaved site by the protease. Pep-MS assay would be useful for evaluating the substrate specificity of a protease simply, quickly, reasonably, and accurately.

MATERIALS AND METHODS

Synthesis of photo-cleavable peptide arrays This synthesis of photo-cleavable peptide arrays was carried out as reported previously (13). A cellulose membrane (grade 542; Whatman, Maidstone, UK) was activated using β-alanine as the N-terminal basal spacer. An Fmoc photo-linker [4-(1-aminoethyl)-2-methoxy-5-nitrophenoxy]-butanoic acid derivative (sc-294977A; Santa Cruz Biotechnology, Dallas, TX, USA) served as a photo-cleavable linker for the Fmoc peptide synthesis (14). The linker conjugated the candidate peptides with cellulose. The Fmoc-activated amino acid (0.25 mol/L) was spotted on the membrane using a peptide auto-spotter (ASP222; Intavis, Cologne, Germany), by following the manufacturer's recommendations but with some modifications. After the addition of the first residue, the remaining amino groups were blocked twice with 5% acetic anhydride for 15 min. At each elongation step, the membrane was deprotected using 20% piperidine and then washed thoroughly with *N,N*-dimethylformamide, followed by a wash with methanol. After the final deprotection, the side-chain-protecting groups were removed over 2.5 h using a mixture of trifluoroacetic acid (TFA, A00025; Watanabe, Hiroshima, Japan), m-cresol (034–04646; Wako, Osaka, Japan), 1,2-ethanedithiol (A00057; Watanabe), and thioanisole (T0191; Tokyo Chemical Industry, Tokyo, Japan) at a ratio of 40:1:3:6. Finally, the membrane was washed thoroughly with diethyl ether and then methanol. By analyzing each peptide residue with bromophenol blue (BPB), we confirmed the quality of the synthesized peptides using the peptide auto-spotter (Fig. 1A).

Hydrolysis reaction to peptide arrays by protease Some peptide arrays were hydrolyzed by stirring them with 2 μg/mL of an *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK)-treated trypsin protease reagent solution (P8101S; New England Biolabs Japan, Tokyo, Japan) at a given temperature for a predetermined time. Other peptide arrays were hydrolyzed by stirring in 5 mg/mL microbial protease solution from *Bacillus subtilis* (76774-43-1; Amano Enzyme, Aichi, Japan) at a given temperature for a predetermined time. After the reaction of the protease, the peptide arrays were rinsed with water followed by ethanol. Finally, they were dried in a warming incubator (Fig. 1B).

Release of peptides from photo-cleavable peptide arrays Each peptide in a photo-cleavable peptide array was cleaved from the solid phase by irradiation with 302-nm UV. The peptide arrays were dried completely at room temperature, then irradiated with 302-nm UV for 2 h using a transilluminator (TM-20; Funakoshi, Tokyo, Japan) (13). It has been already confirmed that UV irradiation at 302 nm for 2 h can release a peptide from arrays as effectively as UV irradiation for 2 h at 365 nm, which has been demonstrated in the past (data not shown). Subsequently, each spot on the array was punched using a biopsy punch. Each resulting peptide-containing disk (peptide spot) was placed in a well of a 96-well plate with a filter (MSRLN0410; Merck Millipore, Darmstadt, Germany). Next, the peptide was released into 200 μL of distilled water (11307-79; Kanto Chemical, Tokyo, Japan) at 25°C for 2 h. After the release of each peptide, the solution containing the peptide was filtered and recovered by centrifugation at 500 × *g* to remove any insoluble materials (Fig. 1C–F) (15).

