ORIGINAL RESEARCH ARTICLE

Comparative analysis of the effects of different storage conditions on major royal jelly proteins

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Received 17 January 2007, accepted subject to revision, 14 March 2007, accepted for publication 21 March 2007.

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Summary

An experiment was carried out to analyse changes in the protein components of royal jelly (RJ) under different storage conditions, based on two dimensional electrophoresis (2-DE). The proteins identified were compared to those proteins already identified in the proteome complement of the RJ. The results showed that the total detected protein spots were 75, 45, 63 and 69, with molecular weight in the range of 7.64-72.33 kDa, isoelectric point 4.95-8.70, in the 2-DE image of RJ protein components stored at -20°C for 80 days, 4°C for 80 days, room temperature for 30 days, room temperature for 80 days, respectively. The spot of major royal jelly protein, apalbumin 1, was saturated in all images in this experiment, indicating that temperature has no significant effects on it. The spots number and the quantity of apalbumin 2 and apalbumin 3 did not increase or decrease following the temperature trend, suggesting they are also sensitive to temperature. However, spots of apalbumin 4 and glucose oxidase were observed only in the image of -20°C for 80 days, and spots of apalbumin 5 were detected in the images of -20°C and 4°C for 80 days, indicating they are the proteins most sensitive to storage temperature and thus may be potential freshness markers for RJ, and that the best way to maintain quality of RJ is under freezing conditions.

Keywords: royal jelly, apalbumins, storage temperature, 2-DE

Introduction

Royal jelly (RJ), secreted from the hypopharyngeal and mandibular glands of worker honey bees mainly between the sixth and twelfth day of their adult life, is fed to worker larvae for three days, and to queen larvae, and it plays a key role in honey bee caste determination (Graham, 1992; Patel *et al*., 1960; Lensky & Rakover, 1983; Knecht & Kaatz, 1990; Evans & Wheeler, 1999). RJ is a white-yellow colloid with pH between 3.6-4.2, and is a mixture with several constituents, containing water, proteins, lipids, carbohydrates, free amino acids, vitamins and minerals (Scarselli *et al*., 2005; Simuth *et al*., 2004; Howe et al., 1985; Koya-Miyata *et al*., 2004). Proteins account for ~50% of RJ dry weight, and important protein components belong to a family named Major Royal Jelly Proteins (MRJPs), now named apalbumins, with molecular weight of 49-87kDa (Hanes & Simuth, 1992; Albert & Klaudiny, 2004).

The fragments of apalbumin 1 and apalbumin 3 exist in RJ and are believed to play a key role in the physiological mechanism of honey bee larval female caste differentiation (Scarselli *et al*., 2005). For human beings, RJ has nutritional, healthy and pharmacological functions, such as hypotensive activities, antitumor activities, anti-inflammatory activities and anti-diabetes with its insulin-like peptide (Tokunaga et al., 2004; Li *et al*., Kohno *et al*., 2004; 2003; Fujii, 1995). So far, the biological functions of some component proteins in RJ have been reported (Simuth *et al*., 2004). Royalisin is an antimicrobial peptide against Gram-positive bacteria and fungi (Fujiwara *et al*., 1990; Bilikova *et al*., 2001); Jelleines are an antimicrobial peptide family against Gram-positive, Gram-negative bacteria and yeasts (Fontana *et al*., 2004); and apisin is a 350kDa glycoprotein that can stimulate proliferation of human monocytes (Watanabe *et al*., 1998; Kimura, 2003).

