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Authentication of shrimp muscle in complex foodstuff by in-solution digestion and high-resolution mass spectrometry

Qing Chen, Xiao-Dong Pan * and Bai-Fen Huang

A method for shrimp muscle identification in complex foods is required to safeguard the shrimp-allergic population. This study described a method for authentication of shrimp in complex foodstuffs (fish balls) by liquid chromatography tandem QTOF mass spectrometry (UPLC-QTOF-MS). The proteins in shrimp muscle were extracted using a Tris-HCl solution and then digested using tryptic protease. The main allergen proteins, tropomyosin (TM) and arginine kinase (AK), were characterized using the 'bottom up' MS approach. After analysis of their peptide mass fingerprinting based on the UniProt database, two specific heat-stable peptides, ALSNAEGEVAALNR for TM and VSSTLSLEGELK for AK, were screened as surrogate (signature) peptides. The detection limit, expressed as shrimp meat per kilogram of food, was 8 g kg⁻¹ (usage of TM) or 5 g kg⁻¹ (usage of AK). The developed method is suitable to screen potential addition of shrimp meat in foodstuffs by detection of allergen proteins.

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Introduction

The consumption of shrimp continuously increases worldwide due to its well-established health benefits. For the majority of world's population, the growing interest can be considered a nutritional advantage. However, for a small but rather significant group of food-allergic individuals, the consumption can pose a severe health problem.¹ Many cases of shellfish allergies have been frequently reported, being currently viewed as an emergent issue of public health.^{2,3}

In the European Union (EU), food labeling regulations have been revised and labeling of several allergenic ingredients is now mandatory (Directives 2003/89/EC and 2007/68/EC).⁴ But different countries mandate a different selection of allergens for food labeling. Despite this regulation, total avoidance might be difficult for the allergic consumer. Furthermore, little is known on threshold doses, *i.e.* the minimum amount of an allergenic food which is able to cause an allergic reaction. Hence, the sensitive and selective analytical methods for allergens are required to protect certain consumers.

The most frequently used analytical methods for allergen detection are either immunological based on antibodies or polymerase chain reactions (PCR).² Many disadvantages of current established methods for allergen analyses are discussed, such as cross-reactivity and potential false-negative results.⁵⁻⁸ Recently, LC/MS technique using protease digestion

was developed to characterize and identify proteins.⁹⁻¹² Different mass analyzer including triple quadrupole (QQQ), time-of-flight (TOF), orbitrap and ion trap (IT) are applied for the allergen test.^{13,14} Because MS identification is a direct type of detection using different principles, it is expected to be an effective method for allergen confirmation. Noticeably, identifying and detecting allergens using MS systems, sample preparation is a critical step. Allergen proteins are usually extracted from food matrices and digested with enzymes generating peptides¹⁵

In this study, we aimed to analysis shrimp allergen protein in complex foodstuffs (fish balls) by liquid chromatography tandem QTOF mass spectrometry (UPLC-QTOF-MS). The allergen protein was prepared by in-solution digestion and (solid phase extraction) SPE clean-up. The main allergen protein in shrimp, tropomyosin (TM) or arginine kinase (AK) was tested by their optimized signature peptides.

Experimental

Chemicals and materials

Ammonium bicarbonate (NH₄HCO₃), dithioerithol (DTT), iodoacetamide (IAA) and hydrochloric acid (HCl, 37%) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Acetonitrile (ACN) and formic acid (FA) were purchased from Merck (Darmstadt, Germany). All the reagents used were analytical or HPLC grade. Sequencing grade modified trypsin was from Shanghai Yaxin Biotechnology Co., Ltd (Shanghai, China). All chemical agents were prepared using ultrapure water and without further purification. Ultrapure water was obtained by

Zhejiang Provincial Center for Disease Control and Prevention, Physical-chemistry Room No. 401, Bin-Sheng Road No. 3399, Binjiang District, Hangzhou, 310051, China. E-mail: zjpanxiaodong@hotmail.com; Fax: +86 571 87115261; Tel: +86 571 87115274



a Milli-Q Gradient A10 water purification system (Millipore, Bedford, MA, USA) during all the experiments.

Samples

The samples (fish balls) were purchased from local supermarket (TESCO, Hangzhou, China). Fish balls are mainly made from fish meat, starch, pork meat or shrimp meat. For validation of developed method, the home-made fish balls containing 10, 50, 100, 150, 200 g kg⁻¹ shrimp meat were prepared by mixture of fish meat (*Anguilla japonica*), starch (*Dioscorea esculenta* Burkill), pork meat (*Yorkshire*), shrimp meat (*Penaeus vannamei*) and water. The home-made fish balls are cooked in boiled water for 30 min. The samples were stored at -20 °C before experimental analysis.