Mass spectrometric analysis of the released peptide Each sample was loaded onto a 2 mm × 250 mm C18 reverse-phase column (TSKgel ODS-100 V 5 μm; Tosoh, Tokyo, Japan). Samples were then analyzed using a mass spectrometer. The peptides were eluted using a gradient of buffer A (0.2% formic acid in water) and

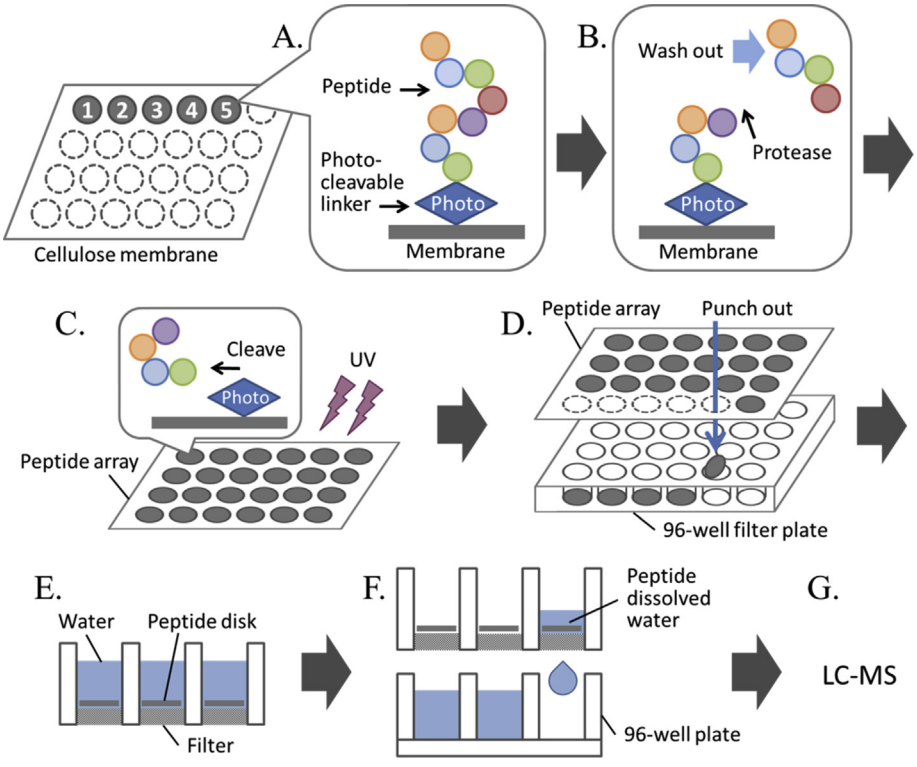

FIG. 1. Design of a Pep-MS assay that combines a photo-cleavable peptide array and a mass spectrometer for confirming the position of protease cleavage: (A) Starting from the peptide synthesis on a cellulose membrane using a peptide array synthesizer, this figure is an overview of how a peptide is spotted on the cellulose membrane. The word "Photo" means photo-cleavable linker. (B) Peptide arrays were hydrolyzed using a protease. (C) Peptides spotted on a membrane were released by UV irradiation. (D) Each peptide spot was punched out as a disk and placed in one well of a 96-well plate. (E) These peptides were dissolved in water, and (F) the resulting solution was filtered. (G) The molecular weight of each peptide fragment was analyzed by LC-MS.

buffer B (0.2% formic acid in acetonitrile) at a flow rate of 200 mL/min. A 30-min gradient was run with 2%–20% B over 7 min, 20%–60% B over 4 min, 60%–80% B over 1 min, 80% B for 3 min, 80%–2% B over 0.1 min, and held at 2% B for 14.9 min. As the peptides eluted from the microcapillary column, they were electrosprayed directly into the mass spectrometer with a distal 4.5-kV spray voltage. MS data analysis was performed using the Xcalibur software (Xcalibur 1.3; Thermo Fisher Scientific, Tokyo, Japan) (Fig. 1G).

