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

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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 VSSTLSSLEGELK 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.

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 matrice and digested with enzymes generating peptides[15]

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 (NH_4HCO_3), dithiotheritol (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

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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 NH_4HCO_3 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 NH_4HCO_3 and 10 μL of 400 μg m L^{-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 13 000*g* 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 cm^3/60 mg, 30 μm). Cartridge was preconditioned 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 300C_{18} 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) guidelines[16] 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 synthesize and unfavorable LC properties.[17,18] Therefore, we selected the peptide VSSTLSSLEGELK, LIDDHFLFK, LTSAVNEIEK and TFLVWVNEEDHLR for AK, and IQLLEEDLER, ALSNAEGEVAALNR, IVELEEELR, LAEASQAADESER 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, IVELEEELR 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 ($n = 8$, $P < 0.05$).

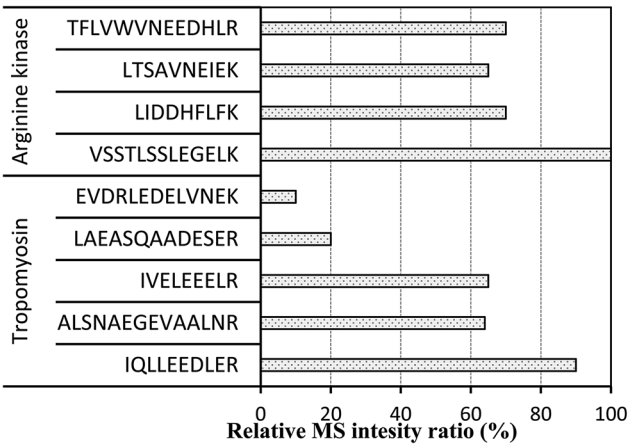

Fig. 1 he intensity of peptides obtained by 2 h digestion.

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
	IVELEEELR	y2y4y6y7y7y8y9	6999	565.3094	1.8585
	LAEASQAADESER	y4y5y7y9y10y13	6492	688.8181	2.8633
	SITDELDQTFSELSGY	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**

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CLUSTAL O(1.2.2) multiple sequence alignment

TR|B4YAH6|B4YAH6_LITVA
60
TR|D3XNS0|D3XNS0_FENME MDAIKKKMQAMKLEKDNAMDRADTLEQQNKEANNRAEKSEEEVHNLQKRMQQLENDLDQV 60
SP|P86704|TPM_PANBO    MDAIKKKMQAMKLEKDNAMDRADTLEQQNKEANNRAEKSEEEVFGLQKKLQQLENDLDSV 60
SP|Q9N2R3|TPM_CHAFE    MDAIKKKMQAMKLEKDNAMDRADTLEQQNKEANLRAEKTEEEIRATQKKMQQVENELDQA 60
SP|A1KYZ2|TPM_PENMO    MDAIKKKMQAMKLEKDNAMDRADTLEQQNKEANNRAEKSEEEVHNLQKRMQQLENDLDQV 60
SP|Q25456|TPM_METEN    ----------MKLEKDNAMDRADTLEQQNKEANNRAEKSEEEVHNLQKRMQQLENDLDSV 50
TR|E7CGC1|E7CGC1_PENMO MDAIKKKMQAMKLEKDNAMDRADTLEQQNKEANNRAEKSEEEVHNLQKRMQQLENDLDQV 60
TR|D7F1J4|D7F1J4_CRACN MDAIKKKMQAMKLEKDNAMDRADTLEQQNKEANNRAEKSEEEVFSLQKRMQQLENDLDSV 60
TR|D2KMW0|D2KMW0_FENCH MDAIKKKMQAMKLEKDNAMDRADTLEQQNKEANNRAEKSEEEVHNLQKRMQQLENDLDQV 60
TR|A4URH3|A4URH3_ERISI MDAIKKKMQAMKLEKDNAMDRADTLEQQNKEANNRAEKTEEEIRATQKKMQQVENELDQA 60
TR|A5D6H8|A5D6H8_LIMPO ---IKNKMQAMKLDKENACDRADIAEQQSRDANARADKAEEEVRSLQKKIQQIENELDQV 57
TR|A7L5V3|A7L5V3_PORTR MDAIKKKMQAMKLEKDNAMDRANTLEQQNKEANLRAEKTEEEIRATQKKMQQVENELDQA 60
TR|V5NBV4|V5NBV4_MACRS MDAIKKKMQAMKLEKDNAMDRADTLEQQNKEANNRAEKSEEEVFSLQKRMQQLENDLDSV 60
TR|M1H607|M1H607_PORPE     MDAIKKKMQAMKLEKDDAMDRADTLEQQNKEANIRAEKAEEEVHNLQKRMQQLENDLDQV
MDAIKKKMQAMKLEKDNAMDRADTLEQQNKEANNRAEKSEEEVHNLQKRMQQLENDLDQV 60
                                **::*:.* *::: . * *.:::* :*:*:::*:   **: * :**:** .