Sample extraction and digestion

Allergen proteins were extracted from 5 g ground matrix in 15 mL Tris-HCl (200 mM, pH 9.2) with 2 M urea by shaking for 30 min. Then, the mixture was sonicated for 15 min at 4 °C to avoid carbamidomethylation with urea. The samples were centrifuged at 5000 g for 15 min at 10 °C. 100 µL supernatant, 100 µL 500 mM NH₄HCO₃ and 665 µL deionized water were mixed in an Eppendorf tube. 10 µL 50 mM DTT solution were added to the mixtures and reduced in 40 °C water bath for 30 min at this stage. In the next step an alkylation was performed by adding 10 µL of 150 mM IAA in the dark for 30 min at room temperature. Immediately prior to the incubation, 100 µL of 500 mM NH₄HCO₃ and 10 µL of 400 µg mL⁻¹ trypsin (freshly prepared) were added and incubated 6 h at 37 °C. The reaction was terminated by addition of 5 µL formic acid. The insoluble substances in tryptic hydrolysates were removed by centrifuging at 13 000g for 10 min. Before analysis of Q-TOF, the supernatant was further cleaned up by SPE.

For screening of the surrogated peptides for the allergens, 0.5 g shrimp muscle sample was directly selected, and prepared by the above method.

Peptides clean-up

SPE purification of digested proteins was performed on Oasis® HLB SPE columns (3 cm³/60 mg, 30 µm). Cartridge was pre-conditioned with 1 mL MeOH followed by equilibration with 1 mL water of 0.1% formic acid. The digested samples were loaded on the column. The samples were washed with 1 mL of 20% MeOH in water. The analytes were eluted with 1 mL MeOH and then dried down under nitrogen. The dried samples were reconstituted into 1 mL of 2% acetonitrile in water with 0.1% formic acid. The mixture was analyzed by UPLC-QToF-MS after passing through a 0.22 µm nylon filter (Agela Technologies).

Instrument conditions

Tryptic hydrolysates were separated using an ACQUITY UPLC System equipped with ACQUITY UPLC binary solvent manager, sample manager, and column manager (Waters, Milford, MA, USA). Chromatographic separation was carried

out on a narrow-bore Acquity UPLC BEH 300C₁₈ column (1.7 µm, 2.1 mm × 100 mm) maintained at 35 °C, equipped with a guard column of the same material (Waters, Milford, MA, USA). The 0.1% FA aqueous solution (solvent A) and 0.1% FA ACN solution (solvent B) were used for the mobile phases. Gradient elution was: 2% B to 40% B for 20 min; 100% B for 1 min; re-equilibration at the initial conditions for 2 min. The flow rate for separations was maintained at 0.3 mL min⁻¹ and a 10.0 µL injection volume was used for all standards and samples.

Quadruple time-of-flight tandem mass spectrometry (QToF-MS) detection was performed on a Synapt G2 HDMS equipped with an electrospray ion (ESI) source (Waters). All data were acquired in the electrospray positive ion (ESI⁺) mode with MS^E mode. Details of TOF conditions were as follows: capillary voltage, 3 kV; sampling cone voltage, 25 V; extraction cone voltage, 4 V; source temperature, 100 °C; desolvation temperature, 400 °C; cone gas flow, 30 L h⁻¹; desolvation gas flow, 800 L h⁻¹; ramp trap collision energy, 15–35 V; and lockspray reference compound, leucine-enkephalin (*m/z* 556.2771 Da).

Allergen database and data processing

The MS^E data were searched against the shrimp allergen database containing TM and AK of *Litopenaeus vannamei* (whiteleg shrimp) (downloaded from <http://www.uniprot.org/> on Jan. 10, 2015) using the IDENTITY^E search algorithm within the ProteinLynx Global Server v. 2.5 (PLGS 2.5; Waters, U.K.). Search parameters included the “automatic” setting for mass accuracy (50 ppm for precursor ions and 0.1 Da for product ions), a minimum of one peptide match per protein, a minimum of three consecutive product ion matches per peptide, and a minimum of seven total product ion matches per protein. The maximum false positive rate (FPR) against the randomized forward database was set to 4%. Only one missed tryptic cleavage site was allowed during the search. Modifications included the following: fixed, carbamidomethylation of Cys; variable, deamidation of Asn and Gln, oxidation of Met, and dehydration of Ser and Thr.

Method validation

Method validation was performed based on the international conference on harmonization (ICH) guidelines¹⁶ for validation of bio-analytical procedures. For linearity measurements, fish balls containing shrimp meat (*Penaeus vannamei*) with six different concentrations (10, 50, 100, 150, 200 g kg⁻¹) along with blank samples were performed for calibration curves over 3 days.

Method acceptance criteria states that the precision of the calibration curve and QC samples (containing 1.5% shrimp meat) are considered to be acceptable if RSD ≤ 15% for intra and inter day precision. Furthermore, the accuracy compared with the nominal value needs to be no more than 15%. Finally, the calibration curves must meet the above criteria and have a correlation coefficient *r* of at least 0.99.



Results and discussion

Analysis of tryptic peptides

Selection of suitable signature peptides for accurate quantitation of targeted protein is a crucial challenge for developing LC-MS/MS approach. Bioinformatics tool is usually adopted to assist the computational prediction of tryptic products. The theoretical tryptic cleavage peptides of allergen proteins were obtained by computational prediction by Waters BiolyNX softwares and online PeptideMass tools provided by UniProt (http://web.expasy.org/peptide_mass).

