Original paper

Identification of Peptides in Sediments Derived from an Acidic Enzymatic Soy Protein Hydrolysate Solution

Toshihiro NAKAMORI^{1*}, Mami NAGAI², Motohiro MAEBUCHI¹, Hitoshi FURUTA¹, Eun Young PARK²,
Yasushi NAKAMURA² and Kenji SATO²

¹Research and Development Division, Fuji Oil Co., Ltd., 4-3 Kinunodai, Tsukubamirai-shi, Ibaraki 300-2497, Japan

²Lab. Food Science, Graduate School of Life and Environmental Sciences Division of Applied Life Sciences, Kyoto Prefectural University, 1-5 Shimogamo, Kyoto 606-8522, Japan

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Soy peptides were produced by microbial protease digestion of soy proteins. Under an acidic condition (pH 3.8), soy peptide solution (5% w/v) yielded sediment during chilled storage. The object of the present study was to identify peptides in the sediment in order to elucidate the sedimentation mechanism. Peptides remaining in solution after chilled storage had a molecular mass of 200 – 5,000 Dalton (Da), based on the elution volume obtained from size exclusion chromatography. However, peptides extracted from the sediment using 30% acetonitrile-0.1% trifluoroacetic acid had a molecular mass larger than 2,000 Da. Only limited numbers of peptides *f* (71 – 88), *f* (340 – 362), *f* (335 – 362) and *f* (310 – 356) of β -conglycinin α subunit were identified in the sediment. These results demonstrated that the sediment was produced from relatively large molecular peptides derived from the C-terminal region of the β -conglycinin α subunit. Moreover, the major peptide in the sediment was sensitive to cleavage by mammalian trypsin.

Keywords: soy peptide, soy protein hydrolysate, sediment, β -conglycinin

Introduction

Since the 1970s, enzymatic soy protein hydrolysate (ESPH) has been prepared on an industrial scale. The nutritional value of peptides in ESPH has been extensively investigated. Attention has recently been focused on the rapid increase of peripheral blood amino acid levels due to ingestion of ESPH compared with the ingestion of intact soy proteins or amino acid mixtures containing the same amino acid composition (Maebuchi *et al.*, 2007). In addition, it has been reported that the peptide transporter (PEPT1) on enterocytes is retained after long-term fasting and treatment with anticancer drugs, although the amino acid transporter level decreases under these conditions (Adibi, 2003). Therefore, these peptides have been considered as a preferred amino acid source over the usual amino acid mixtures and proteins under certain

pathological conditions (Bamba *et al.*, 1992). On the basis of these facts, ESPH has been used as the amino acid source of enteral nutrition, sports drinks, etc. since the 1980s (Masuda *et al.*, 2007). In addition to their nutritional value, those peptides found in ESPH have been demonstrated to exert beneficial functions, including antihypertensive (Matsui *et al.*, 2005), antihypertriglyceridemic (Inoue *et al.*, 2011), and antiinflammatory (Kovacs-Nolan *et al.*, 2012) effects. ESPH has also been investigated as a functional food ingredient to improve mental health (Maebuchi *et al.*, 2013).

As a clinical nutrient, the final products are formulated as an emulsion with lipids; therefore, transparency of the final product is not a necessity. Instead, much effort has concentrated on improving product flavor and taste. However, in the case of beverages in plastic and glass bottles, demand for a transparent final product has

*To whom correspondence should be addressed.

E-mail: nakamori.toshihiro@so.fujioil.co.jp

been increasing. To satisfy consumer preferences, a highly soluble type of ESPH has been developed (Nakamori *et al.*, 2010). Peptides in ESPH are soluble even under acidic conditions, *i.e.*, a beverage storage condition commonly used for sports and energy drinks. However, protein sedimentation is frequently observed upon prolonged chilled storage of acidic beverages containing ESPH, inciting consumer complaints. It is therefore necessary to control sediment formation during storage of acidic beverages. However, as information on the peptides in the sediment is limited, the sedimentation mechanism with respect to ESPH remains unclear.

