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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

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

Qing Chen, Xiao-Dong Pan (1)* 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 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.

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 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), 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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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: zjupanxiaodong@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 μ L 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 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 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 μ m). 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 μ m 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 μ m, 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 μ L 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 \leq 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.

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, LAEASQAA-DESER 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, IVE-LEEELR 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.

Protein	Peptides (*recommended signature peptides)	Matched products string	Precursor intensity	Precursor (m/z)	Mass error (ppm)
B4YAH6 LITVA Lit v	IOLLEEDLER	h2h2h3v1v3v5v6v7v8v9v10	12,938	629 3392	3 5958
1 tropomyosin	*ALSNAEGEVAALNR	b9b14v5v6v7v8v9v10v14	8017	707.8684	2.0720
1 doponijobili	IVELEEELR	v2v4v6v7v7v8v9	6999	565.3094	1.8585
	LAEASQAADESER	v4v5v7v9v10v13	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	LGFLTFCPTNLGTTVR	b16y5y7y9y10y11y12y16	23 790	898.9724	3.3503
arginine kinase	*VSSTLSSLEGELK	b5b6b8b13b13y4y6y8y9y10y11y12y13	16 996	675.3613	5.8746
0	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

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

CLUSTAL O(1.2.2) multiple sequence alignment

TR|B4YAH6|B4YAH6 LITVA

		_		
0				
R	D3XNS0	D3XNS0_FENME	MDAIKKKMQAMKLEKDNAMDRADTLEQQNKEANNRAEKSEEEVHNLQKRMQQLENDLDQV	60
P	P86704	TPM PANBO	MDAIKKKMQAMKLEKDNAMDRADTLEQQNKEANNRAEKSEEEVFGLQKKLQQLENDLDSV	60
P	Q9N2R3	TPM CHAFE	MDAIKKKMQAMKLEKDNAMDRADTLEQQNKEANLRAEKTEEEIRATQKKMQQVENELDQA	60
P	A1KYZ2	TPM_PENMO	MDAIKKKMQAMKLEKDNAMDRADTLEQQNKEANNRAEKSEEEVHNLQKRMQQLENDLDQV	60
P	Q25456	TPM METEN	MKLEKDNAMDRADTLEQQNKEANNRAEKSEEEVHNLQKRMQQLENDLDQV	50
R	E7CGC1	E7CGC1_PENMO	MDAIKKKMQAMKLEKDNAMDRADTLEQQNKEANNRAEKSEEEVHNLQKRMQQLENDLDQV	60
R	D7F1J4	D7F1J4_CRACN	MDAIKKKMQAMKLEKDNAMDRADTLEQQNKEANNRAEKSEEEVFSLQKRMQQLENDLDSV	60
R	D2KMW0	D2KMW0_FENCH	MDAIKKKMQAMKLEKDNAMDRADTLEQQNKEANNRAEKSEEEVHNLQKRMQQLENDLDQV	60
R	A4URH3	A4URH3_ERISI	MDAIKKKMQAMKLEKDNAMDRADTLEQQNKEANNRAEKTEEEIRATQKKMQQVENELDQA	60
R	A5D6H8	A5D6H8_LIMPO	IKNKMQAMKLDKENACDRADIAEQQSRDANARADKAEEEVRSLQKKIQQIENELDQV	57
R	A7L5V3	A7L5V3 PORTR	MDAIKKKMQAMKLEKDNAMDRANTLEQQNKEANLRAEKTEEEIRATQKKMQQVENELDQA	60
R	V5NBV4	V5NBV4_MACRS	MDAIKKKMQAMKLEKDNAMDRADTLEQQNKEANNRAEKSEEEVFSLQKRMQQLENDLDSV	60
R	M1H607	M1H607_PORPE	MDAIKKKMQAMKLEKDDAMDRADTLEQQNKEANIRAEKABEEVHNLQKRMQQLENDL	DQV
DJ	AIKKKMQ/	MKLEKDNAMDRAI	DTLEQQNKEANNRAEKSEEEVHNLQKRMQQLENDLDQV 60	
			**::*::* *::: * *.:::* :*:*::::*: **: * :*:*:	