For further confirmation of tryptic peptides, UPLC-QToF-MS was applied for comparing the endogenous and theoretical peptides from tryptic proteins. These peptides were identified detected in tryptic shrimp muscles after comparing the acquired data and sequence database search (Table 1). The coverage ratios of the searched peptides to targeted protein were all more than 50%. Theoretically, tryptic peptides numbers of TM and AK from *Litopenaeus vannamei* (whiteleg shrimp) were 32 and 29. But, actual detected peptides in MS showed in Table 1 were less than those. Not all the obtained peptides can be used as the surrogate (signature) peptides. The candidate peptides were selected based on several critical factors such as specificity of amino acid sequences, reproducibility in sample preparation, intensity of their MS signal.¹⁷

Selection of signature peptides

Applied criteria for signature peptides are the absence of cysteine and methionine, peptide size between seven and 20 amino acids, and no modification of amino acids. Furthermore, the peptides with more amino acids (>14) may be not used due to their expensive synthesise and unfavorable LC properties.^{17,18} Therefore, we selected the peptide VSSTLSSLEGELK, LIDDHFLFK, LTSAVNEIEK and TFLVWVNEEDHLR for AK, and IQLLEEDLER, ALSNAEGEVAALNR, IVELEEEELR, LAEASQAADSEER and EVDRLEDELVNEK for TM.

With the aim to screening these peptides with the property of easy tryptic digestion and high MS intensity, we reduced the digestion time to 2 h and investigated the MS intensity of these peptides. As showed in Fig. 1, different intensities of peptides were obtained. The signature peptides, IQLLEEDLER, IVELEEEELR and ALSNAEGEVAALNR for TM and VSSTLSSLEGELK and LIDDHFLFK for AK were selected. The difference of tryptic hydrolysis degree within peptides is hard to explain. Although disulfide bonds of proteins were sheared by DTT and IAA in the pretreatment process, the secondary structure main composed of hydrogen bond and van der Waals forces still existed.

For further optimization of signature peptides, the sequences of TM and AK in some crabs and shrimps were aligned (Fig. 2). We selected the peptides, ALSNAEGEVAALNR for TM and VSSTLSSLEGELK for AK, which are not presented in most crabs. In addition, the intensities of the two peptides were not significantly changed after treatment with boiled water for 30 min ($n = 8$, $P < 0.05$).

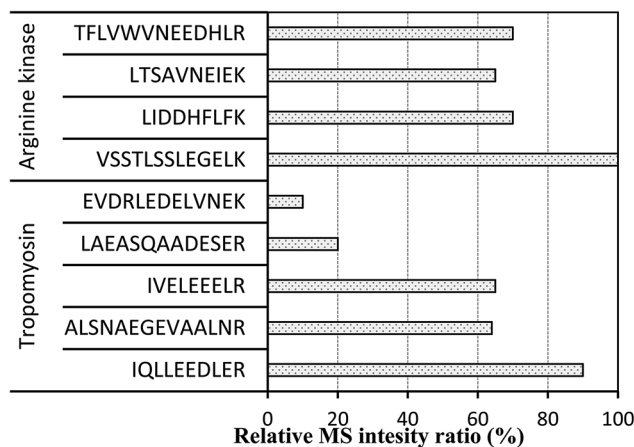


Fig. 1 The intensity of peptides obtained by 2 h digestion.

Table 1 Identified peptides by in-matrix digestion and UPLC-QTOF-MS

Protein	Peptides (*recommended signature peptides)	Matched products string	Precursor intensity	Precursor (m/z)	Mass error (ppm)
B4YAH6 LITVA Lit v 1 tropomyosin	IQLLEEDLER	b2b2b3y1y3y5y6y7y8y9y10	12 938	629.3392	3.5958
	*ALSNAEGEVAALNR	b9b14y5y6y7y8y9y10y14	8017	707.8684	2.0720
	IVELEEEELR	y2y4y6y7y7y8y9	6999	565.3094	1.8585
	LAEASQAADSEER	y4y5y7y9y10y13	6492	688.8181	2.8633
	SITDELDTQTFSELSGY	b10b13b16y10y11y16	5227	902.9098	3.1328
	LAMVEADLER	y5y6y7y8	1250	573.7949	5.1705
	EVDRLEDELVNEK	b7b8y4y5y7y11y12	1040	794.3971	5.6077
Q004B5 LITVA arginine kinase	LGFLTFCPTNLGTTVR	b16y5y7y9y10y11y12y16	23 790	898.9724	3.3503
	*VSSTLSSLEGELK	b5b6b8b13b13y4y6y8y9y10y11y12y13	16 996	675.3613	5.8746
	DFGDVNSFVNVDPEGK	b11y4y5y7y10y16	9333	869.8987	3.5018
	*LIDDHFLFK	b6y5y6y7y9	3888	574.3119	4.2465
	LTSAVNEIEK	y5y6y7y9y10	3506	552.3025	4.9357
	EMQDGILELIK	y5y7y8y9	2755	644.8461	5.3712
	TFLVWVNEEDHLR	y3y7y8y9y10y11y13	2641	829.4190	3.0708
	FLQAANACR	b5y1y5y6y7	2093	1050.5166	1.3153
	GEHTEAEGGIYDISNK	b8b9b10y4y5y9	1279	860.3935	3.2045



