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Digested Ara h 1 Loses Sensitizing Capacity When Separated into Fractions
Katrine L. Bogh,* Vibeke Barkholt,‡ Neil M. Rigby,§ E. N. Clare Mills,‡∥ and Charlotte B. Madsen†

National Food Institute, Division of Toxicology and Risk Assessment, Technical University of Denmark, Mørkhøj Bygade 19, DK-2860 Søborg, Denmark
Department of Systems Biology, Enzyme and Protein Chemistry, Technical University of Denmark, Soltoft Plads, Building 224, DK-2800 Kgs. Lyngby, Denmark
Institute of Food Research, Biochemistry, Norwich Research Park, Colney, Norwich, NR4 7UA, United Kingdom

ABSTRACT: The major peanut allergen Ara h 1 is an easily digestible protein under physiological conditions. The present study revealed that pepsin digestion products of Ara h 1 retained the sensitizing potential in a Brown Norway rat model, while this sensitizing capacity was lost by separating the digest into fractions by gel permeation chromatography. Protein chemical analysis showed that the peptide composition as well as the aggregation profiles of the fractions of Ara h 1 digest differed from that of the whole pool. These results indicate that the sensitizing capacity of digested Ara h 1 is a consequence of the peptides being in an aggregated state resembling the intact molecule or that most peptides of the digests need to be present in the same solution, having a synergistic or adjuvant effect and thereby augmenting the immune response against other peptides.

KEYWORDS: Ara h 1, digestion, animal model, food allergy, peptides, aggregation

INTRODUCTION

Food allergy most often involves an allergen-specific IgE antibody-mediated immunologic response. It is an adverse reaction to an otherwise harmless food or food component that involves an abnormal response of the immune system to specific food proteins. One of the major unanswered questions in food allergy research is what makes a protein a food allergen. Yet, no definite answer to this exists. However, one of the hypotheses has been that for a protein to be a food allergen, it must survive the digestion process through the gastrointestinal tract, to reach the immune system in an intact protein or as large peptide fragments.1,2 The first systematic assessment of food allergen digestibility was conducted in 1996 by Astwood et al.3 They showed that in general food allergens were resistant to pepsin digestion, whereas nonallergenic proteins were more easily digested. Since this, several studies examining the correlation between resistance to digestion and allergenicity have been made, where the correlation between stability and allergenicity was less clear.4—13 However, it may still be reasonable to think that proteins being resistant or at least partially resistant to digestion have an increased probability of reaching the intestinal mucosa in a form that is sufficiently immunologically active to sensitize the mucosal immune system and be sufficient in size to retain the ability to cross-link two IgE molecules and thereby elicit an allergic reaction. The stability to digestion is for those reasons also recommended for use in the safety assessment of newly introduced proteins in genetically modified foods based on a decision tree or a weight of evidence approach, which includes, among a variety of tests, the assessment of resistance to digestion by pepsin.11—13 However, while pepsin stability as a part of an allergenicity assessment would still seem reasonable for the purpose of safety evaluation of most food proteins, we now know that for some allergenic proteins, this approach would be misleading. The milk allergen β-casein (Bos d 8)7,14,15 as well as the peanut allergen Ara h 19,10 have several times been shown to be easily digestible food allergens.

Peanut allergy is one of the most common and serious types of IgE-mediated food allergies in terms of persistency and severity15,16 and seems to be an increasing problem in the western world.18,19 The peanut protein Ara h 1, which is a major allergen,20,21 is a 7S globulin protein belonging to the cupin superfamily of allergens.22 Ara h 1 is a homotrimeric protein, consisting of 63.5 kDa large subunits,20 held together by hydrophobic interactions between amino acids at monomer—monomer contact points.3,23,24 Ara h 1 is a readily digestible allergen, being digested to small peptide fragments by gastroduodenal digestion.3,10 Even though Ara h 1 is a labile protein, Eiwegger et al.9 and Bogh et al.10 showed that the digestion products of Ara h 1 retain allergenic potential, being able to sensitize as well as elicit allergic reactions. These studies indicated that aggregation of peptides may play a major role in maintaining allergenic activity. Epitope mapping studies of this protein have suggested both linear25 and conformational3 IgE-binding epitopes, at least some of which are able to survive the digestion process.10,26

Peptides need to have a certain size to be allergenic, but the exact lower molecular weight (MW) size limit is not known. Yet, many suggestions for such a lower MW size limit have been presented,11,27—31 and the general view appears to be that peptides need to be approximately 3.5 kDa to contain sensitizing and eliciting allergenic capacity. This may be a realistic limit for some allergen-derived peptides. We have previously

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shown that peptide fragments, as small as 2 kDa, have the capacity to sensitize and elicit allergic reactions. In the present study, we focus on examining under which conditions such peptide fragments retain their allergenic potential.