Peptide mapping We hydrolyzed α-casein (C-6780; Sigma–Aldrich, St. Louis, MO, USA) and β-casein (C-6905; Sigma–Aldrich) using a trypsin protease reagent at 25°C for 16 h. The hydrolyzed peptide fragments of α-casein and β-casein were identified by LC separation and MS. Briefly, casein hydrolysates were subjected to LC-MS/MS analysis. Ten microliters of samples were injected into a reverse-phase column (TSKgel ODS-100 V 5 μm; Tosoh) and were separated using solvent A (2% [v/v] acetonitrile and 0.2% [v/v] formic acid in deionized water) and solvent B (90% [v/v] acetonitrile and 0.2% [v/v] formic acid in deionized water) as the mobile phase. The proportion of solvent B was increased linearly from 2% to 45% over 90 min at 25°C at a flow rate of 200 μL/min. The mass spectrometer was set to the data-dependent MS/MS mode (m/z 200–2000), in which 1 full MS scan was followed by 1 MS/MS scans. MS/MS data analysis was performed using the Proteome Discoverer software (Proteome Discoverer 2.1; Thermo Fisher Scientific) with the SEQUEST algorithm. SEQUEST was set up to search the Uniprot *Bos taurus* reference database, assuming a non-specific digestion enzyme. The search parameters included a 2 Da tolerance for precursor ion masses and a 0.6 Da tolerance for fragment ion masses.

Formol titration The α-amino nitrogen (AN) was titrated using formaldehyde as described for the Sorensen method (16). Total nitrogen (TN) was determined by the Kjeldahl method (16). Then, the degree of hydrolysis (DH) was calculated using the ratio between AN and TN according to Eq. 1.

$$\%DH = \frac{AN}{TN} \times 100 \quad (1)$$

RESULTS AND DISCUSSION

Analysis of all positions of amino acids in casein cleaved by the trypsin protease reagent First, to check the analysis accuracy of Pep-MS assay, we evaluated the cleavage positions using a trypsin protease reagent. We synthesized a photo-cleavable peptide array consisting of 8-mer overlapped by 6 amino acids (2 amino acid-offset) and corresponding to the primary sequences of α_{S1}-casein, α_{S2}-casein and β-casein (Table 1), and hydrolyzed the peptide array using a trypsin protease reagent at 25°C for 16 h. Trypsin protease is known to cleave the C terminus of Lys and Arg (except Lys-Pro and Arg-Pro), 19 sites for α_{S1}-casein, 29 sites for α_{S2}-casein, and 15 sites for β-casein are correct cleavage sites (Fig. 2, open triangles).

After the reaction with the trypsin protease reagent followed by release by 302-nm UV irradiation, the recovered peptides on each spot were analyzed by LC/MS. The cleaved fragments were subjected to full scan detection, and the N-terminal side of a detected fragment for which the ion intensity peak area was $\geq n \times 10^6$ (n is the number of amino acids constituting each fragment) was set as a cleavage position. For example, the chromatograph of spot No. A-9 NENLLRFF showed two MS peaks: one with a molecular weight of 312.2 Da corresponding to FF and another with a molecular weight of 1051.8 Da corresponding to NENLLRFF (Fig. S1C). According to this result, we identified the point between NENLLR and FF as the cleavage position of trypsin. The list of analysis results is shown in Table 2. When the data of these cleavage positions were summelized, 19 cleavage sites for α_{S1}-casein, 28 sites for α_{S2}-casein, and 14 sites for β-casein were detected as cleavage sites (Fig. 2, closed triangles), and there was 1 false-positive cleavage site for α_{S1}-casein. Based on the above results, calculation of the ratio of the number of matched sites among the total of the number of sites identified by evaluation and the number of theoretical sites provides a match rate of this evaluation of 96.8% (120/124).

