Original Article

Gel-forming Mucins in Oviductus Ranae Contribute to Swelling Capacity by iTRAQ Proteomics Analysis

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ABSTRACT

Introduction: Oviductus Ranae (OR) is the dried oviduct of female Rana temporaria chensinensis David, which is one of the best-known and highly valued oriental foods and medicines in China. OR has a unique physiological phenomenon that is high swelling capacity. **Objectives:** We aimed to study which components are associated with high swelling capacity in OR. **Materials and Methods:** isobaric tags for relative and absolute quantification (iTRAQ) proteomic methods were used to identify differentially expressed proteins between the OR and three counterfeit oviducts, including *Rana nigromaculata, Bufo bufogargarizans* and *Rana catesbeiana* to explore the mechanism of swelling capacity. **Results:** We found that a total of 1220 proteins were identified from 2149 unique peptide sequences. Comparing with three counterfeit oviducts, 11 differentially expressed proteins were identified in OR, including 9 up-regulated proteins, such as mucins (MUC2, MUC5AC, MUC5AC-like, MUC5B), PREDICTED: a-kinase anchor protein 13-like (AKAP13-like), PREDICTED: IgGFc-binding protein (FcγBP), PREDICTED: IgGFc-binding protein-like (FcγBP-like), *et al.* and 2 down-regulated proteins. Bioinformatics results found that these up-regulated proteins were involved in biological processes, including metabolic process, cellular process, responses to stimulus and biological regulation. **Conclusion:** Mucins, AKAP13-like, FcγBP and FcγBP-like may act as a positive regulator for the innate immune response in oviduct of Chinese brown frog and exceptional swelling capacity of OR is closely related to gel-forming mucins. Taken together, our results provide the basis for further understanding of the unique physiological phenomenon of OR.

Key words: Oviductus Ranae, Swelling capacity, Gel-forming mucins, Counterfeit oviducts, Differentially expressed proteins.

INTRODUCTION

Oviduct of female *Rana temporaria chensinensis* David (Chinese brown frog) is one of the best-known and highly valued oriental foods and medicines, which is also known as oviductus ranae (OR). Chinese brown frog distributed mainly in North-east China. According to Traditional Chinese medicine, OR is natured, sweet and salt in flavor and possesses the functions of nourishing yin, moistening lung and replenishing the kidney essence, for the treatment of debilitated health, neurasthenia, deficiency of kidney qi, poor memory and so on. OR is yellowish-white or milk-white with lipoid gloss, its length is 4~8 times of body length, its weight accounts for 20 %~26 % of body weight.[1] OR is mainly composed of proteins, which are about 56.3 % and water-soluble protein accounts for 13.33 %.[2] Some studies have reported proteins

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of OR as importantly active ingredients have a series of functions, including anti-fatigue, anti-anoxia and immune regulation.[3-5] In particular, OR has the ability to form gels based on their strong water absorbing capacity and the swelling capacity is above 95,[6] Zhang *et al.* reported that distinctive physiological phenomenon of OR might be mainly related to mucins.[7] In the Chinese medicinal market, three counterfeit oviducts of *Rana nigromaculata* (ORN), *Bufo bufogargarizans* (OBBG), *Rana catesbeiana* (ORC) are common and easy to be confused as OR, which have no therapeutic functions. Meanwhile, protein contents of these counterfeits were between 47 %-69 %, but they have lower swelling capacity than OR.[6,8,9]

Transcriptomic analysis has identified a series of genes of OR, including fourteen growth factors, seven types of collagens, several gel-forming mucins, galectin, mimecan, fibulin and antioxidant enzymes, which are involved in cell adhesion, migration, proliferation and differentiation and so on.[7] However, the genomics of OR may not explain the function and the difference between OR and other counterfeits. Proteomics is the large-scale analysis of protein expression, which identifies the main proteins and shows the differentially expressed proteins in different samples. The isobaric tags for relative and absolute quantification (iTRAQ) are a highly sensitive and accurate technique for quantitative examination of proteomics, which quantifies proteins based on peptide labelling and allows large-scale identification of proteins from multiple samples with broad dynamic ranges of protein abundance.[10] At present, few people study differences of protein expression between OR and its counterfeits. So, differentially expressed proteins were screened by using iTRAQ on the proteomics data of OR and proteomics data of ORN, OBBG and ORC, respectively and then to find the specific proteins to be associated with biological activity and swelling capacity of OR in this study.

MATERIALS AND METHODS

Sample Preparation

Ranae temporaria chensinensis samples were collected in November, 2013, from the Changbai Mountain Area, Jilin Province, China (125°16'57"E~131°19'12"E, 40°51'55"N~44°38'54"N). *Rana nigromaculata* samples were collected in October 2013, from Beidahu Township, Yongji County, Jilin Province, China (126°30'14"E, 43°27'53"N). *Bufo gargarizans* samples were collected in November 2013, from Jilin City, Jilin Province, China (126°25'17"E, 44°4'40"N) and Beidahu Township, Yongji County, Jilin Province, China (126°30'14"E, 43°27'53"N). *Rana catesbeiana* samples were collected in November 2013, Zhangzhou City, Fujian Province, China (117°42'35"E, 24°30'11"N). The frogs were anesthetized with eugenol (Sigma-Aldrich, Missouri, USA) solution of bath immersion (0.35 ml/L) for 30 min to immediately obtain the oviducts of frog,[11] which were freeze-dried and ground into powder. The power of each group was mixed from 10 samples with equal amounts. This experiment was approved by the Bioethics Committee of the Changchun University of Chinese Medicine and the Institutional Animal Care (Approval NO. 2013-R0073) was conducted based on the guideline for the use of laboratory animals.