As a functional and healthy food, the storage condition of RJ has drawn considerable attention in recent years, as improper storage conditions will affect its active components. In the 1990s,

Chen *et al*. investigated the quality changes of RJ stored at -20°C, 4°C and room temperatures for up to 7 months and found that the viscosity of RJ increased remarkably when stored at room temperature, with the brown intensities increasing rapidly and water-soluble proteins hydrolyzing, but not at -20°C. When subjected to SDS-PAGE, four to five different protein components, with molecular weights ranging from 50–88kDa, were found degrading gradually with increase in temperature and time period, resulting in the appearance of lower molecular weights (20-40kDa) (Chen & Chen, 1995). The protein components in RJ stored under different conditions have been compared by chromatography and PAGE and a 57kDa glycoprotein was found as a suggested freshness marker for RJ (Kamakura *et al*., 2001). The degradation of the glycoprotein is proportional to storage temperature and time period and the glycoprotein might be derived from subunit of a 350kDa RJ protein stimulating the proliferation of human monocytes. Proteinase inhibitor EDTA (Ethylenediaminetetraacetic acid) suppresses the degradation of 57kDa protein during storage at high temperature (Kamakura *et al*., 2002).

Although previous researchers have carried out a wide spectrum of investigations into the changes of protein components in RJ, and the identification of RJ origin by proteins (Biondi *et al*., 2003), very little is known about the changes in the protein components of RJ stored under different storage conditions by two dimensional electrophoresis (2-DE). The present study was carried out in order to analyse the changes of protein components in RJ under different storage conditions using 2-DE technology, to look for possible markers for assessing RJ freshness and to find the best way of storing it.

Materials and Methods

Chemical reagents

Immobilized pH gradients (IPG) strip (pH range 3-10 linear), two-dimensional gel electrophoresis (2-DE) marker, Bio-lyte (pH3-10), mineral oil were purchased from Bio-Rad Laboratories Ltd. Tris-base, ammonium persulfate(AP), sodium dodecyl sulfate (SDS), N,N,N',N'-tetramethylethylene diamine (TEMED) and glycin were from Sigma. Acrylamide, N, N' methylenebisacrylamide, Bromophenol Blue, Coomassie Brilliant Blue (CBB) G-250, thiourea, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate (CHAPS), glycerol, bovine serum albumin (BSA) were purchased from Amresco. Agarose and urea were from Solarbio. Dithiothreitol (DTT) and iodoacetamide were from Merk. All other chemicals were from Beijing Chemical Reagents Inc.

Royal jelly samples

100 g of RJ was sampled from the apiary of the Honey Bee Research Institute, Chinese Academy of Agricultural Science in June 2006. 20g of RJ from each of 5 colonies were harvested in sterile bottles when the larvae were grafted into the queen cell cups for 72 hours and a unique sample homogenized and subdivided into the different aliquots to be subjected to the various storage conditions of -20°C, 4°C and room temperature for 30 days and for 80 days, respectively.

Preparation of protein samples

The approach of sample preparation was slightly modified according to Zhong et al (Zhong *et al*., 2005). The RJ (1mg RJ/10µl buffer) was mixed in phosphate buffer (PB) pH 7.6, containing 32.5mM K_2HPO_4, 2.6mM KH_2PO_4, 400mM NaCl. The mixture was homogenized for 5 minutes in ice and sonicated for 2 min, then centrifuged at 12,000g and 4°C for 10 minutes, further centrifuged at 15,000g and 4°C for 10 minutes. The supernatant was removed to another tube for use. The pellets (1mg RJ/2µl buffer) were mixed in the PB pH 7.6, and then centrifuged at 15,000g and 4°C for 10 minutes. The supernatant was removed and mixed into a tube containing supernatant as a PB-soluble protein extract, while the pellets (1mg RJ/10µl buffer), PB-insoluble proteins, were mixed in lysis buffer (8M urea, 2M thiourea, 4% CHAPS, 20mM Tris-base, 30mM DTT, 2% Bio-lyte pH 3-10), then the mixture was sonicated for 2 minutes, centrifuged at 15,000g and 4°C for 10 minutes. The supernatant was removed and mixed into the tube containing the PB-soluble protein extract, 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 minutes for precipitating proteins and desalting. Subsequently, the mixture was twice centrifuged at 15,000g and 4°C for 10 minutes. The supernatant was discarded and the pellets (1mg RJ/4µl buffer) were resolved in foregoing lysis buffer, then the mixture was homogenized for 5 minutes in ice and sonicated for 2 minutes, subsequently adjusted to pH 7.0 with 2M NaOH.