TR|B4YAH6|B4YAH6_LITVA QESLLKANIQLVEKDKALSNAEGEVAALNRRIQLLEEDLERSEERLNTATTKLAAESQAA 120
SP|P86704|TPM_PANBO    QEALLKANQHLEEKDKALSNAEGEVAALNRRIQLLEEDLERSEERLNTATTKLAAESQAA 120
SP|Q9N2R3|TPM_CHAFE    QEQLSAANTKLDEKEKALQNAEGEVAALNRRIQLPEEDLERSEERLNTATTKLAAESQAA 120
SP|A1KYZ2|TPM_PENMO    QESLLKANIQLVEKDKALSNAEGEVAALNRRIQLLEEDLERSEERLNTATTKLAAESQAA 120
SP|Q25456|TPM_METEN    QESLLKANNQLVEKDKALSNAEGEVAALNRRIQLLEEDLERSEERLNTATTKLAAESQAA 110
TR|E7CGC1|E7CGC1_PENMO QESLLKANIQLVEKDKALSNAEGEVAALNRRIQLLEEDLERSEERLNTATTKLAAESQAA 120
TR|D7F1J4|D7F1J4_CRACN QEALLKANAHLEEKDKALSNAEGEVAALNRRIQLLEEDLERSEERLNTATTKLAAESQAA 120
TR|D2KMW0|D2KMW0_FENCH QESLLKANIQLVEKDKALSNAEGEVAALNRRIQLLEEDLERSEERLNTATTKLAAESQAA 120
TR|A4URH3|A4URH3_ERISI QEQLSAANTKLDEKEKALQNAEGEVAALNRRIQLLEEDLERSEERLNTATTKLAAESQAA 120
TR|A5D6H8|A5D6H8_LIMPO QEQLVTANAKLEEKDKALQNAEGEVAALNRKVQLLEEDLERSEERLKIATQKLEEATQLA 117
TR|A7L5V3|A7L5V3_PORTR QEQLSAANTKLDEKEKALQNAEGEVAALNRRIQLLEEDLERSEERLNTATTKLAAESQAA 120
TR|V5NBV4|V5NBV4_MACRS QEALLKANQHLEEKDKALSNAEGEVAALNRRIQLLEEDLERSEERLNTATTKLAAESQAA 120
TR|M1H607|M1H607_PORPE QESLLKANTQLEEKDKALSNAEGEVAALNRRIQLLEEDLERSEERLNTATTKLAAESQAA 120
TR|D3XNS0|D3XNS0_FENME QESLLKANIQLVEKDKALSNAEGEVAALNRRIQLLEEDLERSEERLNTATTKLAAESQAA 120
                        .* *   *. :* **:**: ************:** **********: ** ** **:* *
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Align of selected proteins for **arginine kinase**

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CLUSTAL O(1.2.2) multiple sequence alignment