Swelling Capacity Determination

The swelling capacity test was performed, according to Chinese Pharmacopeia. 0.2 g dry samples of OR, ORN, BBG and ORC were cut into 3 mm fragments, added into the expansion tubes and then filled 25 mL of water. The samples were shaken once every hour at the beginning of 6 h and placed stably for 18 h at room temperature (23±1°C). After pouring water away, the volume of swelling (accurate to 0.1 mL) in each group was read to calculate the swelling capacity (S, mL/g), as the following equation: $S=V/W$. V is the volume of swelling capacity of the sample (mL), W is the weight of the sample (g).

Protein Extraction, Digestion and iTRAQ Labeling

The 10 mg of sample correspond to oviduct powder (OR, ORN, OBBG and ORC) was incorporated into 1 mL SDT buffer (4 % sodium dodecyl sulphate (SDS), 100 mM dithiothreitol (DTT), 150 mM Tris-HCl, pH8.0), boiled for 5 min and broken to obtain the homogenate. The crude extract of different samples was incubated in boiling water for 15 min again and centrifuged with 14,000×g for 45 min at 25°C to collect the supernatant. The protein concentration was determined using a bicinchoninic acid protein assay kit (BCA, Beyotime Biotechnology and Shanghai, China).

Taking advantage of the recently developed filter assisted sample preparation (FASP) method for sample preparation,[12] 0.25 mg of proteins for each sample were performed to the reduction and alkylation, followed by overnight digestion with trypsin (Promega Corporation, Wisconsin, USA) at 37°C. According to manufacturer's instructions (Applied Biosystems, Massachusetts, USA), the OR, ORN, OBBG and ORC samples were labeled with iTRAQ reagents as 113 (OR1), 114 (OR2), 115 (ORN), 116 (OBBG) and 117 (ORC), respectively. The labeled samples were kept at room temperature for 2 h and pooled and vacuum-dried.

Peptides Fractio;nation with Strong Cation Exchange (SCX) Chromatography

iTRAQ labeled peptides were fractionated by SCX chromatography using the AKTA Purifier system (GE Healthcare). The collected 30 fractions were finally combined into 10 pools and desalted on C_{18} Cartridges (Empore™ SPE Cartridges C_{18}, Sigma-Aldrich). Each fraction was concentrated by vacuum centrifugation and reconstituted in 40 µl of 0.1 % (v/v) trifluoroacetic acid.

LC-MS/MS Analysis

LC-MS/MS was performed as described previously[13] using a Q Exactive mass spectrometer coupled to Easy-nLC (Thermo Fisher Scientific, Massachusetts, USA). 10 µL of each fraction was injected for nano LC-MS/MS analysis. The peptide mixture (5 µg) was loaded onto a the C_{18}-reversed phase column (Thermo Scientific Easy Column, 10 cm × 75 µm × 3µm) in buffer A (0.1 % formic acid) and separated with a linear gradient of buffer B (80 % acetonitrile and 0.1 % formic acid) at a flow rate of 250 nL/min controlled by Intelli Flow technology over 140 min. MS data were acquired using a data-dependent top 10 method dynamically. The dynamic exclusion duration was 60 s. Survey scans were acquired at a resolution of 70,000 at m/z 200 and resolution for higher energy collisional dissociation (HCD) spectra was set to 17,500 at m/z 200. The normalized collision energy was 30 eV and the underfill ratio. The instrument was run with peptide recognition mode enabled.

Protein Identification and Data Analysis

In our previous study, the OR transcriptome was sequenced to generate the corresponding unigenes.[7] In the present study, the amino acid sequences translated from the coding DNA sequences (CDS) of unigenes were used as the protein database.

The raw files were analyzed using the Proteome Discoverer 1.3 software (Thermo Electron, San Jose, California, USA). Searching for the fragmentation spectra was performed using the Mascot search engine. The results were filtered based on a false discovery rate (FDR) of no more than 1 %. At least two unique peptides supported the protein identification. Isobaric Labeling Multiple File Distiller and Identified Protein iTRAQ Statistic Builder were used to calculate the ratios of protein, in which sample REF was used as the reference, based on the weighted average of the intensities of report ions in each identified peptide. The final ratios were then normalized with the median average protein ratio, assuming that most proteins remained unchanged in abundance. Protein ratio represents the median of the unique peptides of the protein.[14] The proteins ratio meets the fold change (>1.2 or <0.83 and *P*-value <0.05), which was considered as significantly differentially expressed.

The identified proteins were clustered analysis by Cluster 3.0 software. Clustering parameters: filter data ≥ 80 %, logratio transformation, genes cluster and arrays cluster and average linkage.