TR	B4YAH6	B4YAH6 LITVA	OESLLKANI	DLVEKDKALS	NAEGEVAAL	NRRIOLLEEL	LERSEERLNTAT	TKLAEASOAA	120
SP	P86704	TPM PANBO	OFALLKANO	HLEEKDKALS	NARGEVAAL	NERTOLLEEI	LERSERRLNTA'	TTKLARASOAA	120
SP	09N2R3	TPM CHAFE	OEOLSAANT	KLDEKEKAL/	NARGEVAAI	NRRIOLPEEL	LERSEERLNTA	TTKLARASOAA	120
SP	A1KYZ2	TPM PENMO	OESLLKANI	OLVEKDKALS	NAEGEVAAI	NRRIOLLEEI	DLERSEERLNTA	TTKLAEASOAA	120
SP	025456	TPM METEN	OESILIKANN	OLVEKDKALS	NARGEVAAL	NERTOLLEEI	LERSERLNTA'	TTKLARASOAA	110
TR	E7CGC1	E7CGC1 PENMO	OESLLKANI	LVEKDKALS	NAEGEVAAL	NRRIOLLEEL	LERSEERLNTAT	TKLAEASOAA	120
TR	D7F1J4	D7F1J4 CRACN	OEALLKANA	ILEEKDKALS	NAEGEVAAL	NRRIOLLEEL	LERSEERLNTAT	TKLAEASOAA	120
TR	D2KMW0	D2KMW0 FENCH	OESLLKANI	DLVEKDKALS	NAEGEVAAL	NRRIOLLEEL	LERSEERLNTAT	TKLABASOAA	120
TR	A4URH3	A4URH3 ERISI	OEOLSAANT	KLDEKEKALC	NAEGEVAAL	NRRIQLLEED	LERSEERLNTAT	TKLAEASQAA	120
TR	A5D6H8	A5D6H8 LIMPO	OEOLVTANA	KLEEKDKALC	NAEGEVAAL	NRRVOLLEED	LERSEERLKIAT	OKLEEATOLA	117
TR	A7L5V3	A7L5V3 PORTR	OBOLSAANT	KLDEKEKALC	NAEGEVAAL	NRRIOLLEEL	LERSEERLNTAT	TKLABASOAA	120
TR	V5NBV4	V5NBV4 MACRS	QEALLKANO	HLEEKDKALS	NAEGEVAAL	NRRIQLLEED	LERSEERLNTAT	TKLAEASQAA	120
TR	M1H607	M1H607 PORPE	QESLLKANT	LEEKDKALS	NAEGEVAAL	NRRIQLLEED	LERSEERLNTAT	TKLAEASQAA	120
TR	D3XNS0	D3XNS0 FENME	QESLLKANI	LVEKDKALS	NAEGEVAAL	NRRIQLLEED	LERSEERLNTAT	TKLAEASQAA	120
		_	.* * *.	:* **:**:	*******	*** ** ***	********	* ** **:* *	
Align of selected proteins for arginine kinase									