Meanwhile, when trypsin cleavage positions for casein protein were identified by conventional LC-MS/MS-based peptide mapping assay, 20 cleavage sites for α_{S1}-casein, 17 sites for α_{S2}-casein and 25 sites for β-casein were detected as cleavage sites (Fig. 2, shaded triangles), and there were 4 false-positive cleavage sites for α_{S1}-casein, 2 false-positive cleavage sites for α_{S2}-casein, and 13 false-positive cleavage sites for β-casein. Based on these results, the match rate of the evaluation by a conventional mapping assay is 68.8% (86/125). Comparison of the accuracy of the two kinds of assay methods indicates that the Pep-MS assay allows the evaluation of the cleavage sites with much higher sensitivity and accuracy. Unfortunately, even Pep-MS assay resulted in one false-positive and three false negative cleavage sites. Especially in the evaluation of the N-terminal of SPOT No. 1 of α_{S1}-casein, α_{S2}-casein and β-casein (Table 1), the estimated cleavage site was often not coincident with the theoretical cleavage site. This difference might be due to the difference between the peptide conformation when the protease cleaves the interior part of the peptide and when it cleaves the amino acid at the N terminal of the peptide. Further verification of this hypothesis will be a next task in our research.

Effect of temperature conditions on the cleavage positions using a microbial protease from *B. subtilis* Next, to compare the differences in the cleavage positions resulting from differences in the enzymatic degradation conditions, we evaluated using a microbial protease from *B. subtilis*. We synthesized a photo-cleavable peptide array consisting of 7-mer overlapped by 6 amino acids (1 amino acid offset) and corresponding to a part of the primary sequences of α_{S2}-casein (Table 1). We hydrolyzed the peptide array using a microbial protease from *B. subtilis* at 37°C, 50°C, or 63°C for 300 min. After the reaction of microbial protease and release by irradiation with 302-nm UV, the peptides on each recovered spot were analyzed by LC/MS. As mentioned above, the cleavage results of α_{S2}-casein were defined by two levels of scan detection.

Fig. 3 shows that the results under three temperature conditions reveal roughly similar cleavage positions, but some differences in the cleavage strength were confirmed for several sites, such as Tyr-Gln, Lys-Ala, Ala-Met and Val-Arg. This evaluation resulted in the identification of the highest cleavage strength at 50°C. The degree of hydrolysis (DH) of casein hydrolysate treated with this protease also indicated that the cleavage strength at 50°C to be the highest (18.1% at 37°C, 18.9% at 50°C, and 15.0 % at 63°C). The above analysis shows that the Pep-MS assay clarifies the difference in the cleavage

TABLE 1. List of peptides synthesized on peptide arrays.

(A) α_{S1}-Casein		(B) α_{S2}-Casein		(C) β-Casein		(D) (A part of) α_{S2}-casein		(E) β-Lactoglobulin	
Spot No.	Sequence	Spot No.	Sequence	Spot No.	Sequence	Spot No.	Sequence	Spot No.	Sequence
1	RPKHPIKH	1	KNTMEHVS	1	RELEELNV	1	QKFALPQ	1	LIVTQTM
2	KHPIKHQG	2	TMEHVSSS	2	LEELNVPG	2	KFALPQY	2	IVTQTMK
3	PIKHQGLP	3	EHVSSSEE	3	ELNVPGEI	3	FALPQYL	3	VTQTMKG
4	KHQGLPQE	4	VSSSEESI	4	NVPGEIVE	4	ALPQYLK	4	TQTMKGL
96	SEKTTMPL	100	KVIPYVRY	101	VRGPFPII	29	VIPYVRY	155	QLEEQCH
97	KTTMPLWA	101	IPYVRYLA	102	GPFPIIVA	30	IPYVRYL	156	LEEQCHI

(A) 97 spots for α_{S1}-casein, (B) 101 spots for α_{S2}-casein, (C) 102 spots for β-casein, (D) 30 spots for a part of α_{S2}-casein and (E) 156 spots for β-lactoglobulin were synthesized on peptide arrays.