SP|Q95V58|KARG_ARTSF           MVDAGTLEKLEAGFQKLQAATDCKSLVKKYLTREVFDQLKTLKTS-LGATLLDVIQSGVE 59
SP|P51545|KARG_PENJP           -VDAAVLEKLQAGFKKLEAATDCKSLLKKYLSKDIFDKLKQKTS-LGATLLDVIQSGVE 58
SP|Q9NH48|KARG_ERISI           MADAATIAKLDEGFKKLEAATDCKSLLKKYLTKDVFEQLKAKKTK-LGATLLDVIQSGVE 59
SP|Q9NH49|KARG_CALSI           MADAATIAKLEEGFKKLEAATDCKSLLKKYLTKSVFDQLKDKKTS-LGATLLDVIQSGVE 59
SP|P51541|KARG_LIMPO           MVDQATLDKLEAGFKKLQEASDCKSLLKKHLTKDVFDSIKNKKTG-MGATLLDVIQSGVE 59
SP|Q9U9J4|KARG_CARMA           MADAATITKLEEGFKKLEAATDCKSLLKKYLTKSVFDQLKAKKTS-LGATLLDVIQSGVE 59
SP|Q9GYX1|KARG_PACMR           MADAATISKLEEGFKKLQGATDCKSLLKKYLTKDVFDQLKAKKTS-LGATLLDVIQSGVE 59
SP|C7E3T4|KARG_PENMO           MADAAVIEKLEAGFKKLEAATDCKSLLKKYLSKAVFDQLKEKKTS-LGATLLDVIQSGVE 59
TR|E7CGC2|E7CGC2_PENMO         MADAAVIEKLEAGFKKLEAATDCKSLLKKYLSKAVFDQLKEKKTS-LGATLLDVIQSGVE 59
TR|D7F1J5|D7F1J5_CRACN         MVDAEVLEKLEAGYKKLEAATDCKSLLKKYLTKEVFDELKTKKTA-LGATLLDVIQSGVE 59
TR|B1PVZ9|B1PVZ9_METEN         MADAAVIEKLEAGFKKLEAATDCKSLLKKYLTKEVFDKLKDKTS-LGATLLDVIQSGVE 59
TR|Q004B5|Q004B5_LITVA         MADAAVIEKLEAGFKKLEAATDCKSLLKKYLTKEVFDKLKDKRTS-LGATLLDVIQSGVE 59
TR|A0A088FIL9|A0A088FIL9_HALRR MVDTRVLEKLEAGFKKLQDADCKSLLKKYLTQEVFDELKTKEKTS-LGATLLDVIESGVE 59
TR|Q4KY22|Q4KY22_FENCH         MADAAVIEKLEAGFKKLEAATDCKSLLKKYLTKAVFDQLKDKKTS-LGATLLDVIQSGVE 59
TR|G3D692|G3D692_PORTR         MADAATIAKLEEGFKKLEAATDCKSLLKKYLTKSVFDQLKDKKTD-LGATLLDVIQSGVE 59
TR|H6UKS0|H6UKS0_SCYPA         MADAATIAKLEEGFKKLEAATDCKSLLKKYLTKSVFDQLKGKKTS-LGATLLDVIQSGVE 59
TR|C3VUU0|C3VUU0_FENME         MADAAVIEKLEAGFKKLEAATDCKSLLKKYLSKAVFDQLKEKKTS-LGATLLDVIQSGVE 59