This study aimed to isolate and identify peptides in the sediment and to elucidate the sedimentation mechanism of ESPH.

Materials and Methods

Preparation of ESPH ESPH was prepared on a pilot scale by Fuji Oil (Osaka, Japan) by the same method as described previously (Tamaru *et al.*, 2007). Briefly, soy protein isolate (SPI) was digested with an endo-type protease derived from *Bacillus sp.*, at pH 4.5. After harvesting by centrifugation, the supernatant was heated at 90°C for 15 min and filtered through a 0.45-μm membrane-filter before being spray-dried.

Formation of Sediments ESPH (50 g) was dissolved in 800 mL of distilled water, the pH was adjusted to 3.8 using 5% citric acid, and the volume was brought up to 1 L by adding distilled water. This acidic condition has been often used for sports and energy drinks. This acidic solution (100 mL) was poured into a screw-cap glass vial, which was heated in a water bath at 90°C for 15 min before being sealed and stored under darkness at 4°C. The acidic ESPH solution was completely transparent immediately after preparation; however, sediment was observed 5 days after storage at 4°C. The sediment was collected by centrifugation (13,000 rpm, 5 min, 4°C).

Extraction of Peptides from Sediment Peptides in the sediment were extracted using 30% acetonitrile containing 0.1% trifluoroacetic acid (TFA). Soluble (AC-S) and insoluble (AC-INS) fractions were prepared by centrifugation.

Partial Acid Hydrolysis AC-INS was washed twice with distilled water before being partially hydrolyzed using 1M HCl at 100°C for 20 min *in vacuo* (0.1–0.2 Torr).

Isolation of Peptides from Sediment Peptides in the AC-S were fractionated by size exclusion chromatography (SEC) using Superdex Peptide HR 10/30 (GE Healthcare UK Ltd, England). Elution was performed with 30% acetonitrile in water in the presence of 0.1% TFA for over 1 h at 0.5 mL/min. Fractions were collected at 1 min intervals. Peptides in the SEC fractions were further isolated by reversed-phase (RP)-HPLC using a Cosmosil AR-5C18-300 column (50 × 4.6-mm i.d., Nacalai Tesque, Kyoto, Japan). Elution was performed at 1 mL/min using a binary gradient consisting of 10% (A) and 80% acetonitrile (B) in the presence of 0.1% TFA. The gradient profile was as follows (absorbance: 214 nm): 0–30 min, B 0–50%; 30–40 min, B 50–100%;

40–45 min, B 100%, 45.1–60 min, B 0%. The column was maintained at 40°C.

Trypsin Digestion Purified peptide in AC-S (Peak 6) in Fig. 3 was dried *in vacuo*, dissolved in 200 μL of 50 mM Tris-HCl buffer (pH: 8.0), and digested with porcine trypsin (Sigma-Aldrich, St. Louis, MO) with a substrate-to-enzyme ratio of 20:1. This reaction mixture was incubated at 37°C for 4 h with stirring before drying *in vacuo*, and subsequently dissolved in 200 μL of 10% acetonitrile containing 0.1% TFA. The sample was centrifuged (12,000 rpm, 3 min) to yield a clear solution and subjected to reversed-phase (RP)-HPLC as described above.

Other Analytical Procedures Amino acid analysis was performed by the method of Bidlingmeyer *et al.* (Bidlingmeyer *et al.*, 1984) with slight modifications. Treated phenylthiocarbamyl amino acids were resolved using a Superspher RP-18 (e) column (250 × 4-mm i.d., Merck) at 0.8 mL/min via binary gradient elution (Sato *et al.*, 2013). The peptide sequence was identified by the automatic Edman degradation method using a PPSQ-21 (Shimadzu, Kyoto, Japan).