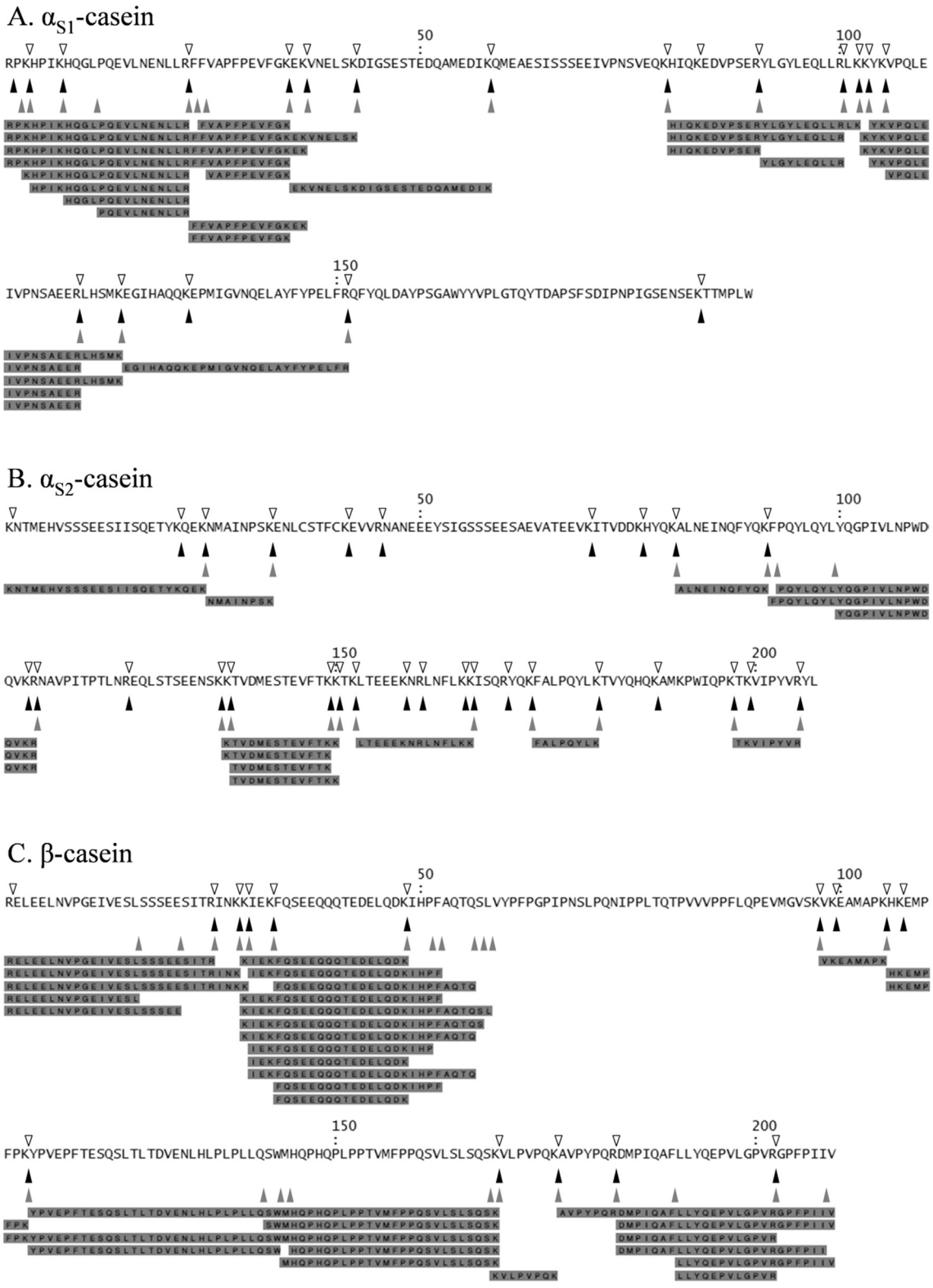

FIG. 2. Evaluated result map for cleavage positions: Cleavage positions in α_{s1}-casein (A), α_{s2}-casein (B), and β-casein (C) were mapped using the trypsin protease reagent. Open triangles indicate the actual cleavage positions, closed triangles indicate the cleavage positions identified by Pep-MS assay, and shaded triangles and bars indicate cleavage positions and peptide fragments identified by the peptide mapping system.

patterns and cleavage strength due to the reaction temperature of the protease. Since it is very important to determine the optimum reaction temperature of the protease when it is used to process proteins (17), we believe that Pep-MS assay would be a convenient and useful tool for understanding the efficient production conditions of peptides.