Align of selected proteins for tropomyosin

CLUSTAL O(1.2.2) multiple sequence alignment

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TR|B4YAH6|B4YAH6_LITVA
60
TR|D3XNS0|D3XNS0_FEMME MDAIKKKQAMKLEKNDNAMRADTLTQQNKANRAKSEEEVHNLKQKMQQLNDLDV 60
SF|P8704|TPM_PANBO MDAIKKKQAMKLEKNDNAMRADTLTQQNKANRAKSEEEVHNLKQKMQQLNDLDV 60
SF|Q2N2R3|TPM_CHAFE MDAIKKKQAMKLEKNDNAMRADTLTQQNKANRAKSEEEVHNLKQKMQQLNDLDV 60
SF|Q25456|TPM_FEMMO MDAIKKKQAMKLEKNDNAMRADTLTQQNKANRAKSEEEVHNLKQKMQQLNDLDV 60
TR|E7CCG1|E7CCG1_FEMMO MDAIKKKQAMKLEKNDNAMRADTLTQQNKANRAKSEEEVHNLKQKMQQLNDLDV 60
TR|D7F1J4|D7F1J4_CRANC MDAIKKKQAMKLEKNDNAMRADTLTQQNKANRAKSEEEVHNLKQKMQQLNDLDV 60
TR|D2R6M0|D2R6M0_FEMCH MDAIKKKQAMKLEKNDNAMRADTLTQQNKANRAKSEEEVHNLKQKMQQLNDLDV 60
TR|A4URH3|A4URH3_ERIS1 MDAIKKKQAMKLEKNDNAMRADTLTQQNKANRAKSEEEVHNLKQKMQQLNDLDV 60
TR|ASD6H8|ASD6H8_LIMPO ---IKNQAMKLEKNDNAMRADTLTQQNKANRAKSEEEVHNLKQKMQQLNDLDV 60
TR|A7L5V3|A7L5V3_PORTR MDAIKKKQAMKLEKNDNAMRADTLTQQNKANRAKSEEEVHNLKQKMQQLNDLDV 60
TR|VSNBV4|VSNBV4_MACRS MDAIKKKQAMKLEKNDNAMRADTLTQQNKANRAKSEEEVHNLKQKMQQLNDLDV 60
TR|MH607|MH607_PORPE MDAIKKKQAMKLEKNDNAMRADTLTQQNKANRAKSEEEVHNLKQKMQQLNDLDV 60
MDAIKKQAMKLEKNDNAMRADTLTQQNKANRAKSEEEVHNLKQKMQQLNDLDV 60
**::*::* *::: *::: *::: *::: *::: *::: *::: *::: *::: *::: *::: *::: *::: *::: *::: *::: *::: *::: *::: *::: *:::

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TR|B4YAH6|B4YAH6_LITVA QEBLLKANIQLVKDKKALSNABGVAAALNRQLLEEDLSEERLNTATTKLABASQA 120
SF|P8704|TPM_PANBO QEBLLKANIQLVKDKKALSNABGVAAALNRQLLEEDLSEERLNTATTKLABASQA 120
SF|Q2N2R3|TPM_CHAFE QEBLLKANIQLVKDKKALSNABGVAAALNRQLLEEDLSEERLNTATTKLABASQA 120
SF|Q25456|TPM_FEMMO QEBLLKANIQLVKDKKALSNABGVAAALNRQLLEEDLSEERLNTATTKLABASQA 120
TR|E7CCG1|E7CCG1_FEMMO QEBLLKANIQLVKDKKALSNABGVAAALNRQLLEEDLSEERLNTATTKLABASQA 120
TR|D7F1J4|D7F1J4_CRANC QEBLLKANIQLVKDKKALSNABGVAAALNRQLLEEDLSEERLNTATTKLABASQA 120
TR|D2R6M0|D2R6M0_FEMCH QEBLLKANIQLVKDKKALSNABGVAAALNRQLLEEDLSEERLNTATTKLABASQA 120
TR|A4URH3|A4URH3_ERIS1 QEBLLKANIQLVKDKKALSNABGVAAALNRQLLEEDLSEERLNTATTKLABASQA 120
TR|ASD6H8|ASD6H8_LIMPO QEBLLKANIQLVKDKKALSNABGVAAALNRQLLEEDLSEERLNTATTKLABASQA 120
TR|A7L5V3|A7L5V3_PORTR QEBLLKANIQLVKDKKALSNABGVAAALNRQLLEEDLSEERLNTATTKLABASQA 120
TR|VSNBV4|VSNBV4_MACRS QEBLLKANIQLVKDKKALSNABGVAAALNRQLLEEDLSEERLNTATTKLABASQA 120
TR|MH607|MH607_PORPE QEBLLKANIQLVKDKKALSNABGVAAALNRQLLEEDLSEERLNTATTKLABASQA 120
TR|D3XNS0|D3XNS0_FEMME QEBLLKANIQLVKDKKALSNABGVAAALNRQLLEEDLSEERLNTATTKLABASQA 120