The mixture, the protein extract of the RJ, was stored at -70°C for further use. The protein concentration was determined according to the method of Bradford (1976) using bovine serum albumin as reference.

2-DE

310 µg of protein sample was suspended in LB and then mixed with rehydration buffer (containing 8mol/L urea, 2% CHAPS, 0.001% Bromophenol Blue, 45mmol/L DTT, 0.2% Bio-lyte, pH3-10). The mixture was loaded on a 17cm IPG strip (pH3-10, linear, Bio-Rad Hercules, CA, USA). IEF was performed at 18°C (PROTEAN IEF Cell, Bio-Rad Hercules,CA, USA) according to the following program: active rehydration for 14 h at 50V; 250V for 30 min x4 times; 1 000V for 60 min; 9 000V for 5h; 9 000V for 60 000V·h. Before SDS-PAGE, the IPG strips were first equilibrated for 15 minutes in equilibration buffer 1(6M urea, 0.375M Tris-HCl (pH 8.8), 20% glycerol, 2%SDS, 2% DTT) and then continued in equilibration buffer 2 (6M urea, 0.375M Tris-HCl (pH 8.8), 20% glycerol, 2%SDS, 2.5% iodoacetoamide) for 15 minutes. After the equilibration, the strip was transferred to SDS-PAGE gel, 12% T separating gel (1.00 mm). Meanwhile, 10µl of 2-DE marker was loaded into 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 PROTEAN® xi Cell (Bio-Rad Hercules, CA, USA) at 25mA/gel for 6.5 hours. The gels were stained with CBB G250 and scanned with transparent model, then analysed with PDQuest V 7.3.0 (Bio-Rad Hercules, CA, USA) (sensitivity 6.86, scale 9). Each sample was replicated five times and the best three with good reproducibility were subjected to analysis. ANOVA (Version 6.12, SAS Institute,

Cary, N.C., USA) was used to compare the mean normalised volume of selected spots in four treatments. In all statistical analysis a probability of $P<0.05$ was considered to be statistically significant.

Results

2-DE images of RJ proteome stored under different conditions

2-DE of each protein sample was performed five times to obtain results with high reproducibility. The total detected protein spots were 75 at -20°C (*Fig.1 d*) for 80 days, 45 at 4°C (*Fig.1 c*), 63 at room temperature for 30 days (*Fig.1 a*), and 69 at room temperature for 80 days (*Fig.1 b*), respectively, with molecular weight ranging from 7.64-72.33kDa and pH 4.95-8.70.

Comparison of apalbumins in RJ stored under different conditions

The proteins identified were compared to those proteins already identified in the proteome complement of the RJ (Scarselli *et al*., 2005; Sano *et al*., 2004). The molecular weight of apalbumin 1-5 ranges from 24.05 to 68.32kDa, with pH 5.17-8.28 (*Table 1*). Apalbumin 1 in all images was a saturated spot (SSP 0406) with no statistical significance (*Table 1, Fig.2*). The volume of apalbumin 2 and apalbumin 3 was irregular under different storage conditions (*Table 1, Fig. 3, Fig. 4*): volume of 18 spots were at -20°C or just appeared at -20°C; volume of 13 spots were significant higher at 4°C or appeared only at 4°C; volume of 5 spots were significant higher or appeared only at room temperature for 30 days, and volume of 14 spots were significant higher or appeared only at room temperature for 80 days (*Table 1*). In contrast, 7 spots of apalbumin 4 (*Table 1, Fig. 5a*) and 3 spots of glucose oxidase (*Table 1, Fig. 6 a*) were clearly detected only at -20°C. 3 spots of apalbumin 5 were detected at 4°C (*Table 1, Fig. 6 b*), and 6 spots of apalbumin 5 were detected at -20°C (*Table 1, Fig.6 a*), no spots of apalbumin 5 were detected at room temperature (*Table 1, Fig. 6 c, d*).