Confirmation of the change in cleavage strength with the protease reaction time Next, to confirm the change in the

TABLE 2. Detected fragments for which ion intensity peak area is $\geq n \times 10^6$.

α_{S1}-Casein		α_{S2}-Casein		β-Casein	
Spot No.	Sequence	Spot No.	Sequence	Spot No.	Sequence
1	R↓PK↓HPIKH	8	ISQETYK↓Q	10	SEESITR↓I
2	KHPIK↓HQG	10	TYKQEK↓NM	11	ESITR↓INK
3	PIK↓HQGLP	11	KQEK↓NMAI	12	ITR↓INK↓K↓I
9	NENLLR↓FF	14	AINPSK↓EN	13	RINK↓K↓IEK
10	NLLR↓FFVA	18	LCSTFCK↓E	14	NK↓K↓IEK↓FQ
11	LR↓FFVAPF	19	STFCK↓EVV	15	KIEK↓FQSE
15	PEVFGK↓EK	20	FCK↓EVVR↓N	23	LQDK↓IHPF
16	VFGK↓EK↓VN	21	KEVVR↓NAN	46	EVMGVSK↓V
17	GKEK↓VNEL	33	ATEEVK↓IT	47	MGVSK↓VKE
19	VNELSK↓DI	34	EEVK↓ITVD	48	VSK↓VK↓EAM
20	ELSK↓DIGS	36	ITVDDK↓HY	49	KVK↓EAMAP
27	AMEDIK↓QM	37	VDDK↓HYQK	50	KEAMAPK↓H
37	PNSVEQK↓H	38	DKHYQK↓AL	51	AMAPK↓HKE
38	SVEQK↓HIQ	39	HYQK↓ALNE	52	APK↓HKEMP
39	EQK↓HIQKE	40	QK↓ALNEIN	53	KHK↓EMPFP
43	DVPSER↓YL	43	INQFYQK↓F	54	KEMPFPK↓Y
44	PSER↓YLGY	44	QFYQK↓FPQ	55	MPFPK↓YPV
45	ER↓YLGYLE	45	YQK↓FPQYL	56	FPK↓YPVEP
48	LEQLLR↓LK	54	NPWDQVK↓R	82	LSLSQSK↓V
49	QLLR↓LKK↓Y	55	WDQVKR↓NA	83	LSQSK↓VLP
50	LRLK↓K↓YK↓V	56	QVKR↓NAVP	84	QSK↓VLPVP
51	LKK↓YKVPQ	60	ITPTLNR↓E	85	K↓VLPVPQK
52	KYK↓VPQLE	61	PTLNR↓EQL	86	LPVPQK↓AV
57	PNSAEER↓L	62	LNR↓EQLST	87	VPQK↓AVPY
58	SAEER↓LHS	66	SEENSK↓KT	88	QK↓AVPYPQ
61	HSMK↓EGIH	67	ENSKK↓TVD	90	PYPQR↓DMP
64	IHAQQK↓EP	68	SKK↓TVDME	91	PQR↓DMPIQ
73	FYPELFR↓Q	72	STEVFTK↓K	99	VLGPVR↓GP
74	PELFR↓QFY	73	EVFTK↓K↓TK	100	GPVR↓GPFP
75	LFR↓QFYQL	74	FTK↓K↓TK↓LT	101	VR↓GPFPII
76	R↓QFYQLDA	77	LTEEEK↓NR		
95	ENSEK↓TTM	78	EEEKNR↓LN		
96	SEK↓TTMPL	79	EKNR↓LNFL		
		80	NRLNFLK↓K		
		81	LNFLK↓K↓IS		
		82	FLKK↓ISQR		
		83	KKISQR↓YQ		
		84	ISQR↓YQK↓F		
		85	QRYQK↓FAL		
		86	YQK↓FALPQ		
		88	ALPQYLK↓T		
		89	PQYLK↓TVY		
		90	YLK↓TVYQH		
		91	K↓TVYQHQK		
		92	VYQHQK↓AM		
		93	QHQK↓AMKP		
		94	QK↓AMKPWI		
		96	KPWIQPK↓T		
		97	WIQPK↓TK↓V		
		98	QPK↓TK↓VIP		
		99	K↓TK↓VIPYV		
		100	KVIPYVR↓Y		
		101	IPYVR↓YLA		

