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Profile Analysis of the Proteome of the Egg of the High Royal Jelly Producing Bees (*Apis mellifera* L.)

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Abstract

The protein composition of the egg development in the high royal jelly producing bees (*Apis mellifera* L.) was investigated. This pioneer study was to separate and quantify the proteins in the egg of the high royal jelly producing worker bees (*Apis mellifera* L.) by using two-dimensional gel electrophoresis along with their three-day development. The results showed that 160, 195, and 176 proteins, with a wide range of molecular weight (17-80 KDa) and relatively narrow scope of pI (4.00-8.40) could be detected on day 1, day 2, and day 3, respectively, during the developmental process of the egg. Meanwhile 44 protein spots were constantly detected along with the egg development. Among them 36% were in the uptrend along with the egg development, 14% were in the downtrend, and 39% were of the largest expressed volume on day 2. In addition, the specific proteins were expressed on day 1, day 2, and day 3 (89, 77, and 80, respectively). Besides the coexistent and specific proteins, 24 proteins were expressed on day 1 and day 2, but silenced on day 3, 49 proteins were expressed on day 2 and day 3, but silenced on day 1, only 3 proteins were expressed on day 1 and day 3, but silenced on day 2. The result indicates that egg development is a sequential and complex gene controlled process, where the eggs of day 2 express the most active proteins. The coexistent proteins suggest that it is conservative and indispensable for this event. These specific proteins suggest that the different developmental stage needs specific proteins to regulate it.

Key words: honeybees, high royal jelly producing, worker bee's egg, two-dimensional gel electrophoresis, proteome

INTRODUCTION

Royal jelly is not only a natural food benefiting human beings' health, but also a lucrative hive-product for beekeepers. Royal jelly, bred in the 1980s in China, is mainly produced in China, with an annual collection of about 2 000 tons, accounting for 90% of the world's total output, which is attributed to the high royal jelly producing bees (*Apis mellifera* L.). Indeed, it is the rare genic resource in China and even in the world. Honeybees (*Apis mellifera*) are eusocial insects belonging to Hymenoptera. They work together in a highly structured social order. Each bee belongs to one of the three specialized groups called castes. The different castes are: queen bees, drones, and worker bees.

All the castes are holometabolic insects with the same four stages, egg, larva, pupa, and adult, during their development. The queen lays the egg and stands at the bottom of the cell. Before hatching into the larval stage, the egg period is approximately 72 h (Graham 1992). To study the egg development of the high royal jelly producing bees, it will be conducive to find out the

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molecular mechanism concerning the high royal jelly production and lay a foundation for molecular breeding.

As the high royal jelly producing bees (*Apis mellifera* L.) were bred from the local Italian bees (*Apis mellifera* L.) in the 1980s, in China (Chen *et al*. 2005; Li and Wang 2005), and has become the best royal jelly producer in the world and gained more and more attention. In the past decades, the high royal jelly producing bees (*Apis mellifera* L.) were studied extensively, ranging from phenotypic investigation to molecular biology assay. During the period from the late 1980s to the late 1990s, a wide spectrum of field investigations showed that the production of the high royal jelly producing bees (*Apis mellifera* L.) was significantly higher than that of their unselected counterpart, Italian bees (*Apis mellifera* L.) (Chen and Han 1992; Shen and Xiao 1993; Chen and Lin 1995; Xu *et al*. 2000, 2001; Liu *et al*. 2001). From the late 1990s to the early 2000s, the bursa number, weight, and length of the hypopharyngeal gland were reported to be the morphological genetic markers of the high royal jelly producing worker bees (*Apis mellifera* L.) (Su 2000; Su and Chen 2003). At the same time, some DNA markers of the high royal jelly producing bees (*Apis mellifera* L.) were also reported by using molecular biological technologies (Zhang *et al*. 2001a, b, c; Jiang *et al*. 2002; Wang *et al*. 2002; Dai *et al*. 2003; Jin *et al*. 2003, 2004).

Meanwhile, the genetic studies show that the royal jelly producing traits were dominated by more than 70% of the genetic component, by employing the genotypic model (Li *et al*. 2003a, b). Subsequently, the DNA microsatellite analysis had indicated that seven alleles were greatly related to the high royal jelly production (Li and Wang 2005). However, no studies have been done on the development process, from zygotes (eggs) to brood emergence, of the high royal jelly producing bees (*Apis mellifera* L.), although a few reports are available on the egg of the honeybees (*Apis mellifera*), which are about expression patterns of deformed proteins during embryogenesis of honeybees (*Apis mellifera* L.) (Fleig *et al*. 1992), the behavior of sperm from egg penetration till the creation of the zygote, the development of the maternal pronucleus, the first two cleavage divisions (Ronglin and Omholt 1999), and the ultrastructure surface of the queen-laid diploid and haploid eggs, and that of worker-laid eggs (Tamar *et al*. 2003).

Therefore, the purpose of this study is to add some data on how protein is expressed during the egg developmental process of the high royal jelly producing worker bees (*Apis mellifera* L.), by using the proteomics approach, which will be conducive to find out the character of protein expression and regulation along with the egg development of the high royal jelly producing bees (*Apis mellifera* L.), and lay a foundation for clarifying the molecular mechanism of high royal jelly production.