CLUSTAL 0(1.2.2) multiple sequence alignment

chosiAL O(1.2.2) multiple sequence alignment

	SP Q95V58 KARG_AR	TSF	MVDAGTLEKLEAGFQKLQAATDCKSLVKKYLTREVFDQLKTLKTS-LGATLLDVIQSGVE	59
	SPICONHAR KARG_PE	NU P TST	- VDRAVDBRUGGERRUBAATDCKSLLKKYLSKDIFDKLKGQKTS-LGATLLDVIQSGVE MADAATTAKLDEGERKUEAATDCKSLLKKYLTKDVEEOLKAKKTK-LGATLDVIGOGVE	59
	SP 09NH49 KARG CA	LSI	MADAATIAKLEEGFKKLEAATDCKSLLKKYLTKSVFDOLKDKKTS-LGATLLDVIQSOVE	59
	SP P51541 KARG_LI	MPO	MVDQATLDKLEAGFKKLQEASDCKSLLKKHLTKDVFDSIKNKKTG-MGATLLDVIQSGVE	59
	SP Q9U9J4 KARG_CA	RMA	MADAATITKLEEGFKKLEAATDCKSLLKKYLTKSVFDQLKAKKTS-LGATLLDVIQSGVE	59
	SP Q9GIX1 KARG_PA	CMR NMO	MADAATISKLEEGFKKLQGATDCKSLLKKYLSKAVFDQLKAKKTS-LGATLLDV1QSGVE MADAAVIEKLEAGEKKLEAATDCKSLLKKYLSKAVFDQLKEKKTS-LGATLLDV1QSGVE	59
	TR E7CGC2 E7CGC2	PENMO	MADAAVIEKLEAGFKKLEAATDCKSLLKKYLSKAVFDQLKEKKTS-LGATLLDVIQSGVE	59
	TR D7F1J5 D7F1J5	CRACN	MVDAEVLEKLEAGYKKLEAATDCKSLLKKYLTKEVFDELKTKKTA-LGATLLDVIQSGVE	59
	TR B1PVZ9 B1PVZ9_I	METEN	MADAAVIEKLEAGFKKLEAATDCKSLLKKYLTKEVFDKLKDKKTS-LGATLLDVIQSGVE	59
	TR Q00485 Q00485	DITVA	MADAAVIEKLEAGFKKLODAKDCKSLLKKYLTORVFDRLKTKKTS-LGATLLDV1QSGVE MVDTRVLRKLEAGFKKLODAKDCKSLLKKYLTORVFDRLKTKKTS-LGATLLDV1QSGVE	59
	TR Q4KY22 Q4KY22	FENCH	MADAAVIEKLEAGFKKLEAATDCKSLLKKYLTKAVFDQLKDKKTS-LGATLLDVIQSGVE	59
	TR G3D692 G3D692_	PORTR	MADAATIAKLEEGFKKLEAATDCKSLLKKYLTKSVFDQLKDKKTD-LGATLLDVIQSGVE	59
	TR H6UKS0 H6UKS0_	SCYPA	MADAATIAKLEEGFKKLEAATDCKSLLKKYLTKSVFDQLKGKKTS-LGATLLDVIQSGVE MADAAVIEKLEAGEKKLEAATDCKSLLKKYLTKEVEDKLKDKKTS-LGATLLDVIQSGVE	59
	SP Q95V58 KARG_AR	TSF	VISTRVRCGRSLQGYPFNPCLTEAQYKEMEDKVSSTLNGLDGELKGTFYPLTGMAKEVQQ	179
	SP P51545 KARG_PE	NJP	VISTRVRCGRSMEGYPFNPCLTEAQYKEMQQKVSSTLSSLEGELKGTYFPLTGMSKEVQQ VIGTVPCGPGMEGYDENDCIJTEAOYKEMPGKVGGTIGNI.BGELKGTYPPLTGMSKEVQQ	178
	SP Q9NH49 KARG_CA	LSI	VISTRVRCGRSMEGYPFNPCLTEAQYKEMESKVSSTLSNLEGELKGTYFPLTGMTKEVQQ	179
	SP P51541 KARG_LI	MPO	IISTRVRCGRSLQGYPFNPCLTAEQYKEMEEKVSSTLSSMEDELKGTYYPLTGMSKATQQ	179
	SP Q90904 KARG_CA	KMA CMR	VISTRVRCGRSMEGYPPNPCLTEAQYKEMESKVSSTLSNLEGELKGTYHALTGMTKDVQQ VISTPVPCOPSMEGYDPNDCLTEACYKEMESKVSSTLSSLEGELKGSEVDLTCMAKDVCQ	179
	SP C7E3T4 KARG_PE	NMO	VISTRVRCGRSMEGYPFNPCLTEAQYKEMEAKVSSTLSSLEGELKGTYYPLTGMSKEVQQ	179
	TR B7CGC2 B7CGC2	PENMO	VISTRVRCGRSMEGYPFNPCLTEAQYKEMEAKVSSTLSSLEGELKGTYYPLTGMSKEVQQ	179
	TR A0A097KV37 A0A TR D7F1J5 D7F1J5	CRACN	VVSTRVRCGRSMEGYPFNPCLTEAQYKEMESKVSSTLSNLEGELKGTYHPLTGMTRDVQQ VVSTRVRCGRSMEGYPFNPCLTEAOYKEMESKVSSTLSSLEGELKGTYYPLTGMSKDVOO	179
	TR B1PVZ9 B1PVZ9	METEN	VISTRVRCGRSMQGYPFNPCLTESQYKEMEAKVSSTLSSLEGELKGTYYPLTGMSKEVQQ	179
	TR A0A097KVS3 A0A	097KVS3_UCACR	SMEGYPFNPCLTEAQYKEMEEKISSTLSNLEGELKGTYYPLTGMTKEVQQ	50
	TR A0A088FIL9 A0A	088FIL9 HALRR	VISINVRCORSEQSIFINPCLIESQINEMEARVSSILSSLEGELKGTYYPLIGMSKEVQQ VVSTRVRCGRSMEGYPFNPCLIEDQYREMEEKVSSILSNLEGELKGTYYPLIGMSKEVOO	179
	TR Q4KY22 Q4KY22	FENCH	VISTRVRCGRSMEGYPFNPCLTEDQYKEMESKVSSTLSSLEGELKGTYYPLTGMGKEVQQ	179
	TR G3D692 G3D692	PORTR	VISTRVRCGRSMEGYPFNPCLTEAQYKEMESKVSSTLSSLEGELKGTYFPLTGMTKEVQQ	179
	TR ADADS / KVD1 ADA TR ADADS 7 KVK5 ADA	097KVK5 PORPR	SMEGIPTNPCLTEAQIAEMESKVSSTLSGLEGELKGSIYPLTGMTKEVQQ SMEGYPFNPCLTEAOYKEMESKVSSTLSNLEGELKGTYPPI.TGMTKRVOO	50
	TR HEUKSO HEUKSO	SCYPA	VISTRVRCGRSMEGYPFNPCLTEAQYKEMESKVSSTLSNLEGELKGTYYPLTGMTKDVQQ	179
	TR G3C6N4 G3C6N4	AUSED	VISTRVRCGRSMEGYPFNPCLTEAQYKEMEDKVSSTLSGLEGELKGTFYPLTGMSKEVQQ	76
	TR C3VUU0 C3VUU0 A0A)	USIKVQ6_RANRA FENME	VISTRVRCGRSMOGYPFNPCLTESOYKEMEAKVSSTLSGLEGELKGTYYPLTGMTKDVQQ	179
	Entry (tropomyosin)	Status	Organism	Length
	B4YAH6	unreviewed	Litopenaeus vannamei (Whiteleg shrimp) (Penaeus vannamei)	284