Bioinformatics Analysis

A Gene Ontology (GO) annotation analysis was performed to analyze the function of the differentially expressed proteins by searching for significantly enriched GO terms compared with enrichment in all identified proteins and three major categories were included: cellular component, molecular function and biological process. An FDR<0.05 was considered as a threshold for significant enrichment of the protein sets. Functional annotations of the proteins were conducted using the Blast2GO program against the Kyoto Encyclopedia of Genes and Genomes (KEGG) and NCBI-nr databases.

RESULTS

Comparative Analysis for Swelling Capacity of Four Kinds of Oviducts

To investigate the difference of swelling capacity of four kinds of oviducts, the swelling capacity was determined by the methods from Chinese Pharmacopeia (Edition 2015). As shown in Figure 1, OR had the maximum swelling capacity with 97.25; however, the swelling capacities of ORN, OBBG and ORC were lower than that of OR, which was 61.12, 18.24 and 12.23,

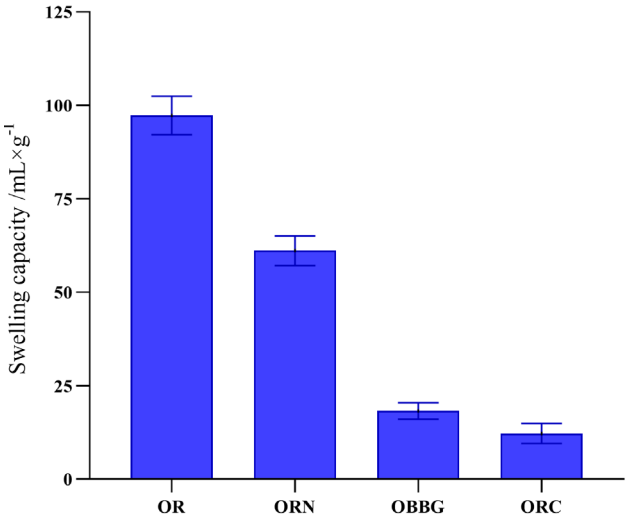

Figure 1: Results of swelling capacity test. OR- Oviduct Rana, ORN- Oviduct of *Rana nigromaculata*, OBBG- Oviduct of *Bufo bufogargarizans*, ORC- Oviduct of *Rana catesbeiana*.

respectively. This data are consistent with the previous reports.[6,9] These results confirm that OR had the high swelling capacity, compared to three counterfeit oviducts.

Primary Data Analysis and Protein Identification

In this study, the iTRAQ technique was performed to obtain a global view of the proteome differences among OR, ORN, OBBG and ORC. In the mass spectrum experiment, a total of 200232 MS spectra were obtained, 5402 spectra were successfully matched to the peptide fragments and 4895 spectra were matched to unique peptide fragments with a Mascot analysis. 1220 proteins were identified from 2149 unique peptide sequences (Table 1, Supplemental files S1 and S2). There were 19, 334, 367, 387, 91 and 22 proteins with a mass of less than 5 kDa, 5-10 kDa, 10-20 kDa, 20-50 kDa, 50-100 kDa, more than 100 kDa, respectively. The molecular weight of most proteins was distributed between 4.0 kDa and 30 kDa, accounting for about 78.10 % (Figure 2A) and the isoelectric point was distributed between 4.4 and 9.8, accounting for about 95.62 % (Figure 2B). Proteins sequence coverage with under 10 %, 10-20 %, 20-30 %, 30-40 %, 40-50 %, 50-100 % variation accounted for 45.08 %, 31.96 %, 13.28 %, 5.41 %, 1.97 % and 2.30 %, respectively. 1011 of 1220 proteins possessed sequence coverage more than 5 % (Figure 2C). The numbers of proteins with a single peptide, 2-6 peptides, 7-10 peptides and more than 10 peptides were 793, 408, 16 and 3, respectively (Figure 2D).

Cluster Analysis of Identified Protein

Hierarchical cluster analysis was used to identify proteins with certain patterns of changes. The differentially regulated proteins were clustered according to similarities in change profiles across all conditions.[15] 1220 proteins were analyzed using hierarchical clustering