MATERIALS AND METHODS

Chemicals

Immobilized pH gradients (IPG) strip, two-dimensional gel electrophoresis (2-DE) marker and Bio-lyte (pH 3-10) were purchased from Bio-Rad Laboratories Inc., USA. Tris-base, ammonium persulfate (AP), sodium dodecyl sulfate (SDS), and glycine were bought from Sigma, USA. Acrylamide, N,N-methylene bisacrylamide, bromphenol blue, coomassie brilliant blue (CBB) R-250, coomassie brilliant blue (CBB) G-250, thiourea, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate (CHAPS), bovine serum albumin (BSA), agarose and urea were purchased from Amresco., USA. 2,3-dihydroxybutane-1,4-dithiol (DTT), and iodoacetoamide were purchased from Merck., Germany. All other chemical reagents were from Beijing Chemical Reagents Inc., China.

Honeybee eggs

Specific age eggs of the worker bees of the high royal jelly producing bees (*Apis mellifera* L.), on day 1, day 2, and day 3, were randomly collected from the queen-controlled frame in the Experimental Apiary of Institute of Apicultural Research, Chinese Academy of Agricultural Sciences in Beijing, China. To guarantee the exact age, the eggs could be sampled. The queen was confined to a confinement chamber (the queen-controlled frame) where only one empty frame could be put in. Then the queen was allowed to lay her eggs

into the cells on the frame for 24 h. Subsequently, the queen was removed from the chamber after 24 h and the worker bees' eggs of day 1 were collected with a plastic transfer tool and put into the frame confinement, to which the queen was forbidden to access. By the same method, the worker bees' eggs of day 2 and day 3 were collected before 48 and 72 h, respectively. A total of 150 eggs of the worker bees were sampled for each age.

Protein extraction

Protein extraction was performed according to the method of Zhong *et al*. (2005), with some improvements. The eggs (1 mg eggs, 10 μL^{-1} buffer) were mixed in a phosphate buffer (PB) pH 7.6, containing 32.5 mM K_2HPO_4, 2.6 mM KH_2PO_4, and 400 mM NaCl. The mixture was homogenized for 20 min in the ice and sonicated for 2 min, then centrifuged at 12 000 g and 4°C for 10 min, and further centrifuged at 15 000 g and 4°C for 10 min. The supernatant was removed to another tube for further use. The pellets (1 mg eggs, 2 μL^{-1} buffer) were mixed in the aforementioned PB pH 7.6, and then centrifuged at 15 000 g and 4°C for 10 min. The supernatant was removed and mixed into the above tube, containing the supernatant as PB-dissoluble protein extraction, whereas, the pellets (1 mg eggs, 10 μL^{-1} buffer) and PB-indissoluble proteins were mixed in a lysis buffer composed of 8 M urea, 2 M thiourea, 4% CHAPS, 20 mM Tris-base, 30 mM DTT, and 2% Bio-lyte (pH 3-10), and then the mixture was homogenized for 10 min in ice, sonicated for 2 min, and then centrifuged at 15 000 g and 4°C for 10 min. The supernatant was removed and mixed into the above-mentioned tube containing PB-dissoluble protein extraction, and the debris was discarded. Trichloroacetic (TCA) was added to the collected supernatants to a final concentration of 10%, and then the mixture was kept in ice for 10 min for precipitating proteins and desalting. Subsequently, the mixture was centrifuged twice at 15 000 g and 4°C for 10 min. The supernatant was discarded and the pellets (1 mg eggs, 5 μL^{-1} buffer) were resolved in the foregoing lysis buffer, and then the mixture was homogenized for 5 min in ice and sonicated for 2 min. Subsequently it was adjusted to pH 7.0 with 2 M NaOH. The mixture, the protein extraction of the worker bees' eggs, was stored at -70°C for further use.

Protein determination

Protein concentration was determined according to the method developed by Bradford (1976), using BSA as the standard. The absorption was measured at 595 nm (Beckman, spectrophotometer DU800).

Two-dimensional gel electrophoresis (2-DE)

Thirty micro liters of the protein extraction of the worker bees' eggs was suspended in 120 µL of the rehydration buffer (8 M urea, 4% CHAPS, 0.001% bromophenol blue, 65 mM DTT, 0.2% Bio-lyte pH 3-10). A mixture of 125-150 µL (each sample containing 77.4 µg of protein) was loaded on a 7 cm immobilized pH gradient (IPG) strip (pH 3-10 L) and isoelectric focusing (IEF) was performed at 18°C on a Protean IEF cell system (Bio-Rad Hercules, CA, USA) according to the following program: 14 h at 50 V, 30 min of a linear gradient at 250 V twice, 30 min at 500 V, 3 h of a linear gradient at 4 000 V, and 20 000 V h^{-1} at 4 000 V. Then the strip was equilibrated in equilibration buffer 1, containing 6 M urea, 0.375 M Tris-HCl (pH 8.8), 20% glycerol, 2% SDS, 2% DTT for 15 min, and then continued in the equilibration buffer 2, containing 6 M urea, 0.375 M Tris-HCl (pH 8.8), 20% glycerol, 2% SDS, 2.5% iodoacetoamide for 15 min. After equilibration, the strip was transferred to SDS-PAGE gel, 12% T separating gel (0.75 mm thick). Meanwhile 5 µL of the 2-DE marker was loaded onto a piece of filter paper, and then it was transferred adjacently to the acid tip of the strip when the filter paper was nearly dry. The second dimension electrophoresis, SDS-PAGE, was performed on a mini protean 3 cell (Bio-Rad Hercules, CA, USA) according to the following program: Ten min at 80 V, and 200 V until bromophenol blue was running out of the gel at room temperature. Then the gel was stained with CBB R-250.