	P86704	reviewed	Pandalus borealis (Northern red shrimp)	284
	A1KYZ2	reviewed	Pengeus monodon (Giant tiger prawn)	284
	025456	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
	1 215 2110			201
	A5D6H8	unreviewed	Limulus polyphemus (Atlantic horseshoe crab)	281
	A5D6H8 A7L5V3 V5NBV4	unreviewed unreviewed	Limulus polyphemus (Atlantic horseshoe crab) Portunus trituberculatus (Swimming crab) (Neptunus trituberculatus) Macraharchium rosenhereii (Giant fresh water rawn)	281 284 284
	A5D6H8 A7L5V3 V5NBV4 M1H607	unreviewed unreviewed unreviewed unreviewed	Limulus polyphemus (Atlantic horseshoe crab) Portunus trituberculatus (Swimming crab) (Neptunus trituberculatus) Macrobrachium rosenbergii (Giant fresh water prawn) Portunus pelagicus (Blue swimmer crab)	281 284 284 284
_	A5D6H8 A7L5V3 V5NBV4 M1H607 D3XNS0	unreviewed unreviewed unreviewed unreviewed unreviewed	Limulus polyphemus (Atlantic horseshoe crab) Portuns trituberculatus (Swimming crab) (Neptunus trituberculatus) Macrobrachium rosenbergii (Giant fresh water prawn) Portomus pelagicus (Blue swimmer crab) Fenencopenaeus merguiensis (Banana prawn) (Penaeus merguiensis)	281 284 284 284 284 284
_	A5D6H8 A7L5V3 V5NBV4 M1H607 D3XNS0	unreviewed unreviewed unreviewed unreviewed unreviewed	Limidus polyphemus (Adantis horseshoe crab) Portmus triubrezidata (Swimning crab) (Neptums triubrezulatus) Macrobrachium rosembergii (Giant fresh water prawn) Portnuns pelagicari (Blue swimmer crab) Penneropenaeus merguiensis (Banana prawn) (Penaeus merguiensis)	281 284 284 284 284 284
	A5D6H8 A7L5V3 V5NBV4 M1H607 D3XNS0	unreviewed unreviewed unreviewed unreviewed	Limilua polyhemus (Alarric horeschoe crab) Portums trihoerudanta (Swimming crab) (Netpmatr Huberulatus) Macrobrachian rosenbergii (Giant fresh water pravn) Portuma pelagicus (Blue swimmer crab) Fenneropenaeus merguiensis (Banana pravn) (Penaeus merguiensis) Orzanism	281 284 284 284 284 284
	A5D6H8 A7L5V3 VSNBV4 M1H607 D3XNS0 Entry (arginine kinase)	unreviewed unreviewed unreviewed unreviewed) Status	Limilua polyphenus (Alamic horeschoe crab) Portuns tribuccidus (Sviminie groh) (Verptanus tribuercultus) Macrobacchian roscobergii (Giant fichs water provn) Pormar polgeica (Blue svimme crab) Ferneroponaesa mergeiensis (Banasa pravn) (Pencesa mergaiensis) Organism	281 284 284 284 284 284
	A5D6H8 A7L5V3 V5NBV4 M1H607 D3XNS0 Entry (arginine kinase) Q95V58	unreviewed unreviewed unreviewed unreviewed unreviewed	Limulua polyphemus (Atlantic horesshoe crab) Portums tribuceulands (Swimmigue crab) (Neptamus tribuerculatus) Macrobrachian resonbergii (Giant fish water pravn) Portums pelagicus (Banana pravn) (Penaeus mergulensis) Fenneropenaeus mergulensis (Banana pravn) (Penaeus mergulensis) Portum pelagicus (Banana pravn) (Penaeus mergulensis) Artenia franciscana (Brine shtimp) (Artenia sanfranciscana)	281 284 284 284 284 284 Length 356
	A5D6H8 A7L5V3 V5NBV4 M1H607 D3XNS0 Entry (arginine kinase) Q95V58 P51545	unreviewed unreviewed unreviewed unreviewed unreviewed) Status reviewed reviewed	Limilia polyhemia (Allaniis horesshoe crab) Portunis trihoeculanda (Sviminia) Macrobrachina rosenbergii (Glant fissh water privin) Macrobrachina rosenbergii (Glant fissh water privin) Fenneropenaeus mergulenstis (Banana pravni) (Penaeus mergulenstis) Organism Artenila franciscoma (Britne shrimp) (Artenila sanfranciscoma) Penaeus japonicus (Kurum prevni) (Marrupenaeus japonicus)	281 284 284 284 284 284 Length 356 355