Results

Sediment formation during storage of ESPH When a 5% soy peptide solution was stored at 4°C under darkness for 5 days, sediment was observed at the bottom of the vial. Moreover, peptide contents in the sediment increased with storage time (Fig. 1).

Isolation and identification of peptides in the sediment Amino acid analysis of soluble and residual fractions revealed that approximately 70% of peptides in the sediment could be extracted with 30% acetonitrile-0.1% TFA (AC-S Fr.). Peptides in the AC-S Fr were first resolved by SEC. For comparison, peptides in the non-sediment fraction of the soy peptide solution (day 20 after chilled storage) were also resolved in the same manner. In the non-sediment fraction, peptides (eluted from 15 to 40 min) yielded eluates of molecular mass 200–5000 Dalton (Da) (Fig. 2). Essentially identical elution patterns were obtained for AC-S fractions from the sediment formed by different storage periods. Most of the peptides in AC-S fractions from the sediment were eluted before 20 min, yielding eluates of molecular mass larger than 2000 Da. SEC fractions eluted from 16 to 18 min were collected, and the peptides were further resolved by RP-HPLC. Intriguingly, the elution patterns were essentially identical to those obtained by injection of the same SEC fraction from sediments formed at different storage times (Fig. 3). Peaks marked as 4–7 expanded after prolonged storage time. However, the same SEC fraction from the non-sediment fraction did not show peaks 3–7 in the sediment fractions. The peak marked as 1 was observed only in the sediment fractions. The peaks marked with numbers were collected and subjected to sequence analysis. Identified sequences are summarized in Table 1. For peaks 3 and 7, sequences not identified as multiple phenylthiohydantoin amino acids appeared in the same cycle. The peptide in peak 1 could be assigned as glycine

Fig. 1. Formation of sediment during storage of acidic soy peptide solution. Sediment content was expressed as constituting amino acids after HCl hydrolysis.

Fig. 2. Comparison of elution patterns of peptides in the non-sediment fraction and the 30% acetonitrile-0.1% TFA (AC-S) fractions from the sediment on days 5, 10 and 20 after storage at 4°C. Determinations were performed by size exclusion chromatography (SEC) using a Superdex Peptide HR 10/30. Elution was performed at a flow rate of 0.5 mL/min. The recorder range of each chromatogram was adjusted to show the same maximum value for comparison of elution patterns. AC-S fractions were eluted before 20 min, yielding eluates of molecular mass larger than 2,000 Da. Non-sediment fraction and 30% AC-S fractions were collected from 16 to 18 min and further isolated by RP-HPLC.

A1a subunit. This peptide was not observed in the sediment fraction. The peptide in peak 2 could be derived from the basic region of the β -conglycinin a subunit, which appeared in both non-sediment and sediment fractions. As for peptides in peaks 4-6, they were tentatively assigned to $f(310-362)$ of the β -conglycinin α subunit.

Identification of peptides in the insoluble fractions (AC-INS)
Peptides in AC-INS Fr. were further solubilized by partial HCl hydrolysis. Peptides in the partial hydrolysate were resolved by RP-HPLC (Fig. 4); a major peak was traced by increasing the acetonitrile concentration (Fig. 4). The peptide in this peak was identified as TKFEEINKVLF, which is also part of $f(310-362)$ of the β -conglycinin a subunit (Sebastiani *et al.*, 1990).

Trypsin digestion of sediment peptides Tryptic digests of the peptide in peak 6 (Fig. 3) were examined. As shown in Fig. 5, peak

6 completely disappeared after tryptic digestion, and fragment peaks with shorter retention times appeared. The peaks marked from 'a' to 'f' were collected and subjected to sequence analysis. Identified sequences are summarized in Table 2. Only peak b could be assigned to $f(310-342)$ (Tables 1, 2). Other peaks (a, c, d, e, f) were assigned to $f(343-356)$, carboxyl region of $f(310-342)$ of the β -conglycinin a subunit. These facts indicate that peak 6 in Fig. 3 covers at least $f(310-356)$ of the β -conglycinin α subunit, whereas direct amino terminal sequence analysis gave only $f(310-342)$ of the β -conglycinin a subunit.