Image analysis

The gel was scanned using a transparency mode

scanner, connected to the PC system, at 32-bit red-green-blue colors and 500 dpi resolution for documentation. The image was analyzed using PDQuest 7.3.0 (Bio-Rad Hercules, CA, USA).

RESULTS

Profile of 2-DE of the eggs of the high royal jelly producing worker bees (*Apis mellifera* L.)

The results were reproducible because they have been duplicated for five times. The three protein patterns corresponding to the eggs of day 1, day 2, and day 3 are shown in Fig.1. All the three gels had been analyzed with the same parameters, 16.93 of sensitivity, 5 of size scale (PDQuest 7.3.0). With the method and condition of the experiment, it can be seen that all the expressed protein spots on the three gels are with the same range of molecular weight (MW) and pI in terms of 17-80 KDa and 4.00-8.40, respectively. However, the detected spot numbers on day 1 (Fig.1-A), day 2 (Fig.1-B), and day 3 (Fig.1-C) are 160, 195, and 176, respectively. From Fig.2, all the detected spots are independent, indicating that the proteins are fully separated.

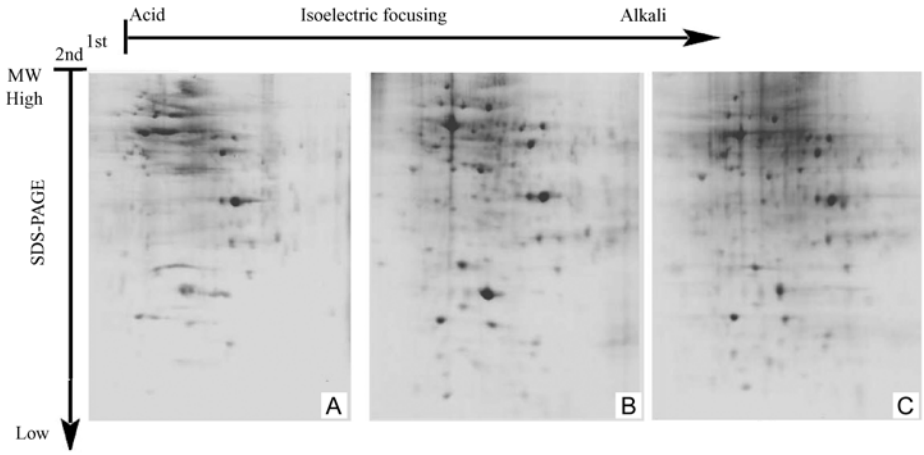

Fig. 1 Profile of the 2-DE analysis of the eggs of the high royal jelly producing worker bees (*Apis mellifera* L.) on different days. A, B, and C are the protein profiles corresponding to day 1, day 2, and day 3, respectively. Each sample of 77.4 μg was subjected to 2-D E and stained with CBB R-250.

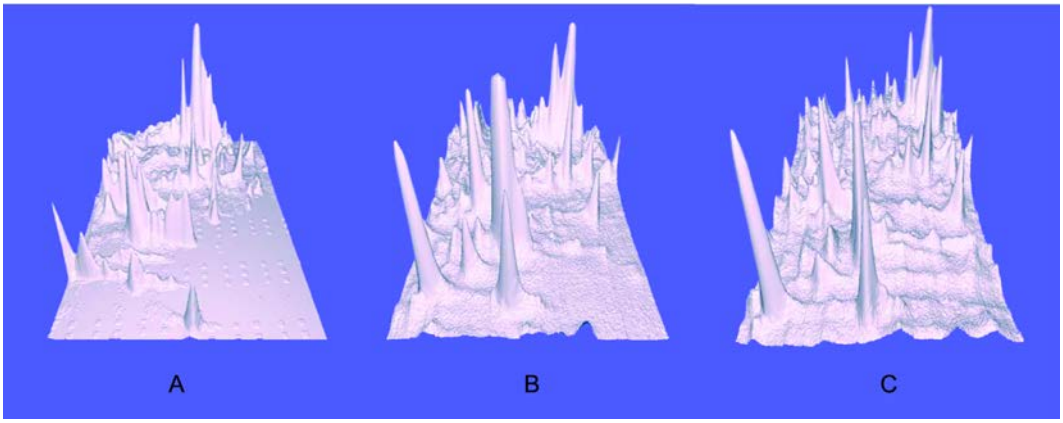

Fig. 2 Three dimensional (3-D) view of the protein pattern and separation based on the same region in the 2-DE profile (Fig.1) on different days of the high royal jelly producing worker bees (*Apis mellifera* L.). A, B, and C are the 3-D patterns of day 1, day 2, and day 3, respectively.