	A5D6H8 A7L5V3 V5NBV4 M1H607 D3XNS0 Entry (arginine kinase) Q95V58 P51545 Q9NH48	unreviewed unreviewed unreviewed unreviewed unreviewed) Status reviewed reviewed reviewed	Limilua polyphemis (Alamic horeschoe crab) Portunis tribucciulus (Sviminia crab) (Vorphani tribucculutas) Macobrochian rosenbergii (Giant fichs water pravn) Porneropenaeus mergeiensis (Banana pravn) (Penaeus mergeiensis) Organism Artenia franciscona (Brine shrimp) (Artenia sanfranciscona) Penaeus japonicus (Kuruma pravn) (Marsupenaeus japonicus) Ericcheir sinansi (Chines mitter cab)	281 284 284 284 284 284 284 284 355 355 355
	A5D6H8 A7L5V3 V5NBV4 M1H607 D3XNS0 Entry (arginine kinase) Q95V58 P51545 Q9NH48 Q9NH49	unreviewed unreviewed unreviewed unreviewed unreviewed) Status reviewed reviewed reviewed reviewed	Limilua polyphemis (Alamic horeschoe crab) Portunis tribucculands (Swimming crab) (Neptanus tribuerculatus) Macrobrachian resonbergii (Giant fieth water pravn) Portunia pelagicae (Ille swimmer crab) Fenneropenaeus mergaiensis (Banana pravn) (Penaeus mergaiensis) Organism Artenila franciscana (Brine shrimp) (Artenila sanfranciscana) Penaeus japonicus (Kuruma pravn) (Marsupenaeus japonicus) Eriocheir sinensis (Chinese milten crab) Calitenetes sandus (Blue crab)	281 284 284 284 284 284 284 356 355 357 357
	A 5D6H8 A 7L5V3 V5NBV4 M1H607 D3XNS0 Entry (arginine kinase) Q95V58 P51545 Q9NH48 Q9NH49 P51541	unreviewed unreviewed unreviewed unreviewed unreviewed reviewed reviewed reviewed reviewed reviewed	Limita polyhemia (Atlanic horeschoe crab) Portuns tribuccidans (Swimming end) (Verfanus tribucculatas) Macrobrachian resonbergii (Claint fiesh water privin) Macrobrachian resonbergii (Claint fiesh water privin) Fenneropenaeus merguleusis (Banaus pravni) (Ponaeus merguleusis) Organism Artemia francisceana (Brine shtimp) (Artemia sanfranciscoma) Penaeus japonicus (Kurum perwin) (Marsupenaeus japonicus) Eriocheir sinensis (Chinese milten crab) Callineetes sapidus (Buer crab) Jimuto norbolemu (Altanic horsebos crab)	281 284 284 284 284 284 356 355 357 357 357 357
	A5D6H8 A7L5V3 V5NBV4 M1H607 D3XNS0 295V58 P51545 Q9NH48 Q9NH48 Q9NH49 P51541 O017211	unreviewed unreviewed unreviewed unreviewed unreviewed reviewed reviewed reviewed reviewed reviewed reviewed reviewed	Limita polyhemis (Atlantic horeschoe erab) Portuns tribucatidas (Swimmig erab) (Rofanus tribuerulatus) Macrobrachian rosenbergii (Giant fieth water pravn) Pormeroponaesa mergaiorusi (Banana pravn) (Ponaesa mergaiorusi) Ponneroponaesa mergaiorusi (Bana pravn) (Marsupenaeus japonicus) Ponicus (Kuruma pravn) (Marsupenaeus japonicus) Eriocheri sinossi (Chinese mittea crab) Calimectes sapidus (Blue crab) Limulus polyhemus (Atlantic horeschoe crab) Comiser samen (Comes thoru the Care at the same state of the sa	281 284 284 284 284 284 356 355 357 357 357 357
	ASD0H8 ATL5V3 V5NBV4 MH1607 D3XNS0 Entry (arginine kinase) Q95V58 P51545 Q9NH48 Q9NH48 Q9NH49 P51541 Q9D9H	unreviewed unreviewed unreviewed unreviewed volumeviewed volumeviewed volumeviewed reviewed reviewed reviewed reviewed reviewed	Limulua polyphemus (Atlantic horseshoe crab) Portunus tribuceulutas (Swimming crab) (Neptanus tribuerculutas) Macrobrachian resource gravity (Giant fresh water pravn) Pornama pelagican (Ellas weinner crab) Fenneropenaeus mergaiensis (Banana pravn) (Penaeus mergaiensis) Organism Artemia franciscana (Brine shrimp) (Artemia sanfranciscana) Penaeus japonicus (Karuma pravn) (Marraynenaeus japonicus) Eriocheir sinensis (Chinase milten crab) Callinetes saylaka (Blue crab) Limulus polyphemus (Atlantic horseshoe crab) Carcinus maenas (Common shore crab) (Green crab)	281 284 284 284 284 284 356 355 357 357 357 357