The objective of this study was to increase our knowledge and understanding of the allergenic capacity of small peptide fragments. This was done by using the known major peanut allergen Ara h 1 as a model allergen, based on the knowledge that this allergen retains its allergenic potential when digested to small peptide fragments while using pepsin as the enzyme for digestion. Digestion products of Ara h 1 and fractions hereof were thoroughly characterized, and examination of sensitizing peptide fragments retain their allergenic potential when digested to small peptide fragments while using pepsin as the enzyme for digestion. Digestion products of Ara h 1 and fractions hereof were thoroughly characterized, and examination of sensitizing peptide fragments retain their allergenic potential. Digestion products of Ara h 1 and fractions hereof were thoroughly characterized, and examination of sensitizing peptide fragments retain their allergenic potential.

MATERIALS AND METHODS

Purification of Peanut 7S Protein Ara h 1. Raw redskin peanuts (Julian Graves LTD, Kingswinford, United Kingdom) were skin peeled. Peanuts were frozen with liquid nitrogen and blended in a steel blender until a fine texture was obtained. Subsequently, the crushed peanuts were taken through two rounds of defatting [peanut:hexane, 1:5, w:v, 1 h, room temperature (RT)] and further homogenized in a coffee grinder.

Proteins were extracted in double-distilled water (peanut:water, 1:5, w:v) with 0.02% sodium azide (v:w), containing a protease inhibitor tablet (Roche complete mini protease inhibitor tablet, Roche, Sussex, United Kingdom) for 1 h at RT. After centrifugation (3000g, 20 min) ammonium sulfate was added to a saturation of 70% and centrifuged (3000g, 30 min). The supernatant was dialyzed against buffer (Tris 20 mM, pH 7.5, 500 mM NaCl) at 4°C, and samples of 40 mL were applied to a column of 10 mL of Con A Sepharose (GE Healthcare, Buckinghamshire, United Kingdom).

Unbound protein was removed by washing with the buffer, while pure Ara h 1 was eluted by addition of 400 mM methyl α-D-mannopyranoside. For further purity, eluted Ara h 1 was applied to a column of Superdex 200 prep grade (HiLoad 16/60 and 26/60 Superdex 200 prep grade, GE Healthcare) and eluted with 25 mM Tris, pH 7, and 150 mM NaCl. Purified Ara h 1 was filtrated through a Millipore filter paper (0.22 μm, Millipore Corp., Bedford, MA) with vacuum and afterward ultrafiltrated through an ultrafilter membrane (pore size, 10 kDa) with gas (argon, 10 psi). The Ara h 1 was dialyzed against 150 mM NaCl, and the concentration was determined by UV absorbance reading at 280 nm. Furthermore, concentration of purified Ara h 1 was determined by amino acid analysis to be 4.38 mg/mL. The purified intact Ara h 1 was analyzed for the presence of endotoxin by Lonza endotoxin testing service (Lonza, Verviers, Belgium).

N-Terminal Sequencing of Intact Ara h 1. To analyze the isotype composition of intact Ara h 1, amino terminal sequencing was performed. Protein sequencing of the intact Ara h 1 (5 μL, 16 pmol/μL) was carried out by automated N-terminal Edman degradation in a Procise 494 sequencer (Applied Biosystems, Foster City, CA) in liquid phase mode.

Simulated Gastric Digestion of Ara h 1. Gastric digestion was performed essentially as described by Bøgh et al.10 In short, pepsin immobilized to agarose (P09069, Sigma, St. Louis, MO) was washed twice (100g, 1 min) in 10 mL of 1 M HCl. Purified Ara h 1 (2.4 mg/mL in 150 mM NaCl) was adjusted to pH 2.5 with 1 M HCl and added to the immobilized pepsin to yield an activity of pepsin of approximately 170 U per mg Ara h 1. The solution was placed in a shaking incubator (200 rpm, 37°C) for 120 min. Reaction was stopped by adjusting the pH to 7 with 1 M NaOH, centrifugation (1000g, RT, 2 min), and filtration of supernatant through a 0.45 μm Millipore filter followed by a 0.22 μm filter.