Discussion

During chilled storage of the acidic ESPH solution, sedimentation occurred 5 days after standing. In the present study, 4 peptides were identified in the sediment. Among them, 3 peptides

were present only in the sediment and the other was present in both the sediment and non-sediment fractions (Table 1). Based on the identified sequences, all identified peptides in the sediment have relatively large molecular mass of 2,700–5,388 Da. SEC analyses also revealed that only negligible amounts of peptides with molecular mass less than 1,500 Da were observed in the sediment. Although the peptide in peak 1 indicated a similar molecular mass, its state (solution) remained unchanged. Therefore, not all peptides forming the sediment were characterized by a large molecular mass. Furthermore, all peptides identified in the sediment were derived from the β -conglycinin α subunit. Fragment *f* (71–88) of the β -conglycinin α subunit (peak 2) was detected in both sediment and non-sediment fractions. Peptides derived from a limited region of the β -conglycinin α subunit (310–362) were specifically present in the sediment recovered from both AC-S and AC-INS fractions (Tables 1, 2), which play a critical role in sedimentation during

chilled storage of acidic ESPH solution.

Undesirable sedimentations have been investigated in the wine and beer industries. In the case of beer, proline-rich proteins (Asano *et al.*, 1982; Siebert, 2006; Jin *et al.*, 2012), and in the case of wine, thaumatin-like proteins and chitinases play a significant role in haze and sediment formations (Marangon *et al.*, 2011; Younes *et al.*, 2013). However, peptides in the sediment of acidic ESPH did not manifest similar homology to the peptides in beer and wine. Furthermore, several studies have reported that the polyphenols contained in wine or beer promote sedimentation via interactions with proteins (Siebert *et al.*, 1996; de Bruijn *et al.*, 2009). It has been demonstrated that removal of polyphenols by polyvinylpyrrolidone (PVPP) resin can suppress formation of haze and sedimentation in wine and beer (Siebert, 1999). However, pretreatment with PVPP resin could not suppress sedimentation in acidic ESPH, which contains significant amounts of isoflavone.

Fig. 3. Elution patterns of peptides in the SEC fraction from the non-sediment fraction and the 30% AC-S fractions on days 5, 10 and 20. Reversed-phase (RP)-HPLC using a Cosmosil AR-5C18-300 column was used to further isolate peptides. Elution was performed at 1 mL/min as described in the 'Materials and Methods'. The same recorder range was used for the three AC-S.

Fig. 4. Elution patterns of peptides in the 30% AC-INS fraction solubilized by partial HCl hydrolysis on reversed-phase (RP)-HPLC using a Cosmosil AR-5C18-300 column. Elution was performed at 1 mL/min as described in the 'Materials and Methods'.

Table 1. Summary of amino-terminal amino acid sequences, pI, and hydrophobicity of isolated peptides. Peptides were isolated by RP-HPLC (Fig. 3, Fig. 4) and identified by the Edman degradation method.

Peak No.	Sequence	Assignment	pI	H.A.
Non-sediment fraction				
1	SVIKPPTDEQQRPQEEE-	Glycinin A1a:263 – 280	4.3	11.1
2	GEIPRPRPRPQHPEREPQ-	β -conglycinin α :71 – 88	9.5	5.6
AC-S fraction				
4	NILEASYDTKFEEINKVLFGREE-	β -conglycinin α :340 – 362	4.4	39.1
5	QGFSRNILEASYDTKFEEINKVLFSREE-	β -conglycinin α :335 – 362	4.7	35.7
6 ^{*1}	AIPVKNKGRFESFFLSSTEAQSYLQGFSRNIL-	β -conglycinin α :310 – 342	8.6	39.4
AC-INS fraction				
8	TKFEEINKVL-	β -conglycinin α :348 – 358	5.8	45.5

Isoelectric point (pI) of identified sequences was calculated accordingly (http://web.expasy.org/compute_pi/).

