ISSN 0006-2979, Biochemistry (Moscow), 2008, Vol. 73, No. 6, pp. 676-680. © Pleiades Publishing, Ltd., 2008.
Published in Russian inBiokhimiya, 2008, Vol. 73, No. 6, pp. 841-847.
Originally published in Biochemistry (Moscow) On-Line Papers in Press, as Manuscript BM07-376, March 30, 2008.

Proteomic Characterization of Royal Jelly Proteins in Chinese (*Apis cerana cerana*) and European (*Apis mellifera*) Honeybees

Ning Qu[1#], Jie Jiang[2#], Liangxian Sun[3], Changcheng Lai[1], Lifang Sun[1], and Xueji Wu[1]*

[1]*School of Life Sciences, Xiamen University, Daxue Road 168, Xiamen 361005, China; fax: +86-592-218-4992; E-mail: xuejiwu@yahoo.com*
[2]*Xiamen Hospital of Traditional Chinese Medicine, Xiamen 361009, China*
[3]*Model Organism Research Center, Quanzhou Normal University, Quanzhou 362000, China*

Received November 19, 2007
Revision received December 27, 2007

Abstract—In this study, the proteins contained in royal jelly (RJ) derived from Chinese and European honeybees have been analyzed in detail and compared. Remarkable differences were found in the heterogeneity of major royal jelly proteins (MRJPs), MRJP2 and MRJP3, in terms of molecular weight and isoelectric points between the two species of RJ. MRJP2 and MRJP3 produced by Chinese honeybee are less polymorphic than those produced by European honeybee. This study is a contribution to the description of the royal jelly proteome.

DOI: 10.1134/S0006297908060072

Key words: *Apis cerana*, *Apis mellifera*, royal jelly, major royal jelly proteins, two-dimensional gel electrophoresis

Honeybees are social insects, living in colonies constituted of different castes: queens, workers, and drones [1]. Though queens and workers develop from the same fertilized eggs, after eclosion, changes occur in behavior, physiology, and morphology. This honeybee polyphenism is mostly due to larval diet rather than genetic predisposition [2-8]. Larvae designated to be queens are fed royal jelly (RJ) their entire lives, while all other larvae are disconnected from royal jelly and are provided a mixture of honey and pollen three days after hatching. It is believed that exclusively feeding a honeybee larva with RJ determines a queen, affecting its cell differentiation and proliferation, and contributes to a queen's longevity and enormous reproductive ability [3, 4].

Royal jelly is synthesized in the glandular cephalic system (hypopharyngeal and mandibular glands) of nurse bees [9-11]. The composition varies with the age and race of nurse bees as well as the seasonal and regional conditions that surrounds them. Fresh RJ is a white-yellow colloid with a pH between 3.6-4.2 and is mainly composed of water (60-70%), proteins (12-15%), carbohydrates (10-16%), lipids (3-6%), and traces of salts, vitamins, and free amino acids [12, 13].

The biological function of some components present in RJ has been previously described. Jelleines are an antimicrobial family of peptides against yeasts and Gram-positive or gram-negative bacteria [14]. Royalisin, a 5.5 kD peptide, was found to have antibacterial and antifungal activities [15].

The important protein components of RJ are those that belong to a homological protein family named major royal jelly proteins (MRJPs). In this family, five species of proteins (MRJP1-5) with molecular weights 49-87 kD have been identified by cDNA cloning, sequencing, SDS-PAGE, two-dimensional gel electrophoresis, and N-terminal sequence analysis [16-18]. MRJP3 and MRJP5 display size polymorphism with molecular weight owing to extensive repetitive regions in the C-terminal region and various sugar chains attached to the protein. The biological function of MRJPs is recognized as nutrition provider for large amounts of essential amino acids. Also, MRJP1 is likely to promote liver regeneration and may have a cytoprotective action on hepatocytes [19-21]. MRJP3 exhibits potent anti-inflammatory effects *in vitro* and *in vivo*.

Royal jelly collected from European honeybees (*Apis mellifera*) has been extensively used as dietary or cosmetic supplements due to the belief that it exerts on human beings similar effects as it does on honeybees. A number of investigative efforts have been made on the protein

Abbreviations: MRJPs) major royal jelly proteins; RJ) royal jelly.
[#] These authors contributed equally to this work.
* To whom correspondence should be addressed.

composition of RJ found in European honeybees. Chinese honeybees (*Apis cerana*), on the other hand, while having been widely bred in China for their resistance to bee mites and acclimatization, have had few substantial biochemical analyses done on their RJ. The only available relevant data is about the genome profile of Chinese honeybees [22-24]. Thus, the aim of this present study was to obtain a proteome profile of RJ taken from Chinese honeybees and to compare the proteins identified with those already described RJ in European honeybees.