Align of selected proteins for arginine kinase

CLUSTAL O(1.2.2) multiple sequence alignment

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SF|Q95V58|KARG_ARTSP MVDAGTLKLEAGPKLAQTDCKSLKKYLTREVPDQLTKLTS -IGATLLDVIQ8V9 59
SF|P5154|KARG_PBNJP ---VDAAVLEKLEAGPKLAQTDCKSLKKYLTREVPDQLTKLTS -IGATLLDVIQ8V9 58
SF|Q9NH48|KARG_ERIS1 MADAATLKELEAGPKLAQTDCKSLKKYLTREVPDQLTKLTS -IGATLLDVIQ8V9 59
SF|Q9NH49|KARG_CALS1 MADAATLKELEAGPKLAQTDCKSLKKYLTREVPDQLTKLTS -IGATLLDVIQ8V9 59
SF|P5154|KARG_LIMPO MDAATLKELEAGPKLAQTDCKSLKKYLTREVPDQLTKLTS -IGATLLDVIQ8V9 59
SF|Q9NH48|KARG_CARMA MADAATLKELEAGPKLAQTDCKSLKKYLTREVPDQLTKLTS -IGATLLDVIQ8V9 59
SF|Q9Y27|KARG_PACMR MADAATLKELEAGPKLAQTDCKSLKKYLTREVPDQLTKLTS -IGATLLDVIQ8V9 59
SF|C7E3T4|KARG_FEMMO MADAATLKELEAGPKLAQTDCKSLKKYLTREVPDQLTKLTS -IGATLLDVIQ8V9 59
TR|D7F1J5|D7F1J5_CRANC MDAVAVLEKLEAGPKLAQTDCKSLKKYLTREVPDQLTKLTS -IGATLLDVIQ8V9 59
TR|B1PZ9|B1PZ9_MBTM MDAVAVLEKLEAGPKLAQTDCKSLKKYLTREVPDQLTKLTS -IGATLLDVIQ8V9 59
TR|Q004B5|Q004B5_LITVA MDAVAVLEKLEAGPKLAQTDCKSLKKYLTREVPDQLTKLTS -IGATLLDVIQ8V9 59
TR|AA0A88F19|AA0A88F19_HALAR MVDVTRLEKLEAGPKLAQTDCKSLKKYLTREVPDQLTKLTS -IGATLLDVIQ8V9 59
TR|Q4KY22|Q4KY22_FBNCH MADAATLKELEAGPKLAQTDCKSLKKYLTREVPDQLTKLTS -IGATLLDVIQ8V9 59
TR|G3D692|G3D692_PORTR MADAATLKELEAGPKLAQTDCKSLKKYLTREVPDQLTKLTS -IGATLLDVIQ8V9 59
TR|H6K206|H6K206_SCTPA MADAATLKELEAGPKLAQTDCKSLKKYLTREVPDQLTKLTS -IGATLLDVIQ8V9 59
TR|C3VU00|C3VU00_FEMME MADAATLKELEAGPKLAQTDCKSLKKYLTREVPDQLTKLTS -IGATLLDVIQ8V9 59