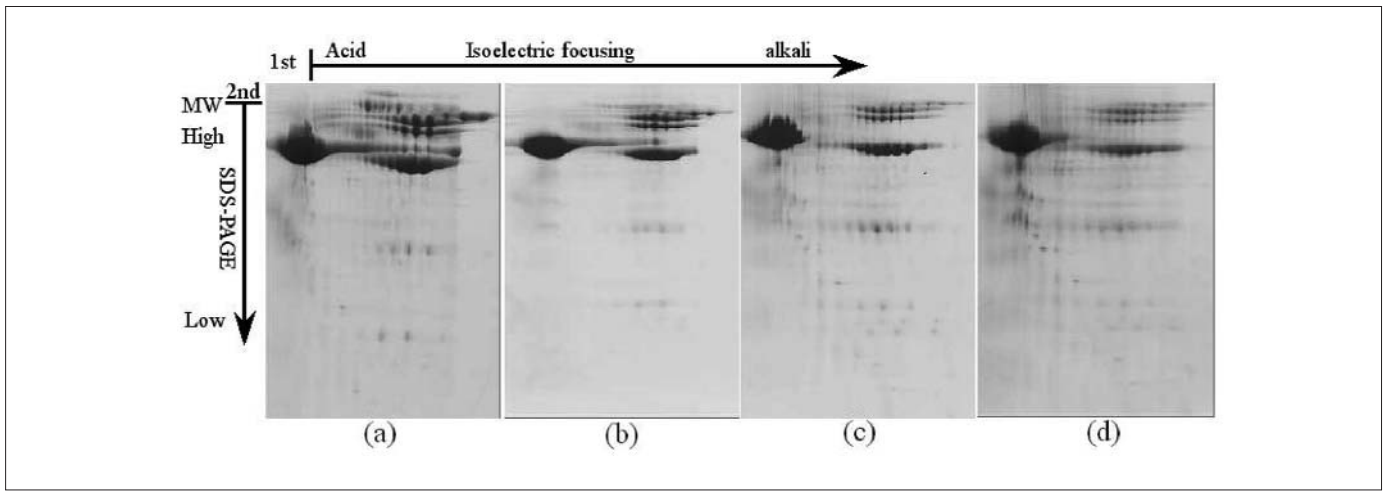

Fig. 1. Representative 2D electrophoresis protein gel of RJ proteome under different storage conditions. 310 µg of each sample was subjected to 2-DE and stained by CCB G-250. (a), (b), (c) and (d) represent 2-DE profile of RJ proteome stored at -20°C for 80 days, 4°C for 80 days, at room temperature for 30 days, and at room temperature for 80 days, respectively.