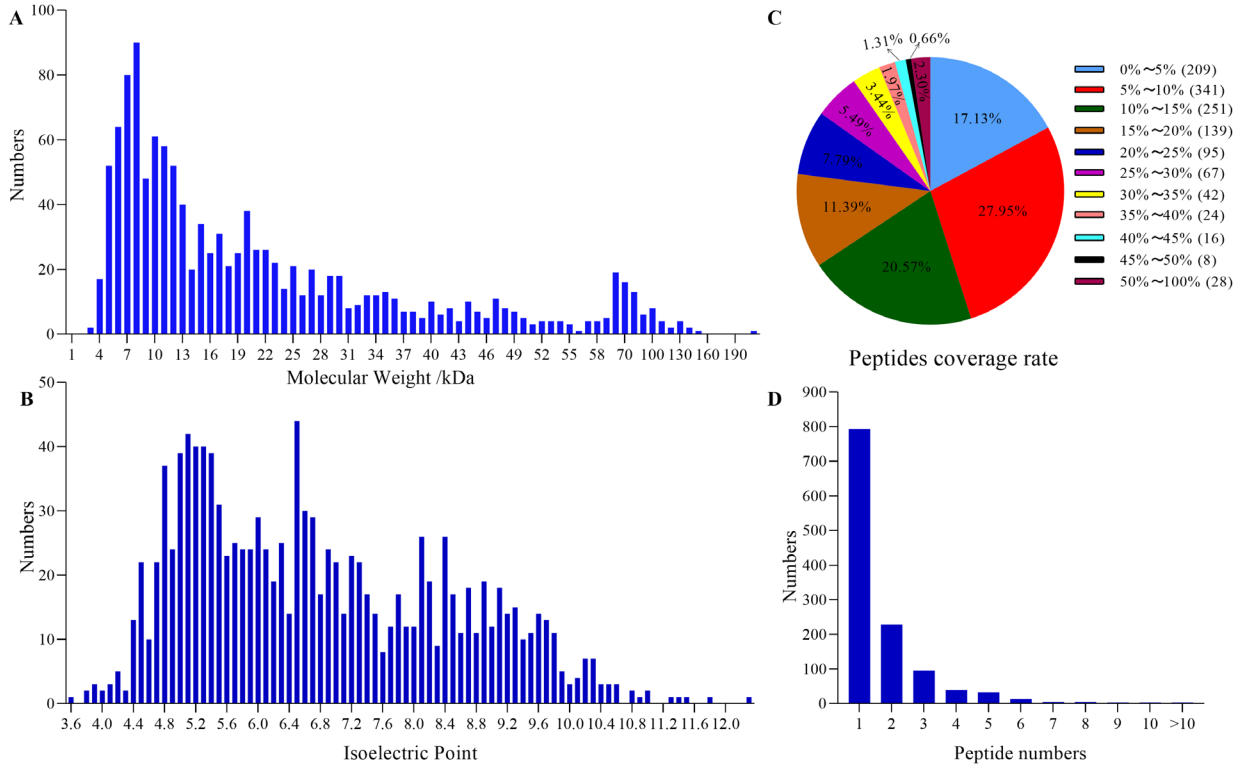

Figure 2: Identification and analysis of the Oviductus Rana Proteome. A, Molecular weight distribution; B, Isoelectric point distribution; C, Coverage of proteins by the identified peptides; D, the numbers of peptides matching to proteins.

Table 1: Summary statistics for iTRAQ proteomic analysis.

Group name	Total spectra	Spectra	Unique Spectra	Peptide	Unique Peptide	Protein
Oviductus Rana	200232	5402	4895	2268	2149	1220

based on the results of proteins quantification by Gene Cluster 3.0 software (Supplemental file S3). As shown in Figure 3, the five samples were divided into 4 clusters, OR cluster (including OR 1 cluster and OR 2 cluster), ORN cluster, OBBG cluster and ORC cluster; and the 1220 proteins were clustered six clusters. Each row of colored boxes was representative of a single protein, red boxes indicated high expressed proteins (log2>0), dark boxes indicated no change (log2=0) and green boxes indicated low expressed proteins (log2<0). Generally, the relatively similar expression patterns were found in OR and ORN. The protein expression in OBBG and ORC was a significant difference with OR and ORN, cluster I showed that most proteins were the high expression in OR and ORN, but they were the low expression in OBBG and ORC. And proteins in cluster VI showed low expressed levels in OR and ORN, which were the relatively high expression in ORC. In cluster II, most of the proteins were highly expressed in OR, ORN and ORC, but these proteins were lowly expressed in OBBG. On the contrary, the great majority of proteins in cluster V were the high expression in OBBG and that were low levels in OR, ORN and ORC. Cluster III included a portion of proteins that showed the low expression levels in ORN and the high expression levels in OR, OBBG and ORC. Cluster IV showed the highly expressed proteins in ORN and relatively low expression in OBBG. Based on the findings of four kinds of frogs, oviductus of *Ranae temporaria chensinensis* had quite different from oviductus of *Rana nigromaculata, Rana catesbeiana* and *Bufo bufogargarizans*.

Differentially Expressed Proteins in Four Kinds of Oviducts

Changes in the protein profile were analyzed and 11 proteins exhibited a difference (*P*-value <0.05) with a FDR of less than 1 %. 9 proteins were increased by more than 1.2-fold and 2 proteins were decreased by less than 0.83-fold in OR, compared with ORN, OBBG and ORC, listed in Table 2. 9 up-regulated proteins were divided into five categories, mucins (MUC2, MUC5AC, MUC5AC-like, MUC5B); PREDICTED: a-kinase anchor protein 13-like (AKAP13-like); PREDICTED: IgGFc-binding protein (FcγBP) and PREDICTED: IgGFc-binding protein-like (FcγBP-like); PREDICTED: similar to Cysteine-rich secretory protein-2 precursor isoform 3 (CRISP3); and metalloproteinase inhibitor 3 precursor (TIMP4), which are high expression in OR. The down-regulated proteins included the MGC68756 protein (CANX) and hydroxyacyl-CoA dehydrogenase trifunctional multienzyme complex subunit beta S