The down arrow denotes the site of cleavage.

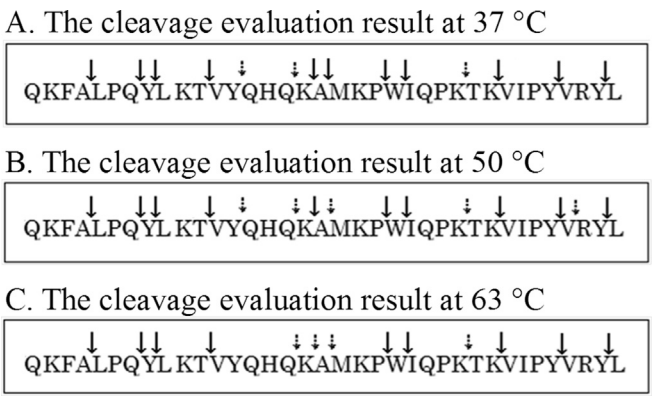

FIG. 3. Evaluated result map for cleavage positions in a part of α_{S2}-casein after hydrolysis with microbial protease from *B. subtilis*. These maps exhibit the result of evaluation at 37°C (A), 50°C (B), and 63°C (C). The N-terminal sides of the detected fragments for which the peak intensity was $\geq 2n \times 10^7$ are indicated by broken-line arrows, while the N-terminal sides of the detected fragments for which the peak intensity was $\geq 4n \times 10^7$ are indicated by solid arrows.

cleavage strength with the protease reaction time, we undertook the following evaluation. We synthesized a photo-cleavable peptide array consisting of 7-mer overlapped by 6 amino acids (1 amino acid offset) and corresponding to the primary sequences of β-lactoglobulin (Table 1), and hydrolyzed the peptide array using a microbial protease from *B. subtilis* at a temperature of 50°C for 3, 30, or 300 min. As mentioned above, cleavage results of β-lactoglobulin were defined by two levels of scan detection.

Fig. 4 shows the evaluation results: the first line (top arrow), second line (middle arrow), and third line (bottom-most of the three arrows) correspond to the hydrolysis of the peptide array using a microbial protease from *B. subtilis* at 50°C for 3, 30, and 300 min, respectively. The results indicate that both the cleavage strength and number of cleavage points increases with the reaction time. The degree of hydrolysis (DH) of casein hydrolysate treated with this protease also indicates that the cleavage strength increases with the reaction time (12.3% for 3 min, 16.3% for 30 min, and 18.9% for 300 min). Interestingly, AA61-70 of β-lactoglobulin was not cleaved in 3 min but cleaved slightly at AA66-67 in 30 min and strongly at AA66-67 and AA69-70 in 300 min. AA58-77 of β-lactoglobulin is known as an epitope in milk protein-reactive patients (18). These results suggest that it is necessary to hydrolyze the protein for a longer time to reduce the allergen content of milk by cleaving the epitope site of AA61-70 with this microbial protease. Thus, Pep-MS assay can be applied not only to understand the cleavage site of the enzyme but also to screen the optimal reaction condition for cleaving a target peptide.