MATERIALS AND METHODS

Chemicals. IPG strips, IPG buffer, Pharmalyte, acrylamide, and Coomassie Brilliant Blue R-250 were purchased from Amersham Biosciences (USA). Trypsin was purchased from Promega (USA). Molecular weight marker proteins and all other chemicals were from Sigma (USA).

Biological samples. Fresh RJ from *A. mellifera* and *A. cerana* were collected from cells containing 72 h queen-designated larvae and quickly stored at −80°C, avoiding heat and oxidation.

SDS-PAGE. RJ (100 mg) was suspended in 1 ml of buffer (50 mM sodium phosphate, pH 7.5, 10 mM NaCl, 2 mM EDTA). After centrifugation at 15,000*g* for 30 min at 4°C, the supernatant was recovered. Protein concentration was determined according to the Bradford method using bovine serum albumin as a reference [25]. Soluble RJ fractions were separated by SDS-PAGE on a 10% polyacrylamide gel and stained with Coomassie Brilliant Blue R-250.

Separation by chromatography. RJ was dissolved in 20 mM Tris-HCl buffer, pH 7.5, 1 mM EDTA; the sample was loaded onto a Q column (Mono Q^{TM}5/50 GL) and then eluted with a linear gradient of 0-1 M KCl.

Two-dimensional gel electrophoresis. RJ (100 mg) was suspended in 1 ml of isoelectric focusing (IEF) sample solution (8 M urea, 2% Chaps, 2% IPG buffer, 1.4 mg PMSF, 20 mg DTT). After centrifugation at 15,000*g* for 30 min at 4°C, the supernatant was collected. Protein concentration was determined using the Bradford method. Supernatant (125 µl) in rehydration buffer contained 8 M urea, 2% Chaps, 0.5% IPG buffer, 0.8% Pharmalyte, and a trace of bromophenol blue. The mixture was loaded on a 7 cm Immobiline Drystrip, pH 3-10. IEF was carried out at 200 V for 2 h, 500 V for 1 h, 1000 V for 30 min, 2000 V for 30 min, and finally 5000 V for 2 h on an Ettan IPGphor apparatus (Amersham Biosciences). Prior to SDS-PAGE, the IPG strip was first equilibrated in 1% DTT, 50 mM Tris-HCl, pH 8.8, 6 M urea, 30% glycerol, 2% SDS, 0.002% bromophenol blue for 10 min and then followed with equilibration in 2.5% iodoacetamide, 50 mM Tris-HCl, pH 8.8, 6 M urea, 30% glycerol, 2% SDS, 0.002% bromophenol blue for 10 min. SDS-PAGE was performed at 10 mA/gel for about 3 h using 10% T, 2.7% C polyacrylamide gel. The gel was stained with Coomassie Brilliant Blue G-250, scanned with a Magicsan, and the images were analyzed with ImageMaster 2D Platinum 6.0.

Tryptic digestion of 2-DE spots. Spots of interest were cut from the gel and destained twice for 30 min using 50 µl of 50% acetonitrile with 50 mM NH_4HCO_3, dried for 30 min with acetonitrile, and incubated with 50 µl 10 mM DTT (in 100 mM NH_4HCO_3) at 56°C for 45 min. The gel pieces were washed with 50 µl 55 mM iodoacetamide in 100 mM NH_4HCO_3 for 30 min at room temperature, washed two times with 50 µl of 100 mM NH_4HCO_3 for 20 min, followed by dehydration with acetonitrile and finally completely dried in a Speed-Vac.

Trypsin solution (10 µl, final concentration 12.5 ng/ml) was pipetted onto the dried gel pieces and incubated at 4°C for 1 h. Excess trypsin solution was discarded and 10 µl of 50 mM NH_4HCO_3 was added. Then the sample was incubated at 37°C overnight. To extract the peptide fragments from the tryptic digest, 50 µl of 50% acetonitrile (containing 2.5% TFA) was added and incubated at 30°C for 1 h. Thereafter, 50 µl of 15% TFA was added and incubated at 40°C for 1 h. After each step, the supernatants were pooled together and dried using the Speed-Vac system.