Separation of Digested Ara h 1 into Fractions. For fractionation of the digested Ara h 1, preparative gel permeation chromatography (GPC) was performed. The digested Ara h 1 (6 mL, 2.3 mg/mL) was loaded onto a Superdex 75 prep grade, HiLoad 26/60 column (GE Healthcare, Uppsala, Sweden) connected to GradiFrac system (Pharmacia GradiFrac FPLC system, GE Healthcare). Peptides were eluted at RT with 150 mM NH4HCO3, pH 7.8, at 1 mL/min and collected in fractions of 4 mL. The eluted peptides were detected by absorbances at 280 and 226 nm. Four runs were made to fractionate all digested Ara h 1. The column was calibrated for MW determination by applying a standard mixture consisting of 1 mg/mL ferritin (440 kDa; F4503, Sigma), 0.75 mg/mL ovaltransferrin (79 kDa; C-0880, Sigma), 1 mg/mL carbonic anhydrase (29 kDa; C-3934, Sigma), 1 mg/mL cytochrome C (14 kDa; C-2506, Sigma), 2 mg/mL apotinin (6 kDa; A-1153, Sigma), and 0.1 mg/mL vitamin B12 (1.3 kDa; V-2876, Sigma).

Fractions from the four consecutive runs were collected and pooled according to the GPC profile (Figure 1) in three different pools

Figure 1. Preparative GPC of Ara h 1 digests. For fractionation of the Ara h 1 digest, preparative GPC was performed in 150 mM HN4HCO3, where Ara h 1 digest was separated into fractions based on the chromatographic profile. From the profile, shown with absorbances at 226 and 280 nm, it was decided to make three different pools, indicated by the vertical dashed lines. The pools are referred to as: digested Ara h 1, large complexes and small complexes, where digested Ara h 1 is the whole pool of digest, consisting of both the fraction of large and the fraction of small complexes. The first double peak had an apparent MW of 60–440 kDa, indicating that some intact Ara h 1 could be left in this fraction. To avoid the possible presence of any intact Ara h 1 in the digests, this double peak was excluded.

Amino Acid Analysis. For examination of amino acid composition and quantification, amino acid analysis was performed according to Barkholt and Jensen32 after hydrolysis overnight in HCl.
MALDI-TOF MS. For analysis of peptide mass distribution in samples of Ara h 1 digests, MALDI-TOF MS was performed on a Bruker MALDI-TOF MS (MALDI TOF/TOF, Ultraflex II, Bruker Daltonik GmbH, Bremen, Germany) equipped with pulsed ion extraction and 200 Hz Smart Beam laser. One microliter of the rediluted fractions from RP-HPLC was loaded onto a MALDI target, followed by addition of 1 μL of 2% TFA and 1 μL of α-cyano-4-hydrocinnamic acid [5 μg/μL in 70% ACN (v:v), 0.1% TFA (v:v)]. All mass spectra were initially calibrated with a tryptic digest of β-lactoglobulin.

GPC Analysis. For analysis of aggregation profiles in samples of Ara h 1 digests, analytical GPC was performed. Samples (40 μL, 1 mg/mL) were applied to a Superdex 75 PC 3.2/30 column (GE Healthcare) connected to a SMART system (GE Healthcare). Chromatography was performed at RT with a flow rate of 50 μL/min using 150 mM HN₄HCO₃ (pH 7.8) as the eluent and 200 Hz Smart Beam laser. One microliter of the rediluted fractions from RP-HPLC was loaded onto a MALDI target, followed by addition of 1 μL of 2% TFA and 1 μL of α-cyano-4-hydrocinnamic acid [5 μg/μL in 70% ACN (v:v), 0.1% TFA (v:v)]. All mass spectra were initially calibrated with a tryptic digest of β-lactoglobulin.