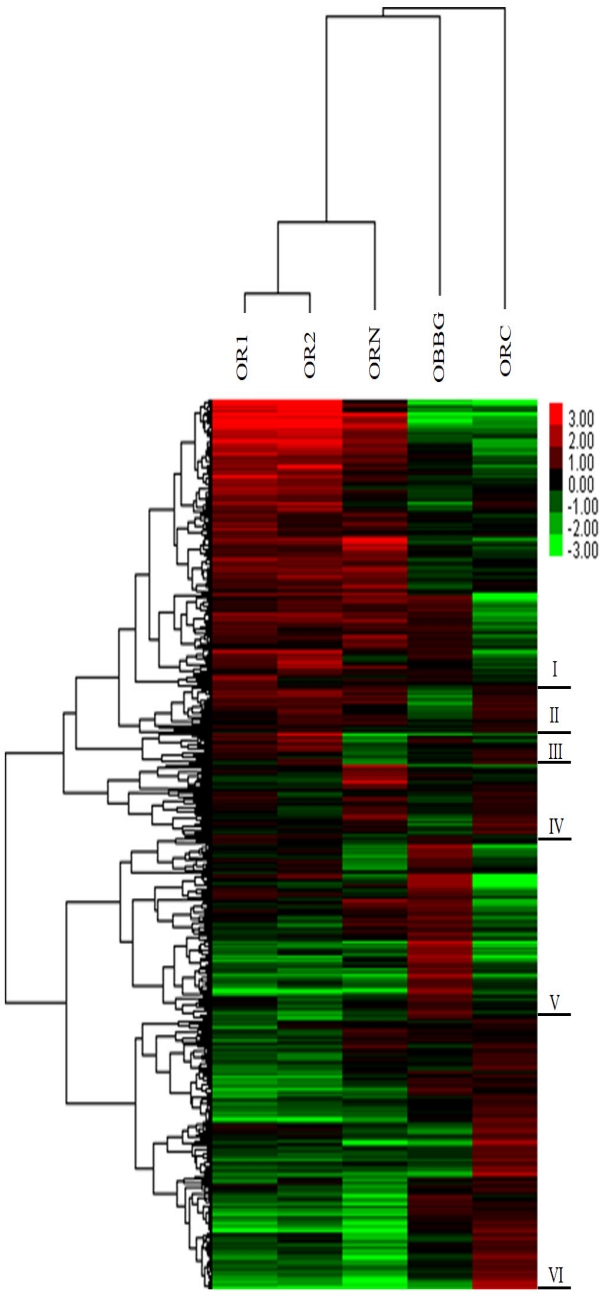

Figure 3: Cluster analysis diagram of identified protein for four kinds of oviducts. OR- Oviduct Rana, ORN- Oviduct of *Rana nigromaculata*, OBBG- Oviduct of *Bufo bufogargarizans*, ORC-Oviduct of *Rana catesbeiana*. Filter criteria: filter genes ≥ 80 %, Log transform data, genes cluster and arrays cluster, and average linkage.

homeolog (HADHB), which are the high expression in ORN, OBBG and ORC (Table 2, Supplemental file S3).

Bioinformatics Analysis of Differentially Expressed Proteins in Four Kinds of Oviducts

The biological functions of these differentially expressed proteins were investigated based on GO database. Of

Table 2: List of differentially expressed proteins implicated the Oviducts of *Ranae temporaria chensinensis*, *Rana nigromaculata*, *Bufo gargarizans*, and *Rana catesbeiana* by iTRAQ analysis.

GenBank® Accession ID	Protein name	coverage	Unique peptides	OR1/ REF	OR2/ REF	OR/REF	ORN/ REF	OBBG/ REF	ORC/ REF	Fold change		
										OR/ORN	OR/ OBBG	OR/ORC
Up-regulated												
ref|XP_002922103.1|	PREDICTED: a-kinase anchor protein 13-like (AKAP13-like)	1.23	1	2.919	6.429	4.674	0.165	0.233	0.255	28.390	20.086	18.361
ref|XP_002936084.1|	PREDICTED: IgGFc-binding protein-like (FcγBP-like)	27.69	2	3.709	4.674	4.192	1.117	0.322	0.351	3.753	13.025	11.957
ref|XP_002940580.1|	PREDICTED: IgGFc-binding protein (FcγBP)	6.87	1	2.525	3.306	2.916	0.911	0.500	0.431	3.200	5.825	6.760
ref|XP_002936083.1|	PREDICTED: mucin-5AC-like (MUC5AC-like)	86	3	3.634	4.371	4.002	1.344	0.364	0.310	2.979	10.985	12.904
emb|CAA06167.1|	mucin, partial (MUC5B)	38.81	2	2.439	3.310	2.874	0.996	0.434	0.569	2.885	6.623	5.052
ref|XP_852687.1|	PREDICTED: similar to Cysteine-rich secretory protein-2 precursor isoform 3(CRISP3)	20.83	1	2.308	3.138	2.723	0.978	0.736	0.570	2.785	3.699	4.778
ref|XP_002198220.1|	PREDICTED: mucin-5AC (MUC5AC)	21.13	1	2.843	3.179	3.011	1.134	0.588	0.504	2.656	5.123	5.977
ref|XP_002667590.1|	PREDICTED: mucin-2, partial (MUC2)	44.93	3	2.564	3.388	2.976	1.394	0.664	0.340	2.134	4.483	8.748
ref|NP_001098327.1|	metalloproteinase inhibitor 3 precursor (TIMP4)	23.81	2	2.383	2.775	2.579	1.299	0.657	0.651	1.986	3.928	3.963
Down-regulated												
gb|AAH60341.1|	MGC68756 protein (CANX)	8.25	1	0.353	0.414	0.384	0.766	1.237	1.177	0.501	0.310	0.326
ref|NP_001080077.1|	hydroxyacyl-CoA dehydrogenase trifunctional multienzyme complex subunit beta S homeolog (HADHB)	5.7	1	0.217	0.217	0.217	0.677	1.651	0.874	0.321	0.131	0.248