	ASD0H8 ATLSV3 V5NBV4 MIH607 D3XNS0 Entry (arginine kinase; Q95V58 P51545 Q9NH48 Q9NH49 P51541 Q9U3J4 Q9U3J4	unreviewed unreviewed unreviewed unreviewed unreviewed) Status reviewed reviewed reviewed reviewed reviewed reviewed reviewed reviewed	Limita polyphemia (Atlantic horeschoe crab) Portunas tribuecidana (Swimming endy) (Vefanata tribuerulatas) Macrobrachian roscobergii (Giant fichs water privon) Fenneropenaeus mergeiotesii (Banasa pravn) (Venaeus merguiensis) Commissional and tribue submitteri (Banasa pravn) (Venaeus merguiensis) Organism Artenia franciscoma (Brine shrimp) (Artenia sanfranciscoma) Penaeus japonicus (Kurum person) (Marrupeneneus japonicus) Eriocheir sinensis (Chinese mitten crab) Calimeters sapidas (Blue crab) Limulus polyphemus (Atlantic horeschoe erab) Carcines menaras (Common shore crab) (Gree roch) Paclograpus marmoratus (Matchel rock crab) (Cancer marmoratus)	281 284 284 284 284 284 356 355 357 357 357 357 357 357
	ASD048 A7L3V3 VSNBV4 MIH607 D3XN80 Entry (arginine kinase) Q95V58 P51545 Q9NH48 Q9NH48 Q9NH48 Q9NH49 P51541 Q9U3/4 Q9GYX1 C7E3T4	unreviewed unreviewed unreviewed unreviewed unreviewed verviewed reviewed reviewed reviewed reviewed reviewed reviewed reviewed	Limilua polyphemis (Alamic horeschoe crab) Portunss tribuccidus (Swimmicz crab) Macrobrachian rosenbergii (Giant fichs water pravn) Pomorphenae Jackgiera (Bue swimmer crab) Pomorphenaesa merguiersisi (Banana pravn) (Ponaesa merguiersis) Pomorphenaesa merguiersisi (Banana pravn) (Ponaesa merguiersis) Organism Artenia franciscana (Brine shrimp) (Artenia sanfranciscana) Ponaesus japonicus (Kuruma pravn) (Marsupenaeus japonicus) Eriocheri sinossi (Chinese mittea crab) Callinectes sapidus (Blue crab) Limulus polyphemis (Alamic horeschoe crab) Carcinas maenas (Comton shore crab) (Green crab) Pachograpusa marroatus (Mathelo cock crab) (Garce marmoratus) Penaesas mondori (Giant tiger pravn)	281 284 284 284 284 284 284 284 284 355 355 355 357 357 357 357 357 357 357
	ASD048 A7L5V3 VSNBV4 MIH607 D3XN807 D3XN807 Q95V58 P51545 Q9NH48 Q9NH48 Q9NH48 Q9NH48 Q90J4 Q9GYX1 C7E3T4 E7CGC2	unreviewed unreviewed unreviewed unreviewed unreviewed reviewed reviewed reviewed reviewed reviewed reviewed reviewed unreviewed unreviewed	Limilua polyphemus (Alamic horeschoe crab) Portunus tribucculands (Swimming crab) (Neptanus tribuderulatus) Macrobrachnus rosenbergii (Giant fiesh water pravn) Porneropenaeus merguiensis (Banua pravn) (Penaeus merguiensis) Ponneropenaeus merguiensis (Banua pravn) (Penaeus merguiensis) Coganism Artemia franciscana (Brins shrimp) (Artenia sanfranciscana) Penaeus japonicus (Kunum a pravn) (Marsupenaeus japonicus) Eriocheir sinensis (Chinese mitten crab) Calineetes sapidas (Blue crab) Limulus polyphemus (Atlantic horseshoe crab) Carcinus maenas (Common shore crab) (Craece mano) Pachegrapus marmoratus (Marbled rock crab) (Cancet mano) Penaeus monodon (Giant tiger pravn) Penaeus monodon (Giant tiger pravn)	281 284 284 284 284 284 284 284 355 355 355 357 357 357 357 357 357 357