Comparison of the protein pattern of the eggs of the high royal jelly producing worker bees (*Apis mellifera* L.) on different days

By comparing the 2-DE profiles of the eggs on different days, 44 spots can be clearly matched to every profile, MW of these spots distributed in a wide spectrum from 19 KDa to 77 KDa, whereas, their pI concentrated within a relatively narrow scope from 5.27 to 8.06 (Table 1). Among these 44 spots, it can be observed that the expressional volume of 16 spots is in an uptrend following the egg developmental process, but the volume of six spots is in a downtrend along with the egg developmental process, whereas, the volume of 17 spots reaches a peak on day 2, and five spots are with a minimal expressional volume on day 2 (Table 1).

Eighty-nine spots were specified on day 1, and their MW and pI ranged between 22-85 KDa and 5.22-8.40, respectively, and the expressional volume ranged from 330 to 46 900 (Table 2). Next, 77 spots were specific to

Table 1 MW, pI, and expressional volume of the coexistent proteins of the eggs of the high royal jelly producing worker bees (*Apis mellifera* L.) on different days

Spot No. (SSP)	MW (KDa)	pI	Expressional volume		
			Day 1	Day 2	Day 3
0304	41.9	5.27	3 291	25 832	27 734.5
1001	25.0	5.44	1 733.7	3 650.7	7 454.7
1504	50.6	5.42	1 431.1	8 992.1	23 427.7
1505	49.3	5.62	2 266.1	12 783.7	35 974
2002	22.5	5.69	10 808.1	26 741.9	27 691.3
2208	32.6	5.86	1 481.5	6 432	12 691.5
2305	33.5	5.63	2 838.1	4 372.6	12 803
2706	68	5.7	3 495.9	9 989.6	11 603.9
3005	19.4	6.19	1 121.7	4 723.7	7 331.1
6104	29.04	6.91	2 237.8	17 287.3	17 639.4
7003	20.8	7.07	5 631.1	6 506.8	7 576.3
7106	29.6	7.11	10 090.3	17 282.4	35 586.8
7205	31.6	7.11	1 161.3	1 742.9	4 092.6
7605	57.4	7.16	11 498.9	19 652.2	35 569.2
8207	30.3	7.99	728.5	3 813.8	5 662.2
8511	52.2	7.59	1 648.8	2 483.4	8 851.2
1102	28.4	5.52	7 787.7	4 813.1	3 817.4
2304	41.7	5.87	14 591.2	13 060.9	6 573
2404	44.7	5.82	18 286.3	17 182.7	8 206.2
4704	66.1	6.39	43 804.3	19 656.5	6 967.1
5605	58.2	6.6	30 090	23 468.8	12 415.8
7306	34.2	7.18	68 345.4	66 994.4	58 078.2
1301	33.8	5.47	2 523.1	4 837.8	1 312.6
2505	49.7	5.85	34 194.7	40 068.1	11 739.4
2604	58.1	5.85	21 265.9	71 430.2	5 984.4
2806	77.3	5.78	3 336.2	10 869.8	8 569.6
3703	67.1	6.02	14 489.7	15 607.7	3 367.2
3704	66.3	6.26	18 947.6	23 446.8	769
4001	24.5	6.38	21 578.8	76 553.8	45 123
4002	22.1	6.44	7 236.3	18 711.9	16 624.7
5405	46.2	6.52	5 960	8 783.9	6 191.6
6306	40.4	6.86	4 099	2 062.5	453.8
6308	34.6	6.83	5 094.2	21 800.9	6 848.9
7105	29.5	7.4	8 602.2	21 394.6	4 592.5
7307	34	7	14 710.5	32 626.5	18 403.1
7506	50.2	7.01	22 661.6	33 963	30 286.7
7509	48.3	7.55	4 707.7	14 516.4	5 124.3
8604	54.3	8.06	1 462.6	8 268.2	948.2
8605	55.5	7.77	1 131.7	5 747.3	4 168.9
5809	79.3	6.56	39 378.7	4 584.4	9 680
6605	58.45	6.77	27 832.8	4 309.6	20 301.8
7006	26.6	7.36	17 605.6	8 498	9 356.8
7308	39.7	7.12	2 525.4	2 216.3	2 524.6
8306	37.1	7.64	4 811.7	3 361.1	5 668.1

the profile on day 2, and their MW scattered from 18 to 80 KDa, whereas, their pI was in the scope of 4.75-8.30, and the expressional volume was 140-39 000 (Table 3). In addition, 80 spots existed on the profile on day 3 specifically, and their MW and pI were 18-80 KDa and 4.70-8.15, respectively, and the expressional volume ranged from 360 to 24 000 (Table 4).

Twenty-four spots with MW 17-70 KDa and pI 5. 09-8.04 were both expressed on day 1 and 2, but silenced on day 3, whereas, 30-50 KDa accounted for 70%. Among them, the expressional volume of the 15 spots with MW 17-70 KDa and pI 5.09-7.98 was in an uptrend on the second day compared with that on day 1. At the same time, the expressional volume of the nine spots with MW 30-55 KDa and pI 5.72-8.04 was in a downtrend on day 2 as compared with that of day 1 (Table 5).