(Note: ID represents accession numbers; Fold change represents the ratio of two samples; OR presents average value of OR1 and OR2)

the 11 differentially expressed proteins, 6 proteins were successfully mapped to one or more GO terms, included 4 up-regulated proteins and 2 down-regulated proteins (Table 3). Among the 6 proteins mapped to GO terms, 5, 6 and 3 are involved in biological processes, molecular functions and cellular components, respectively. For biological process, 4 of differentially expressed proteins were related to metabolic process, followed by the cellular process (3 proteins), single-organism process (2 proteins) and biological regulation (2 proteins). For the molecular function, 3, 3 and 2 proteins were related to catalytic activity, binding and enzyme regulator activity, respectively. For the cellular component, 2, 1, 1 and 1 proteins were related to cell, extracellular region, extracellular matrix and macromolecular complex, respectively.

The biochemical pathways of the differentially expressed proteins were investigated based on the KEGG database. Of 11 differentially expressed proteins, only 2 down-regulated proteins were associated with a KO ID, which involved in 8 pathways. MGC68756 protein participated in Protein processing in the endoplasmic reticulum (ko04141), apoptosis (ko04210), alzheimer's disease (ko05010). Hydroxyacyl-CoA dehydrogenase trifunctional multienzyme complex subunit beta S homeolog participated in fatty acid elongation (ko00062), fatty acid degradation (ko00071), valine, leucine and isoleucine degradation (ko00280), benzoate degradation (ko00362), fatty acid metabolism (ko01212).

DISCUSSION

Ranae temporaria chensinensis, Rana nigromaculata, Bufo bufogargarizans, Rana catesbeiana and their Oviducts

Ranid frogs are regarded as poor thermoregulatory compared with other terrestrial vertebrates,[16] and go into hibernation period on land or underwater from September to April next year in Northeast China when water temperature fell to 10°C and the air temperature was 15°C. *Rana temporaria chensinensis* spends the winter at the bottom of ice-covered ponds that tolerates freezing, hypoxic or anoxic water.[17,18] However, most of *Rana nigromaculata*, *Bufo bufogargarizans and Rana catesbeiana* go deep under the mud that may face the challenges of desiccation, low temperature and anoxia.[19] Maintenance of adequate fuel reserves is also a key requirement for successful overwintering, particularly reproductive activity in the spring, which occurs before feeding and replenishment of reserves.

OR is the oviduct of female *Rana temporaria chensinensis*, processed and dried after collection in the autumn, which is yellowish-white or milk white with lipoid gloss. The length of OR is 4~8 times of body length; its weight accounts for 20 %~26 % of body weight.[1] 56.3 % of components are proteins in the OR, followed by sugars (13.7 %), fatty acid, hormone, etc.,[2] The swelling capacity is 97.25 (Figure 1).[20] The *Rana nigromaculata* is a common frog in North-eastern China. The oviduct of *Rana nigromaculata* (ORN) is oyster white or yellowish-white with lipoid gloss. The protein of ORN accounts for 47.50 %, followed by sugars, fatty acid, hormone, etc.[6] The *Bufo bufogargarizans* belongs to the toad species and distributes throughout the country. The oviduct of *Bufo bufogargarizans* (OBBG) is faint yellow or brown without lipid gloss. The protein of OBBG accounts for 47 %.[8] The *Rana catesbeiana,* often simply known as the bullfrog in Canada and the United States, is a member of the family Ranidae. Oviduct of *Rana catesbeiana*

Table 3: Gene ontology analysis of differentially expressed proteins.

GO ID	GO Term	Protein ID
Biological process		
GO:0008152	metabolic process	AKAP13-like, CANX, HADHB, MUC5B
GO:0009987	cellular process	AKAP13-like, MUC5B, MUC2
GO:0023052	signaling	AKAP13-like
GO:0032502	developmental process	MUC2
GO:0040011	locomotion	MUC2
GO:0044699	single-organism process	AKAP13-like, MUC2
GO:0050896	response to stimulus	AKAP13-like
GO:0051179	localization	MUC2
GO:0065007	biological regulation	AKAP13-like, MUC2
Molecular function		
GO:0003824	catalytic activity	AKAP13-like, HADHB, CANX
GO:0005488	binding	AKAP13-like, MUC5B, MUC2
GO:0030234	enzyme regulator activity	AKAP13-like, TIMP4
Cellular component		
GO:0005576	extracellular region	MUC2
GO:0005623	cell	CANX, AKAP13-like
GO:0031012	extracellular matrix	MUC2
GO:0032991	macromolecular complex	AKAP13-like