SP|Q95V58|KARG_ARTSF           VISTRVRCGRSLQGYPFNPCLTEAQYKEMEDKVSSTLNGLDGELKGTFYPLTGMAKEVQQ 179
SP|P51545|KARG_PENJP           VISTRVRCGRSMEGYPFNPCLTEAQYKEMQSKVSSTLSSLEGELKGTYFPLTGMSKEVQQ 178
SP|Q9NH48|KARG_ERISI           VISTRVRCGRSMEGYPFNPCLTEAQYKEMESKVSSTLSNLEGELKGTYPPLTGMTKEVQQ 179
SP|Q9NH49|KARG_CALSI           VISTRVRCGRSMEGYPFNPCLTEAQYKEMESKVSSTLSNLEGELKGTYPPLTGMTKEVQQ 179
SP|P51541|KARG_LIMPO           IISTRVRCGRSLQGYPFNPCLTAEQYKEMSEKVSSTLSSMEDELKGTYYPLTGMSKATQQ 179
SP|Q9U9J4|KARG_CARMA           VISTRVRCGRSMEGYPFNPCLTEAQYKEMESKVSSTLSNLEGELKGTYHALTGMTKEVQQ 179
SP|Q9GYX1|KARG_PACMR           VISTRVRCGRSMEGYPFNPCLTEAQYKEMEAKVPSTLSSLEGELKGSFYPLTGMAKDVQQ 179
SP|C7E3T4|KARG_PENMO           VISTRVRCGRSMEGYPFNPCLTEAQYKEMEAKVSSTLSSLEGELKGTYYPLTGMSKEVQQ 179
TR|E7CGC2|E7CGC2_PENMO         VISTRVRCGRSMEGYPFNPCLTEAQYKEMEAKVSSTLSSLEGELKGTYYPLTGMSKEVQQ 179
TR|A0A097KVJ7|A0A097KVJ7_METMG --------SMEGYPFNPCLTEAQYKEMESKVSSTLSNLEGELKGTYHPLTGMTKEVQQ 50
TR|D7F1J5|D7F1J5_CRACN         VVSTRVRCGRSMEGYPFNPCLTEAQYKEMESKVSSTLSSLEGELKGTYYPLTGMSKDVQQ 179
TR|B1PVZ9|B1PVZ9_METEN         VISTRVRCGRSMQGYPFNPCLTESQYKEMEAKVSSTLSSLEGELKGTYYPLTGMSKEVQQ 179
TR|A0A097KVS3|A0A097KVS3_UCACR --------SMEGYPFNPCLTEAQYKEMEEKISSTLSNLEGELKGTYYPLTGMTKEVQQ 50
TR|Q004B5|Q004B5_LITVA         VISTRVRCGRSLQGYPFNPCLTESQYKEMEAKVSSTLSSLEGELKGTYYPLTGMSKEVQQ 179
TR|A0A088FIL9|A0A088FIL9_HALRR VVSTRVRCGRSMEGYPFNPCLTEDQYREMEEKVSSTLSNLEGELKGTYYPLTGMSKEVQQ 179
TR|Q4KY22|Q4KY22_FENCH         VISTRVRCGRSMEGYPFNPCLTEDQYKEMESKVSSTLSSLEGELKGTYYPLTGMSKEVQQ 179
TR|G3D692|G3D692_PORTR         VISTRVRCGRSMEGYPFNPCLTEAQYKEMESKVSSTLSSLEGELKGTYYPLTGMTKEVQQ 179
TR|A0A097KVD1|A0A097KVD1_ATEIN ----------SMEGYPFNPCLTEAQYKEMESKVSSTLSGLEGELKGSYYPLTGMTKEVQQ 50
TR|A0A097KVK5|A0A097KVK5_PORPE ----------SMEGYPFNPCLTEAQYKEMESKVSSTLSNLEGELKGTYYPLTGMTKEVQQ 50
TR|H6UKS0|H6UKS0_SCYPA         VISTRVRCGRSMEGYPFNPCLTEAQYKEMESKVSSTLSNLEGELKGTYYPLTGMTKDVQQ 179
TR|G3C6N4|G3C6N4_PANTR         VISTRVRCGRSMEGYPFNPCLTEAQYKEMEDKVSSTLSGLEGELKGTYYPLTGMSKEVQQ 76
TR|A0A097KVQ6|A0A097KVQ6_RANRA ----------SMEGYPFNPCLTEAHYKEMESKVSSTLSGLEGELKGTYYPLTGMTKDVQQ 50
TR|C3VUU0|C3VUU0_FENME         VISTRVRCGRSMQGYPFNPCLTESQYKEMEAKVSSTLSSLEGELKGTYYPLTGMSKEVQQ 179
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Entry (tropomyosin)	Status	Organism	Length
B4YAH6	unreviewed	*Litopenaeus vannamei* (Whiteleg shrimp) (Penaeus vannamei)	284
P86704	reviewed	*Pandalus borealis* (Northern red shrimp)	284
Q9N2R3	reviewed	*Charybdis feriata* (Crucifix crab) (*Cancer feriatus*)	264
A1KYZ2	reviewed	*Penaeus monodon* (Giant tiger prawn)	284
Q25456	reviewed	*Metapenaeus ensis* (Greasyback shrimp) (*Penaeus ensis*)	274
E7CGC1	unreviewed	*Penaeus monodon* (Giant tiger prawn)	284
D7F1J4	unreviewed	*Crangon crangon* (Brown shrimp)	284
D2KMW0	unreviewed	*Fenneropenaeus chinensis* (Fleshy prawn) (*Penaeus chinensis*)	284
A4URH3	unreviewed	*Eriocheir sinensis* (Chinese mitten crab)	284
A5D6H8	unreviewed	*Limulus polyphemus* (Atlantic horseshoe crab)	281
A7L5V3	unreviewed	*Portunus trituberculatus* (Swimming crab) (*Neptunus trituberculatus*)	284
V5NBV4	unreviewed	*Macrobrachium rosenbergii* (Giant fresh water prawn)	284
M1H607	unreviewed	*Portunus pelagicus* (Blue swimmer crab)	284
D3XNS0	unreviewed	*Fenneropenaeus merguiensis* (Banana prawn) (*Penaeus merguiensis*)	284