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SF|Q95V58|KARG_ARTSP VISTVRCRSMGQYFPNCLTBAQYKEMKSVSTLSLSEGLGKTYFPLTQMSKVVQ 179
SF|P5154|KARG_PBNJP VISTVRCRSMGQYFPNCLTBAQYKEMKSVSTLSLSEGLGKTYFPLTQMSKVVQ 178
SF|Q9NH48|KARG_ERIS1 VISTVRCRSMGQYFPNCLTBAQYKEMKSVSTLSLSEGLGKTYFPLTQMSKVVQ 179
SF|Q9NH49|KARG_CALS1 VISTVRCRSMGQYFPNCLTBAQYKEMKSVSTLSLSEGLGKTYFPLTQMSKVVQ 179
SF|P5154|KARG_LIMPO VISTVRCRSMGQYFPNCLTBAQYKEMKSVSTLSLSEGLGKTYFPLTQMSKVVQ 179
SF|Q9NH48|KARG_CARMA VISTVRCRSMGQYFPNCLTBAQYKEMKSVSTLSLSEGLGKTYFPLTQMSKVVQ 179
SF|Q9Y27|KARG_PACMR VISTVRCRSMGQYFPNCLTBAQYKEMKSVSTLSLSEGLGKTYFPLTQMSKVVQ 179
SF|C7E3T4|KARG_FEMMO VISTVRCRSMGQYFPNCLTBAQYKEMKSVSTLSLSEGLGKTYFPLTQMSKVVQ 179
TR|D7F1J5|D7F1J5_CRANC VISTVRCRSMGQYFPNCLTBAQYKEMKSVSTLSLSEGLGKTYFPLTQMSKVVQ 179
TR|B1PZ9|B1PZ9_MBTM VISTVRCRSMGQYFPNCLTBAQYKEMKSVSTLSLSEGLGKTYFPLTQMSKVVQ 179
TR|Q004B5|Q004B5_LITVA VISTVRCRSMGQYFPNCLTBAQYKEMKSVSTLSLSEGLGKTYFPLTQMSKVVQ 179
TR|AA0A88F19|AA0A88F19_HALAR VISTVRCRSMGQYFPNCLTBAQYKEMKSVSTLSLSEGLGKTYFPLTQMSKVVQ 179
TR|Q4KY22|Q4KY22_FBNCH VISTVRCRSMGQYFPNCLTBAQYKEMKSVSTLSLSEGLGKTYFPLTQMSKVVQ 179
TR|G3D692|G3D692_PORTR VISTVRCRSMGQYFPNCLTBAQYKEMKSVSTLSLSEGLGKTYFPLTQMSKVVQ 179
TR|H6K206|H6K206_SCTPA VISTVRCRSMGQYFPNCLTBAQYKEMKSVSTLSLSEGLGKTYFPLTQMSKVVQ 179
TR|C3VU00|C3VU00_FEMME VISTVRCRSMGQYFPNCLTBAQYKEMKSVSTLSLSEGLGKTYFPLTQMSKVVQ 179

Entry (tropomyosin)	Status	Organism	Length
B4YAH6	reviewed	<i>Litopenaeus vannamei</i> (Whiteleg shrimp) (<i>Penaeus vannamei</i>)	284
P8704	reviewed	<i>Penaeus borealis</i> (Northern red shrimp)	284
Q2N2R3	reviewed	<i>Charybdis forsteri</i> (Crucifix crab) (<i>Libinia forsteri</i>)	264
AIKY22	reviewed	<i>Penaeus monodon</i> (Giant tiger prawn)	284
Q25456	reviewed	<i>Metapenaeus ensis</i> (Greasyback shrimp) (<i>Penaeus ensis</i>)	274
E7CCG1	reviewed	<i>Penaeus monodon</i> (Giant tiger prawn)	284
D7F1J4	reviewed	<i>Crangon crangon</i> (Brown shrimp)	284
D2R6M0	unreviewed	<i>Fenneropenaeus chinensis</i> (Fleshy prawn) (<i>Penaeus chinensis</i>)	284
A4URH3	unreviewed	<i>Eriocheir sinensis</i> (Chinese mitten crab)	284
ASD6H8	unreviewed	<i>Limulus polyphemus</i> (Atlantic horseshoe crab)	281
A7L5V3	unreviewed	<i>Portunus trituberculatus</i> (Swimming crab) (<i>Neptunus trituberculatus</i>)	284
VSNBV4	unreviewed	<i>Macrobrachium rosenbergii</i> (Giant freshwater prawn)	284
MH607	unreviewed	<i>Portunus pelagicus</i> (Blue swimmer crab)	284
D3XNS0	unreviewed	<i>Fenneropenaeus merguensis</i> (Banana prawn) (<i>Penaeus merguensis</i>)	284

Entry (arginine kinase)	Status	Organism	Length
Q95V58	reviewed	<i>Artemia franciscana</i> (Brine shrimp) (<i>Artemia salina</i>)	356
P5154	reviewed	<i>Penaeus japonicus</i> (Kuruma prawn) (<i>Marsippenaeus japonicus</i>)	355
Q9NH48	reviewed	<i>Eriocheir sinensis</i> (Chinese mitten crab)	357
Q9NH49	reviewed	<i>Callinectes sapidus</i> (Blue crab)	357
P5154	reviewed	<i>Limulus polyphemus</i> (Atlantic horseshoe crab)	357
Q9U94	reviewed	<i>Carcinus maenas</i> (Common shore crab) (Green crab)	357
Q9GYX1	reviewed	<i>Pachygrapsus marmoratus</i> (Marbled rock crab) (Cancer marmoratus)	357
C7E3T4	reviewed	<i>Penaeus monodon</i> (Giant tiger prawn)	356
E7CCG2	reviewed	<i>Penaeus monodon</i> (Giant tiger prawn)	356
AA0A97KV17	unreviewed	<i>Metacarcinus magister</i> (Dungeness crab) (Cancer magister)	210
D7F1J5	unreviewed	<i>Crangon crangon</i> (Brown shrimp)	356
B1PZ9	unreviewed	<i>Metapenaeus ensis</i> (Greasyback shrimp) (<i>Penaeus ensis</i>)	357
AA0A97KV3	unreviewed	<i>Uca crassipes</i> (Fiddler crab)	210
Q004B5	unreviewed	<i>Litopenaeus vannamei</i> (Whiteleg shrimp) (<i>Penaeus vannamei</i>)	356
AA0A88F19	unreviewed	<i>Halocaridina rubra</i> (Hawaiian red shrimp)	356
Q4KY22	unreviewed	<i>Fenneropenaeus chinensis</i> (Fleshy prawn) (<i>Penaeus chinensis</i>)	356
G3D692	unreviewed	<i>Portunus trituberculatus</i> (Swimming crab) (<i>Neptunus trituberculatus</i>)	357
AA0A97KVD1	unreviewed	<i>Atergatis integerrimus</i> (Red egg crab) (<i>Cancer integerrimus</i>)	210
AA0A97KV5	unreviewed	<i>Portunus pelagicus</i> (Blue swimmer crab)	357
H6K50	unreviewed	<i>Scylla paramamosain</i> (Mud crab)	210
G3C6N4	unreviewed	<i>Austinoebia edulis</i> (Mud shrimp) (<i>Upogebia edulis</i>)	210
AA0A97KV6	unreviewed	<i>Ranina ranina</i> (Spanner crab)	210
C3VU00	unreviewed	<i>Fenneropenaeus merguensis</i> (Banana prawn) (<i>Penaeus merguensis</i>)	356