(ORC) is dark yellow without lipid gloss, the diameter of which is uneven. The protein of ORC accounts for 68.95 %.[8] The swelling capacities of ORN, BBG and ORC were lower 37.17 %, 81.25 % and 87.45 % than that of OR, respectively (Figure 1). Because of the difference in species, living environment and habits, four kinds of oviducts showed significant differences in the swelling capacity and protein content. Modern pharmacological studies have shown that the OR has the functions of antifatigue,[21] enhancing the immune function of organism,[22,23] antitussive,[24] and so on. However, the oviducts of these three counterfeit frogs have no efficacy we mentioned above. Therefore, we used iTRAQ coupled 2D LC-MS/MS to analyze the different expressed proteins in the oviduct of four frogs, which could provide an insight on the explanation of the functional mechanism of OR were found that would be the crucial active ingredient.

Swelling Capacity-Related Differentially Expressed Proteins in OR

OR, one unique physiological phenomenon, possesses high swelling capacity. Zhang *et al.* reported that swelling capacity was closely related to mucins by analyzing the transcriptomic data of OR.[7] Su *et al.* found that swelling capacity was mainly related to extracellular matrix (ECM) and focal adhesion compared to the protein expression profiles of *Rana chensinensis* oviduct during the breeding period and prehibernation.[25] Mucins belong to a family of heavily glycosylated proteins with high molecular weight, secreted by mucosal epithelium. Mucins contribute directly to the composition of the cellular glycocalyx and extracellular matrix.[26,27] The gel-like mucus coat covers mucosal surfaces of the body and forms the most exterior face of the innate immune system.[28] So far, 21 different mucin genes have been described, MUC2, MUC5AC, MUC5B, MUC6 and MUC19, which belong to the gel-forming mucins.[29] In our proteomic analysis, MUC2, MUC5AC, MUC5AC-like, MUC5B were high-level expressed in OR, which were low-level expressed in ORN, OBBG and ORC (Table 2).

The sequence of IgGFc-binding protein (FcγBP) contains repeated cysteine-rich unit, which showed the homology to the sequence of mucin, such as mucin-2, mucin-5AC, mucin-5B and prepro-von Willebrand factor.[30] FcγBP is widely expressed on mucosal surfaces and in external secretions,[31] which may be involved in the maintenance of the mucosal structure as a gel-like component of the mucosa. FcγBP and FcγBP-like were high-level expressed in OR, but which were low-level expressed in ORN, OBBG and ORC (Table 2). The dense "sugar coating" of mucins gives them the considerable water-holding capacity, furthermore, which forms gel through absorbing abundant water. Therefore, the high swelling capacity of OR is closely related to gel-forming mucins.

Immunomodulation-Related Differentially Expressed Proteins in OR

In the proteomic analysis, the high-level expressed proteins participate in the process of immune regulation in OR. A-kinase anchor proteins (AKAPs) are a family of structurally diverse but functionally related proteins, which anchor cAMP-dependent protein kinase A (PKA) as well as other signaling enzymes at focal points within the cell to ensure integration and processing of multiple signaling pathways. AKAP13 (also known as Brx-1, AKAP-Lbc and Ht31) is an important member of the A-kinase anchoring protein family, which expresses in hematopoietic cells, skeletal muscle, lung, heart and estrogen-responsive reproductive tissues, such as breast ductal epithelium. AKAP13 acts as a guanine nucleotide exchange factor (GEF) and participates in metabolic process, cellular process, signaling, single-organism process, responses to stimulus and biological regulation. The GEF domain of AKAP13 was shown to bind RhoA and activate Rho family GTPases,[32] functions as a cytoplasmic integrator or docking platform for multiple signaling cascades including those of the protein kinase A and nuclear hormone receptor signaling pathways. In modulating the immune system, AKAP13, a scaffold protein with GEF activity, is induced by toll-like receptor 2 (TLR2) ligand and mediates NF-κB activation via TLR2. Stimulation of a TLR2 ligand in the human macrophage cell line THP-1 and epithelial cells caused a significant up-regulation in AKAP13 mRNA, corresponding to an increase in protein expression.[33,34] AKAP13-like was high-level expression in OR, which is 28.4-, 20.1-, 18.4-fold in protein expression profile of ORN, OBBG and ORC, respectively (Table 2). Therefore, the AKAP13-like is one of the important ingredients, which may be involved in the immunoregulatory activity of oviduct of female Chinese brown frog.

Furthermore, mucin is composed of a peptide core containing heavily glycosylated regions and forms the most exterior face of the innate immune system.[28] Sugar residues of mucin are responsible for the attachment of parasites and microbes to the mucosal epithelium.[35] Mucins participate in the process of cell differentiation, adhesion, migration, renewal, signal transduction. MUC-5AC and MUC-2 are the major constituents of mucus and confer its viscoelastic properties,[36] which may prevent pathogen penetrance by inhibiting bacterial adhesion

to the mucosal epithelium surface.[37] Van Der *et al.*[38] has reported that MUC-2 can suppress inflammation in the intestinal tract. Shan *et al.* find that the immunoregulatory effects of MUC-2 may be mediated by decreasing IL-6 secretion, inhibiting tumorigenicity and inducing CD8 T cells *in vivo*.[39] Therefore high contents of MUC2, MUC5AC, MUC5AC-like, MUC5B in OR play a critical role in the immunoregulatory function of the oviduct of female Chinese brown frog.