Forty-nine spots with molecular weights 18-71 KDa and pI 4.8-7.67 were silenced on day 1, at the same time both expressed on day 2 and 3. Among these

Table 2 MW, pI, and expressional volume of the specific proteins of the eggs of the high royal jelly producing worker bees (*Apis mellifera* L.) on day 1

Spot No. (SSP)	MW (KDa)	pI	Expressional volume	Spot No. (SSP)	MW (KDa)	pI	Expressional volume
0208	33.8	5.22	1 249.4	5903	85.1	6.43	7 405.9
1006	26.1	5.50	6 008.4	6006	24.5	6.84	15 096.1
1405	47.4	5.56	3 312.6	6007	22.3	6.83	670.1
1610	55.8	5.41	1 824.0	6106	28.8	6.68	1 861.2
1611	55.8	5.66	3 955.1	6205	30.9	6.66	2 176.2
2007	26.6	6.02	7 848.4	6206	32.5	6.87	2 366.1
2008	22.5	5.91	8 088.1	6207	32.2	6.67	701.3
2109	28.8	5.84	2 171.5	6309	34.4	6.68	9 077.1
2210	33.1	5.75	8 263.0	6409	43.4	6.83	5 340.0
2506	49.2	5.67	4 632.6	6410	44.0	6.72	15 596.0
2507	48.6	5.91	28 668.9	6411	46.8	6.68	737.3
2508	51.1	5.83	2 146.2	6506	49.8	6.76	12 035.8
2708	70.0	5.79	7 360.7	6609	57.2	6.92	28 624.9
2709	64.9	6.00	3 216.5	6610	55.1	6.71	5 540.4
2808	79.4	5.85	4 353.0	6611	59.1	6.72	1 145.8
2809	71.8	5.72	1 680.1	7010	24.5	7.02	7 938.1
2810	71.4	5.97	22 383.3	7011	26.0	7.37	831.6
2811	74.6	5.96	3 333.4	7206	31.4	7.23	3 381.2
3006	22.5	6.11	1 366.6	7405	44.5	7.30	2 523.1
3206	32.9	6.16	6 058.3	7406	43.7	7.00	13 799.0
3408	45.6	6.03	8 506.5	7510	51.7	7.29	18 964.0
3506	50.1	6.13	9 135.8	7608	59.1	7.33	9 366.1
3607	57.9	6.21	46 906.8	8112	29.3	8.00	638.0
3608	54.7	6.01	5 674.7	8113	29.3	7.89	6 281.2
3609	57.6	6.01	2 005.5	8208	31.9	7.52	6 809.3
3705	67.0	6.14	33 299.9	8209	33.7	7.70	4 418.3
3806	77.5	6.02	3 198.8	8308	37.6	7.55	4 912.9
3807	77.9	6.14	2 036.7	8309	37.3	7.44	1 172.6
3808	73.2	6.01	921.8	8310	42.1	7.65	1 006.3
3809	73.6	6.14	7 069.4	8311	42.6	7.55	1 088.1
4003	26.9	6.41	25 818.8	8404	45.1	7.55	1 843.4
4309	34.4	6.38	2 793.8	8406	42.9	7.72	5 760.5
4310	39.3	6.37	2 390.3	8512	53.0	7.72	4 918.5
4311	41.8	6.33	4 425.7	8513	48.6	7.72	2 287.0
4405	43.1	6.38	23 569.4	8514	50.8	7.50	991.7
4811	70.5	6.33	3 854.8	8606	55.8	7.67	4 023.7
5003	24.7	6.49	16 498.9	8801	73.6	7.69	8 343.7
5207	32.7	6.49	337.2	8802	74.1	7.55	383.0
5312	42.1	6.49	13 380.6	8803	77.0	7.56	798.5
5406	43.7	6.63	26 478.9	9004	23.4	8.37	1 468.2
5507	49.2	6.52	2 771.7	9302	36.2	8.39	3 750.3
5607	54.1	6.52	21 341.8	9303	35.1	8.32	1 164.7
5708	67.0	6.58	12 057.4	9304	36.2	8.03	1 056.8
5811	80.4	6.43	38 359.2	9509	50.8	8.40	12 125.3
5902	84.6	6.53	4 558.1				

Table 3 MW, pI, and expressional volume of the specific proteins of the eggs of the high royal jelly producing worker bees (*Apis mellifera* L.) on day 2