Mass spectrometry. The sample was dissolved in TFA (0.5%, 2 µl) and mixed with a saturated matrix solution (50 mM α-cyano-4-hydroxycinnamic acid, 60% acetonitrile, 0.1% TFA). The mixture was pipetted onto the MALDI slide sampler and air-dried. The peptide spectra were recorded in the reflector mode and external calibration was performed with a peptide calibration standard. Mass spectra of peptide mixtures were obtained using a Reflex III MALDI-TOF MS (Bruker Daltonics, Germany).

Database research. Peptide mass fingerprinting of the proteins of interest was performed using MASCOT software. Maximum tolerance for masses was adjusted to be 100 ppm and one missed cleavage for tryptic peptides was allowed. SWISS-PORT and NCBIrn databases were used for proteomic research. M_r and p*I* values of each analyzed spot were obtained from the 2D-PAGE gel.

RESULTS

Fractionation of European honeybee and Chinese honeybee royal jellies by SDS-PAGE and chromatography. Electrophoretic analysis of European honeybee RJ showed five protein bands (Fig. 1), which is identical to the results reported by Shmitzova et al. [17]. Chinese honeybee RJ showed three protein bands, two bands of which are similar in molecular weight to those of European honeybee RJ, and one of which has a higher

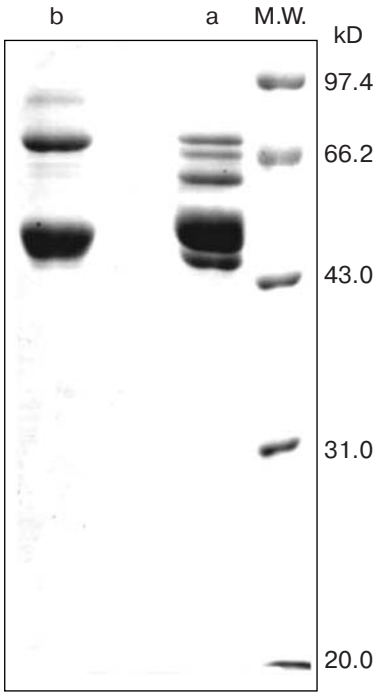

Fig. 1. Soluble RJ proteins from European honeybee (a) and Chinese honeybee (b) were separated by SDS-PAGE on a 10% acrylamide gel and stained with Coomassie Brilliant R-250. M.W., molecular weight standards.

molecular weight than any bands seen in European honeybee RJ.

We further analyzed European honeybee RJ and Chinese honeybee RJ by using ion-exchange chromatography. The chromatographic profiles (Fig. 2) show that there are only three protein peaks present in European honeybee RJ, while four protein peaks are found in Chinese honeybee RJ.

Protein analysis of European honeybee RJ and Chinese honeybee RJ by two-dimensional gel electrophoresis. Figures 3 and 4 show one representative of European honeybee RJ and Chinese honeybee RJ, respectively. Interestingly, the two-dimensional gel electrophoresis patterns between the two honeybee species were substantially different. Particularly marked differences were observed in the p*I* area that ranged from 6 to 9 and had molecular weight range between 60 to 80 kD on the gels. Two-dimensional gel electrophoresis of European honeybee RJ provided identical results to reports [10, 27]; moreover, profiling of the European honeybee RJ proteome has been performed well [27]. Therefore, we decided to identify Chinese honeybee RJ proteins by mass spectrum.

Protein identification. Figure 4 shows the analyzed spots of RJ proteins from Chinese honeybee. All the identified proteins belong to the Chinese honeybee genome. The results of the spot identification are reported in the table. MRJP1, 2, 3, and 4 were identified, whereas MRJP5 was not found in Chinese honeybee RJ. Spots R1 and 2 have been identified to be MRJP1 with MW 51,966 and 51,417, indicating these spots as those identified by cDNA sequencing. Two variants of MRJP1 were identified in Chinese honeybee RJ by cDNA sequencing, while only one isoform was characterized in European honeybee RJ [10, 27]. Spot R3, with p*I* about 7.7, higher than