	ASDOH8 A71,3V3 VUNIN607 D3XNS0	unreviewed unreviewed unreviewed unreviewed reviewed reviewed reviewed reviewed reviewed reviewed unreviewed unreviewed	Limita polyphemia (Atlantic horeschoe crab) Portunas tribuccidana (Swimming endy) (Vefanuar kritheorulatas) Macrobrachian roscobergii (Giant fiesh water prixvn) Penneroponaesa mergelensisi (Banasa pravn) (Penneas mergulensis) Common polyphemia (Banasa pravn) (Penneas mergulensis) Penneus japoniesa (Kuruma person) (Marsupenneasa japoniesa) Penneus japoniesa (Kuruma person) (Marsupenneasa japoniesa) Eriocheir sinensis (Chinese mitten crab) Callinetestes sapidas (Blue crab) Limulus polyphemus (Atlantic horeschoe crab) Carcinos mennas (Common shore crab) (Green crab) Penaesus monodon (Giant igger pravn) Penaesus monodon (Giant igger pravn) Metaecerinos mengister (Dmengenes crab) (Cancer magister)	281 284 284 284 284 356 355 357 357 357 357 357 356 356 210
	ASDOH8 A71,593 VSNBV3 MILR/7 D33N850 D33N850 Q95V38 P51545 Q9NH49 P51541 Q9NH49 P51541 Q90J4 Q9GYX1 C7E3T4 E7CGC2 A0A097KV17 D7F1J5	unreviewed unreviewed unreviewed unreviewed unreviewed reviewed reviewed reviewed reviewed reviewed reviewed unreviewed unreviewed unreviewed	Limita polyhemis (Atlantic horeschoe erab) Portuns tribuccidus (Swimmicz erab) (Vorpanus tribucerulatus) Macrobrachna roschogica (Elus winner erab) Pomeroponaea mergalensis (Banana parany) (Peraeas mergalensis) Pomeroponaeas mergalensis (Banas parany) (Marsupenneus japonicus) Peraeas japonicus (Kuruma parany) (Marsupenneus japonicus) Ericcheir sinossis (Chinese mitten erab) Callinecters sapidus (Blue erab) Limulta polyhemis (Atlantic horoschoe erab) Carcinus maenas (Common shore erab) (Green erab) Penaeas monodon (Giant tiger pawn) Penaeas monodon (Giant tiger pawn) Matacarcinus magister (Dangeness erab) (Career magister) Caragon erangon (Brown shrimp)	281 284 284 284 284 356 355 357 357 357 357 357 357 356 356 210 356
	ASDOH8 A71,594 MTL494 WH14607 D3XNS0 D3XNS0 D3XNS0 D3XNS0 P51545 Q9N1448 Q9N1448 Q9N1448 Q9N1448 Q9N144 Q9GYX11 C7E374 E7CGC2 A0A097KV17 D7F1J5 B1PV79	unreviewed unreviewed unreviewed unreviewed unreviewed reviewed reviewed reviewed reviewed reviewed unreviewed unreviewed unreviewed unreviewed	Limilia polyphenia (Alamic horeschoe erab) Portunis tribuccidans (Swimming erab) (Verfount artibuccidans) Macrobrachian resonbergii (Giant fiesh water privin) Macrobrachian resonbergii (Giant fiesh water privin) Penneers particulti (Banata prasvn) (Pennears merguiensis) Organism Artemia franciscoma (Brine shrimp) (Artrapeneares japonicus) Penneers japonicus (Kurum previn) (Marrapeneares japonicus) Eriocheir sinersis (Chinese mitten crab) Callineteets sapidus (Bue crab) Limulas polyphemes (Atamic horeschoe crab) Cacines maense (Common shore crab) (Creen crab) Pencies polyphemes (Atamic horeschoe crab) Cacines maense (Marrab previnte) Pences monodon (Giant tige prawn) Metacarchus megister (Dangeness crab) (Cancer magister) Crangon crangon (Brown shrimp) Metaneares pensi (Gianscheire wirthing)	281 284 284 284 284 284 356 355 357 357 357 357 357 357 356 356 210 356 357
	ASD048 A71,594 Williams D3XNS0	unreviewed unreviewed unreviewed unreviewed unreviewed reviewed reviewed reviewed reviewed reviewed reviewed unreviewed unreviewed unreviewed unreviewed unreviewed unreviewed	Limita polyhemis (Atlantic horeschoe crab) Portuns tribuccidans (Swimming endy) (Vefnant withorwaldras) Macrobrachian roscobergii (Giant fichs water provi) Penneroponaesa mergelensisi (Banasa pravi) (Pennera progenesi Organism Artenia franciscani (Brine shrimp) (Artenia sanfranciscani) Pennera japonicus (Kurum privin) (Marsupenness japonicus) Eriocheir sinessi (Chinese milten crab) Callinetes sapidas (Biue crab) Limulus polyhemis (Atlantic horeschoe crab) Carcina mondon (Giant tiger pravi) Pennesus monodon (Giant tiger pravi) Pennesus monodon (Giant tiger pravi) Pennesus monodon (Giant tiger pravi) Metacarcinus megister (Dugness cash) (Cancer magister) Carging magister (Dugness cash) (Cancer magister) Carging negas (Bravon shrimp) Metaponesus entiti (Grasyback shrimp) (Pennesus entit)	281 284 284 284 284 284 356 355 357 357 357 357 357 356 356 210 356 210 356 210