Spot No. (SSP)	MW (KDa)	pI	Expressional volume	Spot No. (SSP)	MW (KDa)	pI	Expressional volume
0001	17.9	5.34	3 567.5	4810	70.8	6.39	15 924.6
0102	29.3	4.75	908.8	5001	25.9	6.63	1 431.9
0206	31.1	5.30	141.0	5102	28.7	6.60	2 061.5
0606	58.4	5.27	2 357.5	5308	39.4	6.52	3 563.1
1002	21.7	5.57	67.5	5309	35.8	6.50	3 137.4
1004	26.0	5.60	499.4	5310	35.3	6.62	1 384.6
1005	26.5	5.46	1 396.5	5311	33.6	6.56	6846.0
1302	35.6	5.48	695.9	5504	49.4	6.47	8 934.8
1606	60.3	5.50	9 190.3	5506	50.0	6.64	3 933.0
1608	54.1	5.39	5 884.1	5604	58.4	6.51	234.3
1707	69.4	5.57	10 256.5	5606	55.7	6.61	18 880.4
1708	60.8	5.40	4 998.7	5706	68.7	6.55	20 978.0
1806	79.1	5.60	686.9	5810	80.9	6.61	5 009.0
1807	78.9	5.40	2 250.7	6003	17.5	6.92	4 011.8
2005	26.8	5.82	2 101.8	6005	19.2	6.89	1 207.3
2206	33.3	5.74	5 960.0	6105	30.1	6.75	3 552.8
2207	32.7	5.66	4 713.1	6203	32.0	6.76	5 314.5
2306	41.0	5.71	4 193.8	6407	47.1	6.95	2 320.5
2605	53.0	5.85	5 709.4	6505	47.8	6.76	11 566.4
2707	69.8	5.87	39 066.3	6606	56.1	6.74	397.4
2807	71.7	5.66	9 578.8	6608	57.9	6.90	18 822.3
3406	43.8	6.03	18 188.0	7004	21.2	7.10	1 831.5
3407	43.1	6.17	30 970.5	7009	24.4	7.39	863.4
3504	52.7	5.99	1 038.1	7508	50.5	7.37	881.9
3505	52.5	6.10	28 360.9	7606	57.7	7.35	3 427.6
4104	28.9	6.33	1 306.0	7607	53.8	7.18	1 189.2
4208	32.3	6.38	8 260.1	8001	20.7	8.03	704.5
4505	51.9	6.35	2 320.3	8110	28.7	7.88	4 426.3
4506	52.1	6.45	2 211.4	8111	27.7	7.84	5 110.6
4606	60.2	6.36	20 620.1	8510	50.5	7.72	3 868.2
4607	54.2	6.30	20 023.1	9001	20.9	8.08	2 821.9
4608	54.2	6.38	21 431.3	9002	20.9	8.24	5 534.0
4705	70.5	6.29	17 161.2	9003	21.0	8.30	338.4
4706	60.4	6.42	22 951.0	9102	29.4	8.08	7 557.1
4806	77.4	6.29	3 488.7	9207	30.7	8.24	14 089.5
4807	77.9	6.37	4 663.1	9208	32.2	8.26	7 878.7
4808	78.5	6.42	4 020.3	9209	30.4	8.12	7 686.5
4809	80.8	6.46	20 188.8	9508	51.0	8.26	11 915.6

spots, the expressional volume of the 28 spots with molecular weight 18-71 KDa and pI 4.8-7.64 were upexpressed on day 3 as compared with those on day 2, and the expressional volume of the 21 spots with molecular weight 19-71 KDa and pI 5.28-7.67 were downexpressed on day 3 compared with those on day 2 (Table 6).

In addition, there were three spots that were silenced on day 2, whereas, expressed both on day 1 and 3. Among the three spots, the expressional volume of only one spot (MW 45.4 KDa, pI 7.62) increased on day 3 compared to that on day 1, and the volume of two spots (MW 45.4 KDa, pI 6.17; MW 53.0 KDa, pI 6.38) decreased on day 3 as compared to that on day 1 (Table 7).

DISCUSSION

The 2-DE-based proteome analysis has been successfully used to detect and characterize marker proteins of a cell or tissue (Zivy and de Vienne 2000). To date, only five articles are available, obtained by employing the proteomic approach, to detect and identify the proteins of royal jelly proteome (Sano *et al*. 2004; Santos *et al*. 2005; Scarselli *et al*. 2005), the spermathecal gland secretion, as well as spermathecal fluid in the queen bees (Klenk *et al*. 2004), and the proteins of the honeybee venom (Peiren *et al*. 2005). The present study is a pioneer one that uses the proteomic assay to compare the proteome complement in the developmental process of the worker bees' egg

Table 4 MW, pI, and expressional volume of the specific proteins of the eggs of the high royal jelly producing worker bees (*Apis mellifera* L.) on day 3