Fig. 2 Align of selected proteins for tropomyosin and arginine kinase.

The specificity of targeted signature peptides was confirmed by the Basic Local Alignment Search Tool (BLAST) of Uniprot (<http://www.uniprot.org/blast>). It is critical that signature

peptides are unique to the target protein and detectable by the MS systems of choice.¹⁰ The more information is available, the higher the certainty that the peptide represents the allergen and species of interest. Unfortunately, because of the sequence homology of allergens in different species, it is not always possible that signature peptides are species specific. The results of cross-reaction with TM and AK from other organisms by BLAST were listed in Table 2.

The selected signature peptides do not need to be the most intense signals found in MS spectra, but they do need to be sufficiently intense to allow clear separation from other peptides or MS background (Fig. 3). The heat stability of the selected signature peptides were tested at 100 °C for 0.5 h. There is no significant change ($P < 0.05$, data not shown here) to the intensity of each peptide.

Digestion by trypsin

When identifying and detecting allergen proteins using MS systems, sample preparation is a critical step. Allergen proteins are usually required to be extracted from food matrix and digested with enzymes generating peptides. For comparison, molecular cut-off filter was used after the protein extraction with Tris-HCl solution as described by previous reports.^{19–21} However, there is no obvious difference in the numbers of obtained peptides. For clean-up of obtained peptides, SPE was adopted. A systematic investigation of orthogonal SPE clean-up of digested samples was performed by Yuan *et al.*²² In this study, Oasis® HLB SPE module was used for purification.

Various enzymes are available with specific cleavage sites. Trypsin was adopted in our experiment. It is the most commonly used enzyme due to the well-known cleavage sites between the amino acid arginine (R) and lysine (K). Carrera *et al.*¹⁹ has approved that more peptides could be identified when fish species were digested with trypsin compared with Glu-C.

Method validation

For validation of the detection ability, the home-made fish balls containing 1.5% shrimp meat were selected as the QC sample.

Table 2 Results of surrogate peptides by BLAST in UniProt

Protein	Surrogate peptide	Cross-reaction from other organism by BLAST
Tropomyosin	ALSNAEVEVAALNR	<i>Sinonovacula constricta</i> , <i>Tyrophagus putrescentiae</i> , <i>Portunus pelagicus</i> , <i>Paralithodes camtschaticus</i> , <i>et al.</i>
Arginine kinase	VSSTLSSLEGELK	<i>Drosophila mojavensis</i> , <i>Portunus trituberculatus</i> , <i>Macrophthalmus japonicus</i> , <i>Portunus trituberculatus</i> , <i>Larinus</i> sp. BHJ-2011, <i>Callinectes laevimanus</i> , <i>Aegla neuquensis</i> , <i>Gomezia bicornis</i> , <i>Dotilla myctiroides</i> , <i>Eplumula phalangium</i> , <i>Hylaena elegans</i> , <i>Orithyia sinica</i> , <i>et al.</i>