	ASDOH8 A71,593 VSNBV3 VSNBV3 D33N850 D33N850 Q95V38 P51545 Q9NH49 P51541 Q9NH49 P51541 Q90J4 Q9GYX1 C7E3T4 E7CGC2 A0A097KV17 D7F1J5 B1PVZ9 A0A097KV33	unreviewed unreviewed unreviewed unreviewed reviewed reviewed reviewed reviewed reviewed reviewed unreviewed unreviewed unreviewed unreviewed unreviewed unreviewed	Limita polyhemis (Atlanic horeschoe enb) Dertums tribuezulatus (Swimmier enb) Macrobrachian rosenbergii (Giant fich water pravn) Pormeryponaeae merguleisti (Banana ptenvn) (Poraeae merguleisti) Organism Artenia franciscana (Brine shrimp) (Artenia sanfranciscana) Artenia franciscana (Brine shrimp) (Atlanic horaeus japonicus) Eriocheri shumsi (Chinese mitten enb) Callinectes sapidus (Blue enab) Limita polyhemis (Atlanic horaeschoe enab) Callinectes sapidus (Blue erab) Limita polyhemis (Atlanic horaeschoe enab) Carcinas maenas (Common shore enab) (Graeer mamoratus) Penaeus monodon (Giant tiger pravn) Metacarcinus magiater (Dangeness erab) (Cancer mamoratus) Metacarcinus magiater (Dangeness erab) (Cancer magister) Caragon erangon (Brown shrimp) Metageneue ensis (Greaeysheck shrimp) (Penaeus ensis) Uca erasiypes (Fiddle renb)	281 284 284 284 284 284 356 355 357 357 357 357 357 357 357 357 357
	ASDOH8 A71,594 A71,594 WiH607 D3XNS0 D3XNS0 D3XNS0 P51545 Q9NH49 Q9NH49 Q9NH49 Q9NH49 Q9SY1541 Q9U9J4 Q9GYX1 Q9GYX1 C7E374 E7CGC2 A0A097KVJ7 D7F1J5 B1PVZ9 A0A097KVS3 Q004B5	unreviewed unreviewed unreviewed of Status reviewed reviewed reviewed reviewed reviewed reviewed unreviewed unreviewed unreviewed unreviewed unreviewed unreviewed unreviewed unreviewed unreviewed unreviewed unreviewed	Limilia polyphemia (Alamic horeschoe erab) Portunis tribuccidans (Swimming erab) (Verfanue tribucculatus) Macrobrachian eraschorgii (Giant fich water privin) Macrobrachian eraschorgii (Giant fich water privin) Penneus apparlessit (Banata prasvi)/Fenaesa merguiensis) Organism Artemia franciscoma (Brine shtring) (Artengenesses japonicus) Eriocheir sinensis (Chinese milten erab) Callineteris sapidus (Bue erab) Limulas polyphemis (Alamic horeschoe erab) Carcinus maenza (Common shore erab) (Green erab) Penaesus mondon (Giant igge prawn) Metacarcinus maenza (Ommon shore erab) (Cancer marmentus) Penaesa mondon (Giant igge prawn) Metacarcinus magister (Dangeness erab) (Cancer magister) Carcings erasing (Brown shrimp) Metapanease ensis (Greavyback shrimp) (Penaesa vanion) Lita presses fordidler erab) Litopenaesa vanima (Whiteleg shrimp) (Penaesa vanima)	281 284 284 284 284 284 356 355 357 357 357 357 357 356 356 210 356 357 210 356
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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 Result	s of surrogate peptide	s 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.



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

	Surrogate peptide	Precursor $\binom{2^+}{product}$ ion $\binom{1^+}{product}$	Correlation coefficient (r)	Intraday $(n = 3)$		Interday $(n = 3)$	
Protein				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, ALS-NAEGEVAALNR 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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