RESULTS

Characteristics of Purified Ara h 1. From N-terminal sequencing of the purified Ara h 1, it was evident that both known isoforms of Ara h 1 were present, in the ratio of approximately 1:1 (data not shown). The sequences identified were RHPPGER and RSPPGER, demonstrating that the purified isoforms of Ara h 1 start at amino acid residue 79 (RHPPGER, Ara h 1, clone P17, SwissProt no. P43237) or 85 (RSPPGER, Ara h 1, clone P41B, SwissProt P43238), a confirmation of a study by Wichers et al., showing that Ara h 1 is expressed as a truncated protein, in which the first 78 and 84 amino acids, respectively, are cleaved off. The endotoxin analysis of the purified intact Ara h 1 was <2 endotoxin units (EU)/mg of Ara h 1. From RP-HPLC analysis (Figure 2A), Ara h 1 was calculated to be >98% pure.

Characteristics of Digested Ara h 1 and Fractions Hereof. From RP-HPLC analyses, it was evident that no residual intact Ara h 1 was left in the three pools of Ara h 1 digests, since no detectable peak at the elution time for intact Ara h 1 was seen in the chromatography profiles (Figure 2A vs B–D). When comparing the RP-HPLC profiles for digested Ara h 1, large complexes and small complexes (Figure 2B–D), no significant differences are shown, indicating no apparent variation in peptide composition. However, when comparing...
the total amino acid distribution of the peptides present in the three different pools (Figure 3), it was revealed that differences did exist. While the amino acid distribution of digested Ara h 1 represented the amino acid distribution for intact Ara h 1, the large complexes and the small complexes were found to have an amino acid distribution different from that of the intact Ara h 1. That digested Ara h 1 had an amino acid distribution similar to the distribution of intact Ara h 1 confirms that this pool contains a peptide composition representative of the intact Ara h 1, where hydrophobic amino acids are responsible for approximately 40%, the polar for approximately 12%, and the charged for approximately 48% of total amino acids. In contrast, the large complexes contain approximately 35% hydrophobic amino acids, approximately 9% polar, and approximately 56% charged amino acids, while the small complexes contain approximately 48% hydrophobic amino acids, approximately 15% polar, and approximately 37% charged amino acids. This shows that peptides constituting the two fractions of digested Ara h 1 are different from each other and thereby different from the whole pool of digested Ara h 1 and, therefore, do not contain peptides representing the intact Ara h 1.

For examination of the peptide mass distribution profiles of the three different pools of Ara h 1 digests, MALDI-TOF MS was performed and demonstrated that Ara h 1 was digested to small peptide fragments of sizes ≤ $M_r$ 4,000 (Figure 4), of which more than 75% had apparent $M_r$ between 500 and 2,000. As in the whole pool of digested Ara h 1, the peptides in the large complexes had sizes up to $M_r$ 4,000, while in the small complexes, the peptides were ≤ $M_r$ 3,000. So while the digested Ara h 1 and the fraction of large complexes contained peptides that were up to 33 amino acids, the longest peptides in the fraction of small complexes were up to 25 amino acids. However, for all three pools of Ara h 1 digests, by far, most peptides were between 4 and 16 amino acids.

Realizing from the preparative GPC profile (Figure 1), based on which the fractions of Ara h 1 digests were made that the peptides were in some kind of aggregated state, we analyzed in more detail the aggregation profiles of the three different pools. From the analytical GPC profiles shown in Figure 5, it is evident that the peptides in all three pools were indeed aggregated into complexes of larger sizes. However, from the profiles, it is revealed that the state of aggregation is very different for the three different pools. From the area under the 220 nm GPC absorbance curves, it was indicated that 25% of the peptides in digested Ara h 1 were aggregated into complexes of up to $M_r$ 104,000, that 53% of the peptides in the large complexes were aggregated into complexes of up to $M_r$ 56,000, and that 7% of the peptides in the small complexes were aggregated into complexes of up to $M_r$ 9,000. So, depending on the peptide composition profiles of the digested Ara h 1 and fractions hereof, the aggregation profiles differed significantly. See Table 1 for a summary of protein-chemical characteristics of the three different pools of Ara h 1 digests.