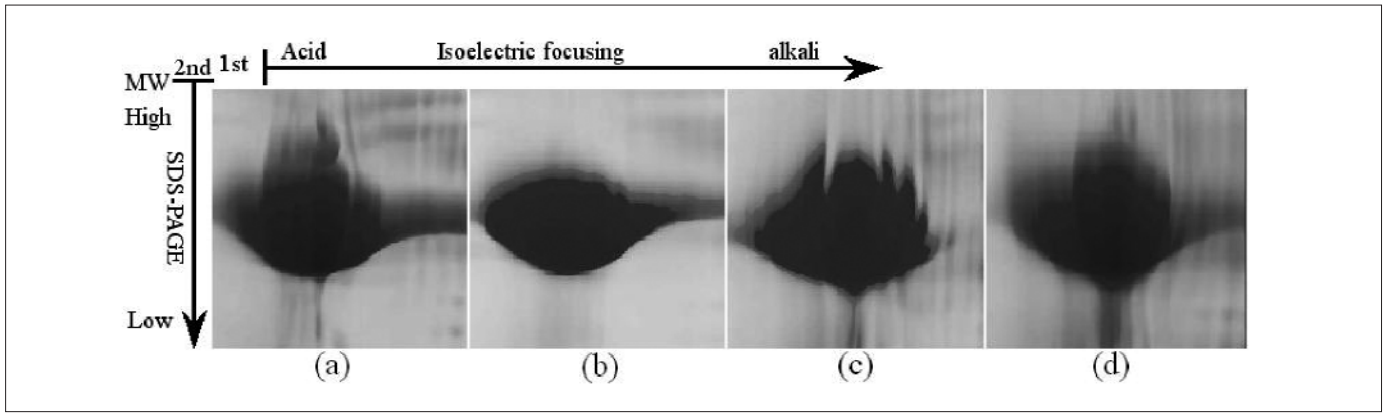

Fig. 2. Apalbumin 1 in all gels (cropped from the corresponding whole image in Fig. 1). 310 µg of each sample was subjected to 2-DE and stained by CCB G-250. (a), (b), (c) and (d) represent the image of apalbumin 1 in RJ proteome stored at -20°C for 80 days, 4°C for 80 days, at room temperature for 30 days, at room temperature for 80 days, respectively.

Table 1. Quantity of apalbumins in all gels.

SSP	Protein Name	Mr (kDa)	pI	Quantity (PPM)			
				-20°C 80	4°C 80	RT 30	RT 80
0406	"apalbumin1"	49.42	5.17	282971.5[a]	261491.5[a]	329834.3[a]	253885.3[a]
1406	"apalbumin2"	45.37	6.15			4150.9[a]	7835.5[b]
2302	"apalbumin2"	44. 79	6.42			6651.8[a]	18880.0[b]
2403	"apalbumin2"	45.17	6.27	4209.7[b]	7729.2[c]	1825.2[a]	1805.6[a]
2903	"apalbumin2"	66.65	6.47	4360.9			
2904	"apalbumin2"	65.62	6.40		5364.3		
3101	"apalbumin2"	24. 28	6.60	1011.4[a]	564.6[b]	7096.9[c]	5217.2[b]
3301	"apalbumin2"	44.14	6.57	15568.0[b]	3573.6[a]	14008.5[b]	3456.7[a]
3304	"apalbumin2"	43.99	6.69	19496.7[c]	3505.4[a]	3367.1[a]	13001.0[b]
3902	"apalbumin2"	66.07	6.71		4090.1		
3908	"apalbumin2"	65.78	6.53		3471.3		
3910	"apalbumin2"	63.96	6.71	1977.0			
3911	"apalbumin2"	64.81	6.58	1621.2			
4103	"apalbumin2"	24.05	6.84	1988.5[b]	680.6[a]	6372.9[d]	4610.7[c]
4202	"apalbumin2"	43.80	7.00	2537.4[b]	3571.3[c]	3381.7[c]	14295.3[a]
4203	"apalbumin2"	43.80	6.85	2520.2[a]	3495.2[b]	3378.9[b]	12325.8[c]
4903	"apalbumin2"	66.95	6.98		1536.6		
5101	"apalbumin2"	24. 09	7.10	2376.0[b]	907.1[a]	3526.7[c]	3811.3[c]
6202	"apalbumin2"	43.80	7.27	2484.6[a]	3689.4[b]	3474.2[b]	17881.4[a]
7101	"apalbumin2"	24.12	7.51	1850.9[b]	538.1[a]	2489.8[c]	3178.9[c]
7302	"apalbumin2"	43.99	7.52	14930.5[b]	31143.2[a]	3517.9[a]	6173.0[b]
8301	"apalbumin2"	44. 90	7.83		9032.7[a]	8555.1[a]	23752.0[b]
9403	"apalbumin2"	45. 37	8.22			1052.5[a]	8066.4[b]
2602	"apalbumin3"	56.88	6.42			1765.1[a]	1983.6[a]
2603	"apalbumin3"	57.57	6.49	1899.5			
2702	"apalbumin3"	60. 39	6.42			1635.0[a]	2151.4[a]
2705	"apalbumin3"	58.33	6.36	1718.7			
2706	"apalbumin3"	58.33	6.26	1407.0			
2906	"apalbumin3"	61.32	6.22		1470.7		
2908	"apalbumin3"	62.03	6.24	312.5			
2910	"apalbumin3"	61. 76	6.35	1210.7			
2911	"apalbumin3"	61. 76	6.47	1654.0			
3601	"apalbumin3"	56.21	6.54			3080.2[a]	2502.3[a]
3602	"apalbumin3"	55.73	6.67	2025.7[a]	4470.1[b]	5176.6[c]	4211.5[b]
3603	"apalbumin3"	55.42	6.79	6592.3[b]	4135.5[a]	5183.0[b]	3332.7[a]
3701	"apalbumin3"	59.75	6.54			2524.8[a]	3579.3[b]
3702	"apalbumin3"	59.42	6.67	1248.0[a]	3011.7[b]	7265.7[b]	4322.5[b]