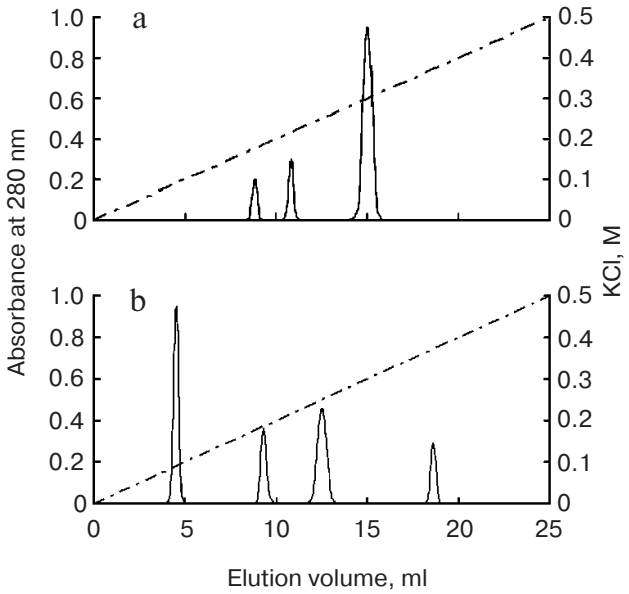

Fig. 2. Chromatographic profiles of RJ proteins from European honeybee (a) and Chinese honeybee (b) from FPLC using a Q column (Mono Q^{TM}5/50 GL) eluted with a linear gradient of 0-1 M KCl in Tris-HCl buffer.

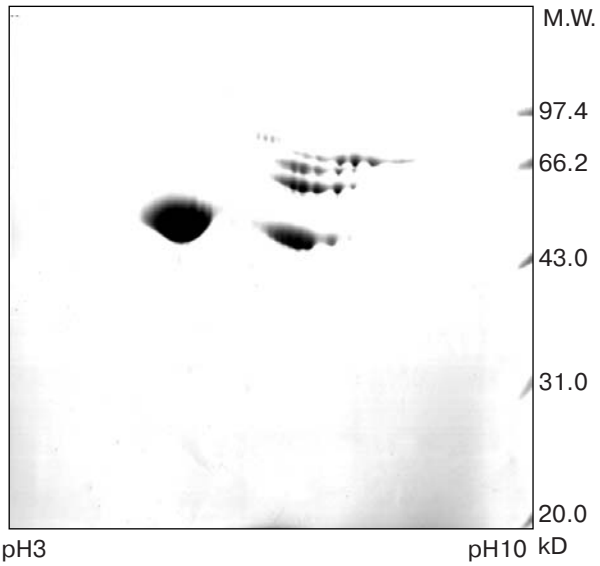

Fig. 3. Two-dimensional electrophoresis analysis of European honeybee RJ proteins that were stained with Coomassie Brilliant Blue G250.

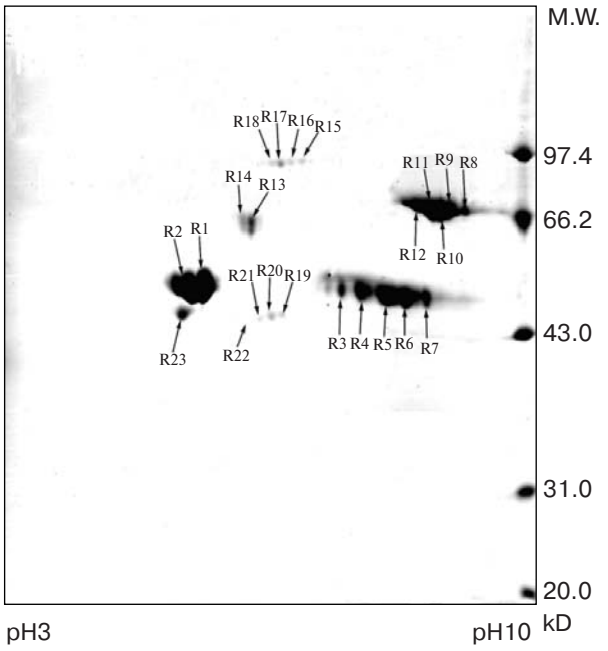

Fig. 4. Spots of two-dimensional electrophoresis-resolved Chinese honeybee RJ proteins that were subjected to tryptic digestion before MS analysis.

in spots R1, 2, was also identified as MRJP1. It was supposed to be deduced from the C-terminal of MRJP1 according to the matched sequence.