**Sensitizing Capacity of Digested Ara h 1 and Fractions Hereof.** Sera from individual BN rats dosed with either PBS (control), 200 μg of intact Ara h 1, 200 μg of digested Ara h 1, 200 μg of large complexes, or 200 μg of small complexes were evaluated for specific antibodies against both intact Ara h 1, digested Ara h 1, and fractions hereof. Looking at the antibody response, it was evident that while both intact Ara h 1 and whole pool of digested Ara h 1 could induce specific IgG response, neither the large complexes nor the small complexes could induce specific IgG antibodies. Analyses of the specific IgG1 (Figure 6) and IgG2a, which revealed similar...
results (data not shown), showed that antibodies raised against intact Ara h 1 were able to recognize both intact Ara h 1, digested Ara h 1, and both fractions of the digested Ara h 1, all to a statistically significant level. Although it is seen from Figure 6 that all animals immunized with intact Ara h 1 could react with all four samples of allergens, it is seen that the binding capacity was different (although not statistically significantly). IgG1 antibodies from the rats immunized with intact Ara h 1 had the highest binding capacity toward the intact Ara h 1, followed by the whole pool of digested Ara h 1 and then the fraction of large complexes. The lowest binding capacity was toward the fraction of small complexes. Contrary to antibodies raised against intact Ara h 1, the antibodies raised against the whole pool of digested Ara h 1 could only react with intact Ara h 1 and the whole pool of digested Ara h 1, the latter being the only one that was statistically significant. The specific antibody responses showed no statistically significant differences between rats immunized with intact and digested Ara h 1.

From Figure 7, it is seen that both intact and digested Ara h 1 could induce specific IgE, although the intact Ara h 1-specific IgE response was only significant for antibodies raised against intact Ara h 1. However, no statistically significant difference was seen between rats immunized with intact and digested Ara h 1, respectively, by use of a multiple comparison test. Because it was not possible to couple the small peptide fragments in digested Ara h 1 to a coupling protein, which could be detected by commercially available secondary antibodies, we could not determine digested Ara h 1-specific IgE responses. We anticipate, however, that the specific IgE response of rats immunized with digested Ara h 1 would be higher for digested Ara h 1 than the one shown for intact Ara h 1. These speculations are based on our knowledge that specific IgE follows the specific IgG1 and IgG2a.10,34

**DISCUSSION**

The present study confirms that Ara h 1 retains both the sensitizing and the reacting potential, when digested to small peptide fragments. This signifies that digestion of Ara h 1 is not an effective approach for significant reduction of either sensitizing nor IgE binding capacity and manifest that a correlation between resistance to digestion and allergenicity is not a general parameter. While Ara h 1 does not need to survive the digestion process as an intact protein or as large fragments to react with the immune system for induction of a specific immune response, this could still be the case for other food allergens. Previous studies examining the influence of digestion on the allergenic potential of other
We have previously shown that mixtures of peptides smaller than 2–5 kDa, which was generally thought to be the lower size limit for a peptide with inherent sensitizing capacity, may still act as a “complete” allergen, being able to sensitize, elicit allergic reaction, and react with IgE. The aim of this study was to further examine how small peptides retain their sensitizing capacity. This was done by studying the specific antibody responses in BN rats immunized with digested Ara h 1 and fractions thereof, separated on the basis of the aggregation profile of the peptides.

From the GPC analysis of the digested Ara h 1, it was evident that peptides did aggregate into complexes of larger sizes. This may be a result of noncovalent interactions, like hydrophobic interactions, since the single cysteine residue present in the Ara h 1 molecule cannot account for the amount and sizes of the complexes. That the sensitizing capacity of digested Ara h 1 is a result of the peptide fragments forming aggregates was hypothesized in earlier studies. That aggregation may enhance the immune response toward antigen subunits was already recognized in 1978, where Morein et al. showed that aggregation of subunits by hydrophobic interactions induced a significant higher immune response as compared to free subunits, suggesting an importance of multimeric structures. The same has been shown with the allergen melittin, a bee venom protein of 2.8 kDa (26 amino acids), with one B cell epitope and one T cell epitope. Melittin was able to induce specific IgG and IgE responses in humans and animals. The immunogenicity and allergenicity of the melittin were found to correlate with oligomerization of the molecules. Also, the state of aggregation may influence the way in which proteins are presented to the immune system in the gastrointestinal tract. This was indicated in a study by Roth-Walter et al., which showed that aggregation of proteins from milk inhibited their uptake by intestinal epithelial cells and redirected uptake to Peyer’s patches, promoting a significantly higher Th2-associated antibody response. This supports the theory that aggregation is a plausible explanation for the allergenic potential of digested Ara h 1.