In addition, FcγBP is widely expressed on mucosal surfaces and in external secretions,[31] which interacts with the Fc portion of IgG and with MUC-2.[40] The Fc region is responsible for many of the biological functions of IgG. It is the site of complement activation as well as the IgG region, which combines with specific receptor sites on macrophages and lymphocytes and thereby modulates the immune response to bound antigens. In inflammatory disease, abnormal production of auto-antibodies reflects the increased generation of FcγBP in goblet cells. FcγBP may prevent injurious complement mediated reactions from occurring by binding any available IgG and inhibit complement-mediated hemolysis of sheep red blood cells (SRBC). So FcγBP and FcγBP-like with high expressions are important components, which participate in the immunoregulatory process of the oviduct of female Chinese brown frog.

OR is a valuable Chinese crude drug as a tonic food. Some studies have found that OR could enhance immune-suppressed mice.[41,42] Protein hydrolysate of OR stimulated macrophage-derived TNF-α, IL-1β, IL-6 and NO production by activating the NF-κB pathway and upregulated the mRNA and protein expression of iNOS in immune response.[3] These Immunomodulation-related differentially expressed proteins, such as AKAP13, mucins, FcγBP and FcγBP-like in OR could involve in immune regulation *in vitro* and *in vivo*.

CONCLUSION

This study combined the transcriptome and proteome approaches for analyzing 11 differentially expressed proteins in OR and three counterfeit oviducts, on the basis of GO and KEGG analysis. Compared with protein expression profiles of ORN, OBBG and ORC, AKAP13-like, MUC2, MUC5AC, MUC5AC-like, MUC5B, FcγBP and FcγBP-like are higher expressed in OR, which are important active components of the innate immune response in the oviduct of female Chinese brown frog. Meanwhile, these differentially expressed proteins of OR could involve in immune regulation *in vitro* and *in vivo*. The exceptional swelling capacity of OR is closely related to gel-forming mucins. The present study provides the first report of vital active proteins in OR, which could help to elucidate the detailed mechanisms of OR for medicinal efficacy.

Data Availability

The data and materials supporting the conclusions of this article are included within the article.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

Supplementary Material

To view the supplementary material for this article, please visit https://doi.org/

S1: Peptide quantification;

S2: Protein quantification;

S3: Differentially expressed proteins.

ABBREVIATIONS

OR: Oviductus Ranae; **iTRAQ:** isobaric tags for relative and absolute quantification; **ORN:** oviduct of *Rana nigromaculata*; **OBBG:** oviduct of *Bufo bufogargarizans*; **ORC:** oviduct of *Rana catesbeiana*; **MUC2:** PREDICTED: mucin-2, partial; **MUC5AC:** PREDICTED: mucin-5AC; **MUC5AC-like:** PREDICTED: mucin-5AC-like; **MUC5B**: mucin, partial; **AKAP13-like:** PREDICTED: a-kinase anchor protein 13-like; **FcγBP**: PREDICTED: IgGFc-binding protein; **FcγBP-like**: PREDICTED: IgGFc-binding protein-like; **GO:** Gene Ontology; **KEGG:** Kyoto Encyclopedia of Genes and Genomes; **GEF:** guanine nucleotide exchange factor; **TLR2:** toll-like receptor 2; **TNF-α:** tumor necrosis factor-a; **IL-1β:** interleukin-1β; **IL-6:** interleukin-6.

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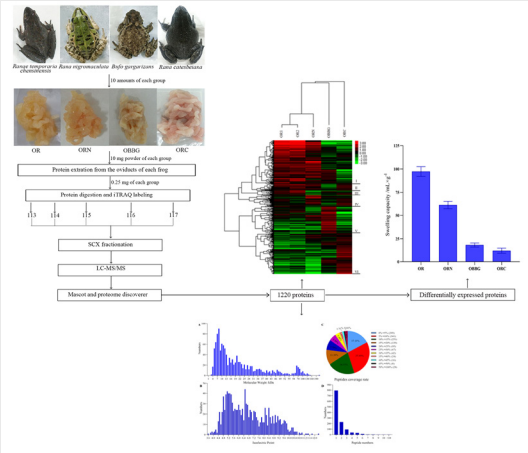
PICTORIAL ABSTRACT



SUMMARY

OR has high swelling capacity based on its strong water absorbing. AKAP13-like, MUC2, MUC5AC, MUC5AC-like, MUC5B, FcγBP and FcγBP-like are higher expressed in OR compared with three counterfeit oviducts by using the iTRAQ proteomic methods. MUC2, MUC5AC, MUC5B belong to the gel-forming mucins. Mucins, AKAP13-like, FcγBP and FcγBP-like may act as a positive regulator for the innate immune response in oviduct of Chinese brown frog and exceptional swelling capacity of OR is closely related to gel-forming mucins. Taken together, our results provide the basis for further understanding of the unique physiological phenomenon of OR.

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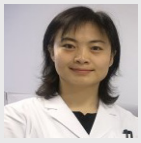

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