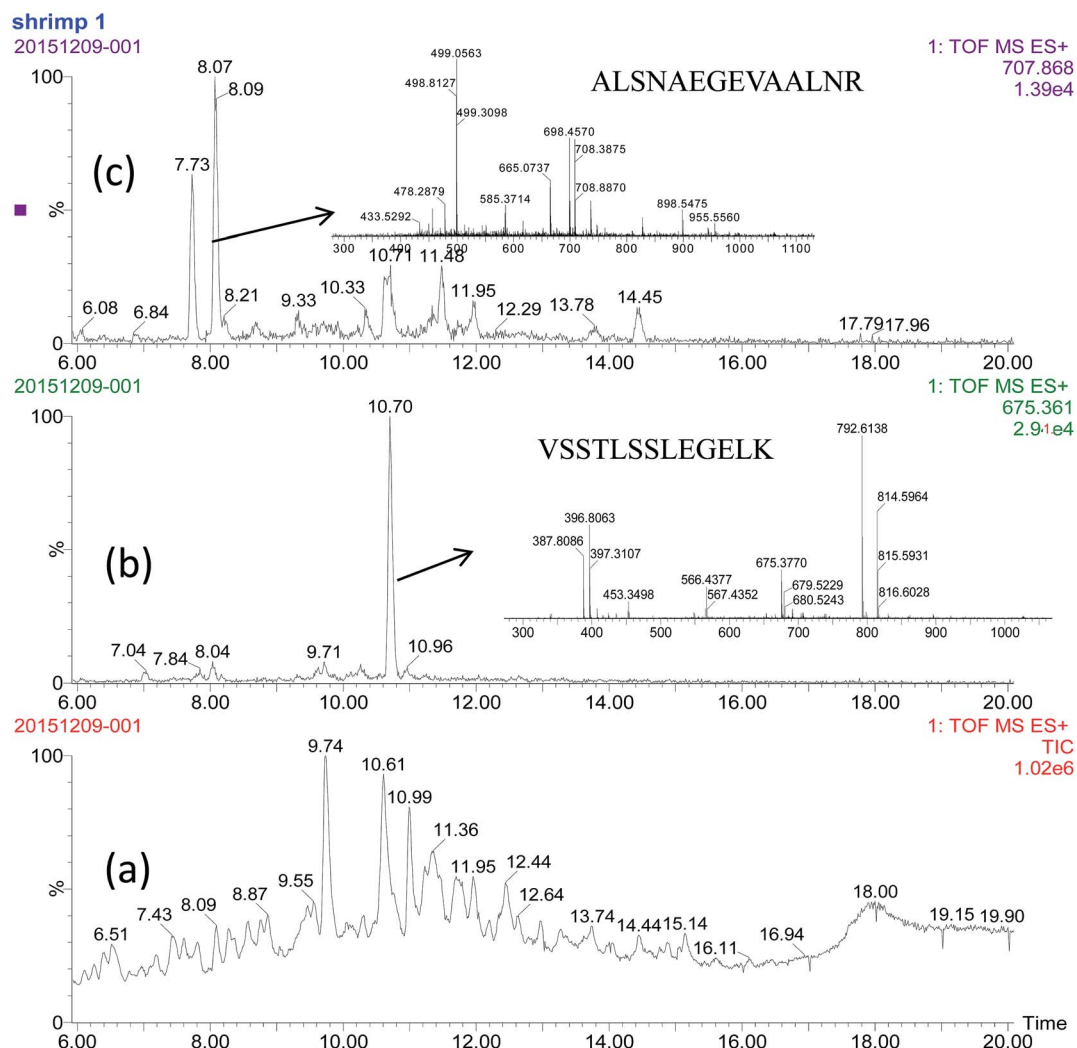


Fig. 3 The chromatogram of total iron (a), selected peptides (b) and (c) by UPLC-QTQF-MS.

Table 3 The precision, accuracy and linearity of surrogate peptide for quantification in UPLC-QToF-MS

Protein	Surrogate peptide	Precursor ($^{2+}$)/product ion ($^{1+}$)	Correlation coefficient (r)	Intraday ($n = 3$)		Interday ($n = 3$)	
				R	RSD	R	RSD
Tropomyosin	ALSNAEGEVAALNR	707.868/829.452	0.9912	86	9.5	90	7.5
Arginine kinase	VSSTLSSLEGELK	675.361/862.451	0.9909	88	11.3	92	8.1

As shown in Table 3, the RSDs of inter- and intra-day were all less than 15%, and the recoveries were more than 80% (Table 3). Usually, the level owning mass response of a signal-to-noise ratio (S/N) of 3 is considered as limit of detection. In this study, the detection limit, expressed of shrimp meat per kilogram of food, was 8 g kg^{-1} (usage of TM) or 5 g kg^{-1} (AK).

Conclusion

The shrimp allergen proteins (tropomyosin and arginine kinase) in complex foodstuffs can be fast detected by in-solution

digestion and UPLC-QTQF-MS. The surrogate peptides, ALSNAEGEVAALNR for TM and VSSTLSSLEGELK for AK were screened. The developed method is suitable to screen potential addition of shrimp muscle in foodstuffs by detection of allergen proteins.

Furthermore, it should be noted that the present method was only used for screening shrimp muscle *via* allergenic proteins in complex foodstuffs. Although different crustacean have species-specific peptides for TM, AK or other proteins,²³ selected surrogate peptides of ALSNAEGEVAALNR and VSSTLSSLEGELK have cross-reaction with other organisms,



especially for some crabs (for example *Portunus trituberculatus* and *Calcinus laevimanus*) (Table 2). Our further work will focus on the screening of a generic surrogate peptide in TM or AK for quantifying most of crustacean foods.

Author contributions statement

X.-D. P, Q. C and B.-F. H conceived the experiment(s), X.-D. P, conducted the experiment(s), X.-D. P and B.-F. H analyzed the results. All authors reviewed the manuscript.

Conflicts of interest

The authors declare no competing financial interests.

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