H.A. : Hydrophobic amino acid (%) = $(N_h/N) \times 100$

N_h : Number of hydrophobic amino acid residues present in the sequence

N : Sequence length

^{*1} : Following experiment revealed that peak 6 consisted of at least *f* (310–356) (Fig. 5).

pI : 5.1, H.A. : 40.4

Fig. 5. Elution patterns of tryptic digests of peak 6 on reversed-phase (RP)-HPLC using a Cosmosil AR-5C18-300 column. Elution was performed at 1 mL/min as described in the ‘Materials and Methods’.

These facts indicate that the mechanism of sedimentation from soy peptide under acidic conditions differs from that of beer and wine.

Although peptides in the sediment of soy peptide solution were initially soluble under acidic conditions, they formed insoluble sediments during storage. Therefore, these peptides may tend to aggregate on standing. The peptides specifically present in sediments, *f* (340 – 362), *f* (335 – 362) and *f* (310 – 356), are rich

in hydrophobic amino acids compared to the peptides present in solution, as indicated in Table 1. In addition, compared to peaks 3–5 in the sediment, peaks 6 and 7 increased after prolonged storage. Identified peptides in peaks 4 and 5, *i.e.*, *f* (340 – 362) and *f* (335 – 362), have isoelectric points similar to pH values of the acidic peptide solution, while peptides in peak 6, or *f* (310 – 356), is a basic peptide. On the basis of these facts, a hypothesis for the

Table 2. Summary of amino-terminal amino acid sequences of tryptic digests of peak 6. Fragment peptides were isolated by RP-HPLC (Fig. 5) and identified by the Edman degradation method.

Peak No.	Sequence	Assignment	pI	H.A.
a	DKFEEINKVLF-	β -conglycinin α :348-355	4.7	45.5
b	AIPVNKPGRFES-	β -conglycinin α :310-321	8.8	33.3
c	SYDTKFEEINK-	β -conglycinin α :346-355	4.7	27.3
d	EASYDTKFEEINK-	β -conglycinin α :343-355	4.4	30.8
e	SYDTKF EEINK-	β -conglycinin α :346-355	4.7	27.3
f	N ILEASYDTKF EEINKV-	β -conglycinin α :346-356	4.4	41.2

Isoelectric point (pI) of identified sequences was calculated accordingly (http://web.expasy.org/compute_pi/).

H.A. : Hydrophobic amino acid (%) = $(N_h/N) \times 100$

N_h : Number of hydrophobic amino acid residues present in the sequence

N : Sequence length

mechanism of sedimentation is proposed as follows. Sediments *f* (340–362) and *f* (335–362) of the β -conglycinin α subunit might initially form a core of sediment by isoelectric precipitation, and the other hydrophobic peptides in peaks 6–7 then bind to the core via hydrophobic interaction and electrostatic forces, thus accelerating aggregation to eventually form insoluble sediments. To confirm this hypothesis, a further study using synthesized peptides is now in progress (Tables 1, 2).

Notably, these peptides in the sediment can be digested by mammalian trypsin, which suggests that these peptides can be degraded in the digestive tract upon ingestion.

Based on the present results, degradation or removal of the β -conglycinin α subunit-derived peptides may suppress sedimentation during chilled storage of soy peptides under acidic conditions. These peptides might be degraded by extensive digestion, which in turn increases bitterness. Alternatively, the peptides could be removed by ultrafiltration. In fact, a solution filtered through a membrane filter (Amicon YM3; > 3,000 Da) did not form any sediment under the above-mentioned condition (Materials and Methods).

To the best of our knowledge, the present study is the first report to attempt to elucidate sediment formation of peptide-based beverages and to put forth practical methods to prevent sediment formation in soy peptide solutions.

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