Conclusion In this study, we developed the Pep-MS assay to identify the protease cleavage positions. There might be some opinions that it is better to use directly mass spectrometer than indirectly it via LC for high-throughput analysis. For example using SELDI plate we can directly and immediately detect sequence molecular weight. However, in this paper, in order to more clearly distinguish cleaved peptides and uncleaved peptides after enzyme treatment, LC separation process was incorporated. In Fig. S1, we showed raw data (some excerpts) of LC chromatograph. As shown in Fig. S1C–E, depending on SPOT, cleaved peptide fragments and uncleaved peptide fragments are mixed. To accurately qualify and quantify all these peptide fragments, we thought that it is necessary to separate each peptide fragment by LC.

The Pep-MS assay could evaluate the protease cleavage positions at a higher accuracy than the conventional LC-MS/MS-based peptide mapping assays, and be used to examine the effects of temperature and time on cleavage positions during protease treatment. In the conventional LC-MS/MS-based mapping assay, the theoretical MS/MS data of a possible peptide fragment is extracted comprehensively from the amino acid sequence data of the target protein and the amino acid sequence of the peptide fragment is identified by comparing the experimental data with the theoretical one. The analysis software ranks the peptide sequences of the candidates being identified according to the unique scoring rule, but it is not able to comprehensively and exactly identify all the peptide fragments because of insufficient LC-separation or the presence of peptides with similar molecular weights and protease peptides produced by autolysis. In contrast, the Pep-MS assay enables separate analysis of the peptide fragment for each SPOT. The Pep-MS assay is able to comprehensively and exactly identify all the peptide fragments, because it has the LC system that can easily separate peptide fragments for each spot and the removal system

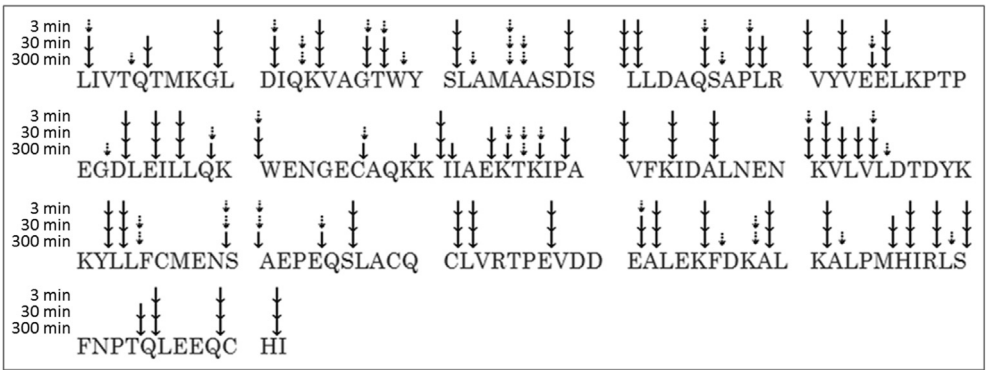

FIG. 4. Evaluated result map for cleavage positions in β-lactoglobulin after hydrolysis with microbial protease from *B. subtilis*. This map shows the result of evaluation for 3 min (first row), 30 min (second row), and 300 min (third row). The N-terminal sides of the detected fragments for which the peak intensity was $\geq 2n \times 10^7$ are indicated by broken-line arrows, while the N-terminal sides of the detected fragments for which the peak intensity was $\geq 4n \times 10^7$ are indicated by a solid arrow.

that can wash out impurities for example protease peptides. Therefore, it is considered to have higher accuracy than conventional methods.

We believe that the Pep-MS assay is a promising method for evaluating the substrate specificity of proteases, which will be useful to find effective production conditions for functional peptides from foods and effective hydrolysis conditions for decreasing the allergen of food proteins.

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