Spot No. (SSP)	MW (KDa)	pI	Expressional volume	Spot No. (SSP)	MW (KDa)	pI	Expressional volume
0002	18.2	5.32	1 247.8	5709	63.5	6.58	6 711.0
0003	22.7	5.11	1 183.5	5710	66.4	6.58	6 796.1
0209	31.8	5.07	3 080.3	5711	69.8	6.55	3 689.7
0210	33.6	5.12	2 756.5	5812	72.9	6.53	945.0
0306	42.5	5.08	2 634.2	6008	22.1	6.68	1 516.1
0504	50.3	4.70	8 945.1	6107	29.8	6.81	5 006.5
0607	59.6	4.84	7 842.0	6310	34.0	6.63	3 896.1
0608	54.2	5.13	14 044.0	6412	43.5	6.66	8 590.1
1007	23.2	5.37	678.0	6612	56.3	6.85	19 112.1
1008	22.6	5.50	7 623.4	6613	56.0	6.72	14 619.0
1303	35.6	5.66	7 189.2	6614	58.5	6.70	13 866.5
1406	44.7	5.63	17 613.0	6705	68.9	6.75	2 359.2
1506	53.9	5.41	16 930.9	6804	80.7	6.68	1 261.5
1507	48.8	5.50	5 613.8	6805	73.4	6.71	1 415.9
1612	59.2	5.51	4 556.4	6806	71.1	6.86	14 721.7
1613	56.3	5.55	3 152.0	7012	21.6	7.37	3 599.8
2009	17.8	5.84	14 622.0	7013	26.6	7.05	3 984.1
2010	21.3	5.80	757.1	7107	30.3	7.37	10 701.0
2110	29.7	5.74	3 466.6	7207	33.9	7.33	22 872.4
2211	33.7	5.85	9 754.8	7407	43.8	7.02	3 987.5
2308	35.6	5.84	10 739.1	7609	56.0	7.00	6 830.6
2405	44.1	5.71	4 477.1	7705	67.2	7.21	3 901.2
2606	57.8	5.70	19 979.8	7804	79.2	7.33	4 754.2
2710	69.4	5.91	20 486.6	7805	76.7	7.15	1 387.7
2812	80.2	5.87	5 453.1	7807	72.5	7.01	2 668.2
3007	21.0	6.13	3 483.9	8002	16.8	7.52	1 956.8
3507	52.6	6.13	8 830.5	8003	17.4	7.50	267.5
3610	59.2	6.05	10 001.1	8004	19.2	7.54	1 698.7
3706	60.0	6.16	23 564.1	8005	23.9	7.67	3 690.7
3707	68.1	6.16	18 477.0	8006	24.6	7.44	7 806.8
3810	80.7	6.17	9 700.3	8114	28.9	7.88	4 305.3
3811	70.2	6.02	5 655.2	8115	30.0	7.74	7 730.3
4004	20.6	6.36	2 753.2	8210	32.2	7.91	2 277.5
4107	28.9	6.36	2 465.8	8312	34.0	7.51	2 770.7
4609	56.7	6.38	2 146.9	8407	45.8	8.02	2 379.1
5004	20.1	6.60	1 663.6	8515	51.2	7.87	4 485.8
5005	24.5	6.61	3 342.9	8516	52.9	7.67	364.6
5208	30.5	6.53	3 299.3	8607	56.0	7.51	821.9
5313	37.0	6.50	7514.9	9103	29.9	8.14	6 130.1
5608	59.2	6.50	11 045.7	9510	51.6	8.13	4 742.5

Table 5 MW, pI, and the expressional trend of the eggs of the high royal jelly producing worker bees (*Apis mellifera* L.) expressed on day 1 and day 2, but silenced on day 3

Spot No. (SSP)	MW (KDa)	pI	Expressional volume		
			Day 1	Day 2	Day 3
0605	55.9	5.09	1 173.1	2 374.9	
1706	67.6	5.41	3 020.7	15 095.8	
2004	17.5	5.78	3 739.1	7 590.0	
2307	33.9	5.83	1 681.5	5 769.1	
3004	26.8	6.19	7 877.3	9 377.2	
3307	39.3	6.18	627.3	1 134.5	
3805	70.8	6.24	3 607.4	21 319.8	
4404	43.1	6.27	4 000.0	30 685.9	
5705	70.5	6.47	10 346.0	17 649.6	
6607	56.0	6.87	738.4	5 483.1	
7204	33.5	7.51	1 299.2	3 222.2	
7703	63.5	7.31	919.8	1 506.9	
8206	30.3	7.77	10 775.7	21 064.6	
8307	38.8	7.98	1 918.7	3 809.7	
8509	51.8	7.84	7 932.9	9 194.5	
2106	29.5	5.85	7 796.5	2 588.8	
3606	58.1	6.10	58 270.6	15 715.4	
5206	30.7	6.52	4 274.5	2 487.6	
6001	26.6	6.85	6 300.3	946.0	
6204	31.1	6.96	3 617.2	1 301.7	
8205	33.6	8.04	7 899.2	6 923.7	
2504	52.5	5.72	2 809.0	2 516.1	
6504	50.5	6.91	3 046.1	2 861.2	
7309	34.6	7.38	20 039.4	19 789.8	

Table 6 MW, pI, and the expressional trend of the eggs of the high royal jelly producing worker bees (*Apis mellifera* L.) expressed on day 2 and day 3, but silenced on day 1