Entry (arginine kinase)	Status	Organism	Length
Q95V58	reviewed	*Artemia franciscana* (Brine shrimp) (*Artemia sanfranciscana*)	356
P51545	reviewed	*Penaeus japonicus* (Kuruma prawn) (*Marsupenaeus japonicus*)	355
Q9NH48	reviewed	*Eriocheir sinensis* (Chinese mitten crab)	357
Q9NH49	reviewed	*Callinectes sapidus* (Blue crab)	357
P51541	reviewed	*Limulus polyphemus* (Atlantic horseshoe crab)	357
Q9U9J4	reviewed	*Carcinus maenas* (Common shore crab) (Green crab)	357
Q9GYX1	reviewed	*Pachygrapsus marmoratus* (Marbled rock crab) (Cancer marmoratus)	357
C7E3T4	reviewed	*Penaeus monodon* (Giant tiger prawn)	356
E7CGC2	unreviewed	*Penaeus monodon* (Giant tiger prawn)	356
A0A097KVJ7	unreviewed	*Metacarcinus magister* (Dungeness crab) (Cancer magister)	210
D7F1J5	unreviewed	*Crangon crangon* (Brown shrimp)	356
B1PVZ9	unreviewed	*Metapenaeus ensis* (Greasyback shrimp) (*Penaeus ensis*)	357
A0A097KVS3	unreviewed	*Uca crassipes* (Fiddler crab)	210
Q004B5	unreviewed	*Litopenaeus vannamei* (Whiteleg shrimp) (*Penaeus vannamei*)	356
A0A088FIL9	unreviewed	*Halocaridina rubra* (Hawaiian red shrimp)	356
Q4KY22	unreviewed	*Fenneropenaeus chinensis* (Fleshy prawn) (*Penaeus chinensis*)	356
G3D692	unreviewed	*Portunus trituberculatus* (Swimming crab) (*Neptunus trituberculatus*)	357
A0A097KVD1	unreviewed	*Atergatis integerrimus* (Red egg crab) (*Cancer integerrimus*)	210
A0A097KVK5	unreviewed	*Portunus pelagicus* (Blue swimmer crab)	210
H6UKS0	unreviewed	*Scylla paramamosain* (Mud crab)	357
G3C6N4	unreviewed	*Austinogebia edulis* (Mud shrimp) (*Upogebia edulis*)	210
A0A097KVQ6	unreviewed	*Ranina ranina* (Spanner crab)	210
C3VUU0	unreviewed	*Fenneropenaeus merguiensis* (Banana prawn) (*Penaeus merguiensis*)	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 significantly 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 matrice 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 column 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	ALSNAEGEVAALNR	*Sinonovacula constricta*, *Tyrophagus putrescentiae*, *Portunus pelagicus*, *Paralithodes camtschaticus*, *et al.*
Arginine kinase	VSSTLSSLEGELK	*Drosophila mojavensis*, *Portunus trituberculatus*, *Macrophthalmus japonicas*, *Portunus trituberculatus*, *Larinus* sp. BHJ-2011, *Calcinus laevimanus*, *Aegla neuquensis*, *Gomeza bicornis*, *Dotilla myctiroides*, *Eplumula phalangium*, *Hylaeus elegans*, *Orithyia sinica*, *et al.*