It is well recognized that small peptides in general are poor immunogens and that peptides in general need to be of a certain size to behave as sensitizing allergens. Muller stated that it is commonly assumed that peptides in the range of 2–5 kDa behave like haptons and are not immunogenic. It is known that for a protein to induce an allergic response, it requires the presence of both T and B cell epitopes. However, immunization with free peptides as small as 6–14 amino acid residues long has been reported to induce acceptable antibody responses. This is in concordance with the earlier study of sensitizing capacity of digested Ara h 1, where peptides of less than 2 kDa were able to induce a statistically significant antibody response without the use of additional adjuvant. This may indicate that the sensitizing capacity of digested Ara h 1 could also be an intrinsic feature of the free peptides themselves. It has been shown several times that induction of antibodies does not require covalent linkage between the

food proteins have revealed digestion to be an effective approach for a significant impairment of sensitization and IgE-binding capacity.
peptide vaccine development. Mixtures of peptides have been shown to induce more B cell epitopes than did the very same peptides when fused or administered alone, indicating that peptides may function as adjuvant or in a synergistic way. Accordingly, the present study suggests that most peptides in the digest need to be present to serve as adjuvant augmenting the immune response against other peptides and therefore need to be administered together. In addition, it has been demonstrated that peptides representing different T cell epitopes varied significantly in their ability to provide help to B cells. This suggests that the inherent property of the peptides constituting T cell epitope peptides differ in efficacy and that this feature may be independent of the protein from where they originate.

The present study revealed that different requirements are needed for a protein to retain sensitizing and antibody binding capacity. While the fractions of digested Ara h 1 had no sensitizing potential, both fractions retained reactivity with antibodies raised in rats immunized with intact Ara h 1. This shows that there are larger requirements for peptides to sensitize than for the peptides to retain reacting activity. These results seem reasonable, since reacting activity only needs an amino acid sequence resembling an antibody epitope, while sensitizing capacity requires the ability to be recognized by the immune system de novo, priming of specific B cells as well as activation of T cells, providing the additional help needed for proper differentiation and proliferation of antibody secreting plasma cells. If most B cell epitopes of Ara h 1 are conformational, this leaves us to explain how antibodies directed against intact Ara h 1 are able to react with peptides from the small complexes. Peptides in the small complexes do not aggregate to an extent where they could represent conformational epitopes but must instead be epitopes derived from the linear sequence of the allergen. Aalberse states that the main factor is the huge difference in binding affinity between antibodies interacting with intact protein versus interacting with peptides from the very same protein. This means that the peptides are much less efficient as compared to the intact protein for antibody binding. The peptide may for instance represent only a fraction of the epitope or may only be a mimic of the epitope for which the antibody was originally directed against, with only a certain degree of resemblance. The strength of interaction with the peptide could be decreased even more because of the higher flexibility of free peptides as compared to the complete protein. This is in agreement with our own unpublished data, demonstrating a higher avidity between the binding of antibodies and intact protein as compared to the binding of the antibodies and digests. It is suggested that about 10% of antibodies directed toward conformational epitopes are able to react with linear peptide fragments of the protein, which correlates well with the present study.

In summary, the current study showed that while digested Ara h 1 has sensitizing capacity, this capacity was lost after separation of the peptides in the digest into fractions. The sensitizing capacity of the digest was not dependent on single peptides but rather the sum of peptides. However, to unravel if the sensitizing capacity is a result of mixtures of free peptides or is a result of the peptides being in a defined aggregated state, further studies are needed. On the basis of the present study, we may conclude that the way in which the digests are presented to the immune systems is of significant importance.
for the outcome and confirms the complexity of the mechanisms involved in sensitization.

**AUTHOR INFORMATION**

*Corresponding Author*

Tel: +45 35887092. Fax: +45 35887001. E-mail: kalb@food.dtu.dk.

**Present Address**

University of Manchester, Manchester Interdisciplinary Bio-centre, Manchester Academic Health Science Centre, School of Translational Medicine, 131 Princess Street, Manchester M1 7DN, United Kingdom.

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The authors declare no competing financial interest.

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**ABBREVIATIONS USED**

ACN, acetonitrile; BN, Brown Norway; EU, endotoxin unit; GPC, gel permeation chromatography; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; MS, mass spectrometry; MW, molecular weight; RP-HPLC, reverse phase high-performance liquid chromatography; SGF, simulated gastric fluid; TFA, trifluoroacetic acid

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