Spot No. (SSP)	MW (KDa)	pI	Expressional volume		
			Day 1	Day 2	Day 3
0101	27.1	4.80		2 879.2	5 593.3
0204	32.1	5.14		1 741.9	2 112.8
0205	30.6	5.13		1 236.1	5 813.3
0305	34.5	4.79		2 423.5	2 872.2
1204	32.9	5.37		1 804.9	2 660.6
1607	56.2	5.61		3 691.8	37 258.6
1609	57.0	5.40		3 390.9	19 987.9
2001	19.9	5.67		3 482.0	7 182.9
2006	24.6	5.83		3 763.2	5 377.6
2101	26.9	5.64		1 977.4	6 108.9
2107	28.1	5.83		3 537.7	7 987.2
2108	28.1	5.68		1 137.0	1 937.6
3003	24.6	6.08		7 809.0	10 498.5
3101	27.0	6.00		33 823.0	39 523.9
3104	29.0	6.11		1 361.1	5 794.2
3205	30.6	6.03		1 006.6	2 175.0
3308	34.0	6.18		5 092.8	24 758.2
3605	55.5	6.17		5 139.3	12 156.7
4105	27.5	6.36		3 994.9	11 682.3
4207	33.6	6.37		6 319.6	12 189.9
4306	41.1	6.27		8 793.8	10 558.2
5205	32.0	6.59		3 267.4	9 810.6
5505	51.7	6.56		7 107.0	8 762.0
6004	18.2	6.71		563.7	1 079.8
6408	43.7	6.76		26 528.7	33 206.0
7001	20.1	7.04		2 550.2	10 334.3
7008	24.7	7.11		1 120.9	2 760.0
8109	29.7	7.64		10 032.1	13 252.3
0207	30.5	5.28		2 416.6	1 647.3
1003	21.9	5.59		1 519.4	1 019.4
2003	18.8	5.77		2 397.4	1 616.0
2209	30.6	5.87		12 305.8	2 166.7
3001	20.4	6.00		1 778.3	1 380.9
3002	23.5	6.02		10 767.6	10 296.1
3804	71.2	6.16		10 780.2	1 580.6
4209	31.2	6.27		8 131.0	2 109.7
4307	41.4	6.40		10 308.9	3 184.6
4308	37.1	6.33		16 388.1	3 548.7
4507	48.8	6.34		2 184.2	1 287.3
4605	57.6	6.29		5 981.6	4 034.8
5707	68.5	6.65		16 047.8	3 167.0
6002	20.9	6.89		1 549.9	871.4
6307	38.2	6.71		11 531.2	4 499.4
6503	50.5	6.82		13 998.5	10 902.1
7007	19.1	7.23		3 246.2	306.8
7404	45.9	7.30		6 266.6	2 989.2
7507	49.9	7.26		9 015.5	3 737.6
7704	67.6	7.41		3 843.3	3 774.6
8702	67.8	7.67		5 568.3	1 620.3

Table 7 MW, pI, and the expressional trend of the eggs of the high royal jelly producing worker bees (*Apis mellifera* L.) expressed on day 1 and day 3, but silenced on day 2

Spot No. (SSP)	MW (KDa)	pI	Expressional volume		
			Day 1	Day 2	Day 3
8405	45.4	7.62	3 706.0		10 789.0
3409	45.4	6.17	11 551.8		5 327.6
4508	53.0	6.38	9 969.6		2 588.1

of the high royal jelly producing bees (*Apis mellifera* L.).

Royal jelly production is a colony performing trait of the worker bees (Graham 1992). Previous studies showed that royal jelly production is dominated by genetic components (Li *et al*. 2003a, b; Li and Wang 2005). Therefore, this genetic process must begin from the egg development of the worker bees. The egg's cleavage begins from when it is laid to the cell. The egg divides to initiate a succession of nuclear cleavage divisions and forms cellular layers. The organs of the bees are derived from these layers, and help to develop the embryo. By the time of day 3 after the egg is laid, embryological development is complete and the egg now contains a larva. But, the change of modality of the worker bees is insignificant along with the process of egg development (Harry and Robert 1996).

On the basis of the detected protein spots along with the egg developmental process, it can be seen that egg development is a sequential and complex gene controlled process, which involves many proteins, although the modal change is insignificant. It can be seen that the eggs of day 2 express most actively, 160, 195, and 176 proteins expressed on day 1, day 2, and day 3, respectively, indicating that the genes in the egg have been activated in an environment at 34°C when the egg is laid to the cell, and activated genes are less on day 1 and more on day 2 and 3, and prepare to hatch, along with the three-day development of the high royal jelly producing bees (*Apis mellifera* L.).

The 44 protein spots are constantly detected along the process of the development of the eggs indicate that these proteins are conservative, and are indispensable for this program. But these coexisting proteins are not all the same; their expressional volume has some changes. Thirty-six percent of the coexisting proteins are in the uptrend along with the egg development, and 39% of them are of the largest expressed volume on day 2. It can be seen that these coexisting proteins regulate the development of the egg by way of altering the expressional volume. It also indicates that egg development is a sequential and complex gene controlled dynamic process.

Nowadays, the reports concerning the basic research on the egg development of the bees are not enough, and the relationship between the change of modality and the structure of the egg, with the expression and the regulation of the proteins is still not clear. The present experiment just lays a preliminary foundation in the proteome of the egg development of the high royal jelly producing bees (*Apis mellifera* L.). If all the proteins can be sequenced by mass spectrometry (MS) and identified by database querying, it will benefit to further understand the mechanism of egg development of the high royal jelly producing bees (*Apis mellifera* L.). In addition, if the differential proteome can be compared between the high royal jelly producing bees (*Apis mellifera* L.) and the unselected ones (*Apis mellifera* L.), the functional gene will be found. All of these will be conducive to elucidate the molecular mechanism concerning the high royal jelly production and will lay a foundation for molecular breeding.

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