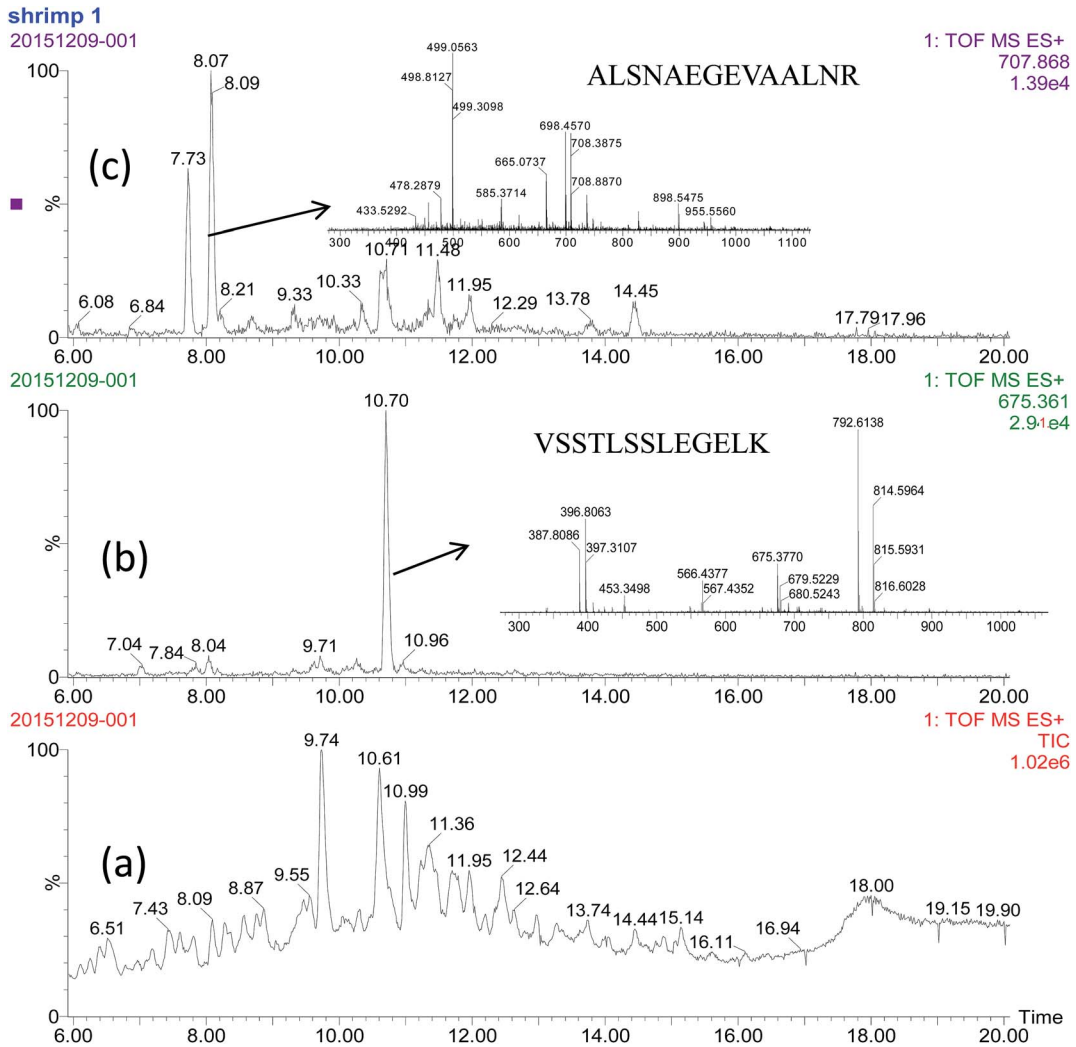

Fig. 3 The chromatogram of total iron (a), selected peptides (b) and (c) by UPLC-QTQF-MS.

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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