MALDI-TOF analysis revealed that spots 4-7 are all MRJP2. The molecular weight of MRJP2 from Chinese honeybee RJ was similar to that of European honeybee RJ, but the p*I* value was higher than that from European honeybee RJ [28]. Twelve isoforms of MRJP2 were identified in European honeybee RJ [27]. Spots 8-12 with molecular weights from 65,919 to 68,074 and p*I* ranging from 8.8 to 9.5 were assigned to be MRJP3. The molecular weights and p*I* values of MRJP3 from Chinese honeybee RJ were identical to indications from cDNA sequencing, and were higher than that of European honeybee RJ [24, 29, 30]. Sano et al. have identified 24 MRJP3 variants in European honeybee RJ, and some spots were C-terminal region degradation isoforms. MRJP2 and MRJP3 derived from Chinese honeybee RJ showed less heterogeneity than those identified in European honeybee RJ.

MRJP4 has not been identified by the proteomic approach and was inferred from the European honeybee genome. Interestingly, spots R13 and 14 present in this experiment were revealed to be MRJP4. In particular, the average molecular weight of MRJP4 identified by our experiment was higher than that predicted from the

Identification of proteins of Chinese honeybee RJ

Spot number	p*I*	Mass (daltons)	Protein identified	Accession
R1	5.78	51 966	MRJP1	Gi 40557703
R2	5.51	51 417	MRJP1	Gi 40557703
R3	7.76	51 417	MRJP1	Gi 40557703
R4	8.03	50 941	MRJP2	Gi 46358503
R5	8.40	50 173	MRJP2	Gi 46358503
R6	8.70	49 668	MRJP2	Gi 46358503
R7	8.90	50 047	MRJP2	Gi 46358503
R8	9.54	66 200	MRJP3	Gi 56422037
R9	9.30	65 919	MRJP3	Gi 56422037
R10	9.20	65 919	MRJP3	Gi 56422037
R11	9.03	66 060	MRJP3	Gi 56422037
R12	8.84	68 074	MRJP3	Gi 56422037
R13	6.46	64 124	MRJP4	Gi 42601244
R14	6.30	64 261	MRJP4	Gi 42601244
R15	7.19	94 716	not identified	
R16	7.03	94 279	not identified	
R17	6.87	92 972	not identified	
R18	6.74	93 406	not identified	
R19			not identified	
R21			not identified	
R22			not identified	
R23	5.45	46 625	MRJP7	Gi 57546160

cDNA sequence of Indian honeybees. The difference may originate from the difference in species, potential N-glycosylation sites, or different degrees of glycosylation.

Spot R23 was identified as MRJP7, which was reported in the molecular cloning of *mrjp* from Chinese honeybee [24]. Spots numbered from 15 to 22 were not identified by MALDI-TOF analysis because of the deficiency in protein quantity. They were possibly some oxidases or MRJP5 corresponding to two-dimensional gel electrophoresis result of European honeybee RJ. However, in the EST library established from the hypopharyngeal gland of Chinese nurse bees, MRJP5 was not found either [27].

DISCUSSION

In summary, European honeybee RJ and Chinese honeybee RJ were analyzed by two-dimensional gel electrophoresis. When the identified Chinese honeybee RJ proteins were compare to those previously identified in European honeybee RJ, we observed considerable heterogeneity within each protein of the MRJP family. However, some proteins—MRJP2 and MRJP3—exhibited less heterogeneity than those from European honeybee RJ in terms of molecular size as well as p*I* values. In agreement with our findings, MRJP3 protein derived from Africanized honeybee RJ was less polymorphic in size than that from European honeybee RJ. These results prompt us to speculate that the DNA sequence of the MRJP coding region may also be less polymeric in Chinese honeybees compared with that of European honeybees. These results further imply that the differences between the RJ proteins produced by Chinese and European honeybees may be ascribed to genetic differences in addition to posttranslational modifications. Further studies are necessary to confirm these possibilities.

This research was supported by the Scientific Research Foundation for Returned Overseas Chinese Scholars, State Education Ministry (to X. W.), and National Natural Science Foundation of China 30300183 (to L. S.).

REFERENCES

1. Ohashi, K., Natori, S., and Kubo, T. (1997) *Eur. J. Biochem.*, **249**, 797-802.
2. Brouwers, E. W. M., Ebert, R., and Beetsma, J. (1987) *J. Apic. Res.*, **26**, 11-23.
3. Beetsma, J. (1979) *Bee Wld.*, **60**, 24-39.
4. Evans, J. D., and Wheeler, D. E. (2000) *Genome Biol.*, **2**, 1-6.
5. Evans, J. D., and Wheeler, D. E. (1999) *Proc. Natl. Acad. Sci. USA*, **96**, 5575-6535.
6. The Honeybee Genome Sequencing Consortium (2006) *Nature*, **443**, 931-949.
7. Pothichot, S., and Wongsiri, S. (1993) *Asian Apic.*, 128-133.
8. Tan, K., Hepburn, H. R., He, S., Radloff, S. E., Neumann, P., and Fang, X. (2006) *Naturewissenschaften*, **93**, 315-320.
9. Kubo, T., Sasaki, M., Nakamura, J., Sasagawa, H., Ohashi, K., Takuchi, H., and Natoris, S. (1996) *J. Biochem.*, **119**, 291-295.
10. Santos, S. K., Santos, D. L., Mendes, A. M., Souza, M. B., Malaspina, O., and Palma, S. M. (2005) *Insect Biochem. Mol. Biol.*, **35**, 85-91.
11. Lensky, Y., and Rakover, Y. (1983) *Comp. Biochem. Physiol.*, **75**, 607-615.
12. Chen, C., and Chen, S. Y. (1995) *Food Chem.*, **54**, 195-200.
13. Simuth, J. (2001) *Apidology*, **32**, 69-80.
14. Fontana, R., Mendes, M. A., de Souza, B. M., Konno, K., Cesar, L. M., Malaspina, O., and Palma, M. S. (2004) *Peptides*, **25**, 919-928.
15. Bilikova, K., Gusui, W., and Simuth, J. (2001) *Apidologie*, **32**, 275-283.
16. Klaudiny, J., Hanes, J., Kulifajova, J., Albert, S., and Simuth, J. (1994) *J. Apic. Res.*, **33**, 105-111.
17. Schmitzova, J., Klaudiny, J., Albert, S., Schroder, W., Schreckengost, W., Hanes, J., Judova, J., and Simuth, J. (1998) *Cell. Mol. Life Sci.*, **54**, 1020-1030.
18. Albert, S., Klaudiny, J., and Simuth, J. (1999) *Insect Biochem. Mol. Biol.*, **29**, 427-434.
19. Fujii, M., Yonekura, M., Higuchi, T., Morimitsu, K., Yoshino, I., Mukai, S., Aoki, T., Fukunaga, T., Inoue, Y., Sato, M., and Kanaeda, J. (1996) *Food Sci. Technol. Int.*, **2**, 223-225.
20. Kimura, Y., Washino, N., and Yonekura, M. (1995) *Biosci. Biotechnol. Biochem.*, **59**, 507-509.
21. Kucharski, R., Malezska, R., Hayward, D. C., and Ball, E. E. (1998) *Naturwissenschaften*, **85**, 343-346.
22. Su, S. K., Albert, S., Chen, S. L., and Zhong, B. L. (2005) *Apidologie*, **36**, 389-401.
23. Albertova, V., Su, S. K., Brockmann, A., Gadau, J., and Albert, S. (2005) *J. Agric. Food Chem.*, **53**, 8075-8081.
24. Imjongjirak, C., Klinbunga, S., and Sittipraneed, S. (2005) *J. Biochem. Mol. Biol.*, **38**, 49-57.
25. Bradford, M. M. (1976) *Analyt. Biochem.*, **72**, 248-254.
26. Laemmli, U. K. (1970) *Nature*, **227**, 680-685.
27. Sano, O., Kunikata, T., Kohno, K., Iwaki, K., Ikeda, M., and Kurimoto, M. (2004) *J. Agric. Food Chem.*, **52**, 15-20.
28. Bilikova, K., Klaudiny, J., and Simuth, J. (1999) *Biologia*, **54**, 733-739.
29. Su, S. K., Chen, S. L., Zhong, B. X., Zheng, H. Q., and Albert, S. (2005) *Scientia Agricultura Sinica*, **38**, 612-618.
30. Scarselli, R., Donadio, E., Giuffrida, M. G., Fortunato, D., Conti, A., Balestreri, A., Felicioli, R., and Felicioli, A. (2005) *Proteomics*, **5**, 769-776.