4601	"apalbumin3"	55.38	6.92	8893.9[a]	9916.5[b]	11265.8[c]	7849.9[a]
4701	"apalbumin3"	58.81	6.79	4926.1[a]	10551.0[c]	4387.0[a]	5333.9[b]
4702	"apalbumin3"	58.81	6.93	13038.8[b]	24270.8[c]	9568.8[a]	9203.3[a]
4905	"apalbumin3"	62.21	6.97				1982.1
5601	"apalbumin3"	55.44	7.10	11761.9[c]	3334.2[a]	7433.9[c]	3964.6[a]
5701	"apalbumin3"	58.79	7.10	23479.5[c]	3570.6[a]	6356.0[b]	6460.6[b]
5902	"apalbumin3"	62. 06	7.13			1168.6[a]	2301.2[b]
5904	"apalbumin3"	62.96	7.14		2474.1		
6601	"apalbumin3"	55.52	7.33	9950.6[c]	2553.9[a]	7916.3[b]	3972.7[a]
6702	"apalbumin3"	59.05	7.34	19585.6[b]	23753.4[c]	6275.6[a]	5272.8[a]
6901	"apalbumin3"	62.02	7.38			1517.3a	2717.7[b]
7601	"apalbumin3"	55. 47	7.59	15064.5[b]	21691.0[c]	2258.2[a]	3358.1[a]
7701	"apalbumin3"	58.90	7.60	13216.1[b]	11721.7[b]	3500.3[a]	4112.3[a]
7902	"apalbumin3"	61.6	7.64	11848.2[c]	12325.9[c]	867.9[a]	2967.6[b]
8602	"apalbumin3"	56.27	7.91			1192.6[a]	2220.1[b]
8703	"apalbumin3"	59.66	7.92			1266.3[a]	2149.4[b]
8704	"apalbumin3"	59. 46	7.94		5719.2		
8902	"apalbumin3"	61.82	7.94	21249.8[c]	12701.0[b]	1560.7[a]	1855.2[a]
9901	"apalbumin3"	61.77	8.28	8639.8[c]	1538.3[a]	2256.2[b]	2308.3[b]
1602	"apalbumin4"	55.54	6.14	5895.4			
1603	"apalbumin4"	56.07	6.02	1111.2			
1604	"apalbumin4"	56. 56	5.91	2333.8			
1701	"apalbumin4"	59.10	5.71	1447.6			
2501	"apalbumin4"	55.06	6.20	2476.9			
2502	"apalbumin4"	54.57	6.28	4116.9			
2506	"apalbumin4"	55.09	6.37	974.0			
1903	"apalbumin5"	67.24	6.08		2204.4		
1905	"apalbumin5"	63.13	5.89	930.7			
1906	"apalbumin5"	62.85	6.11	841.4			
1907	"apalbumin5"	68.02	5.98	1314.5			
1908	"apalbumin5"	68.32	6.07	1377.0			
2901	"apalbumin5"	66.95	6.23	10979.0			
2902	"apalbumin5"	66.95	6.36	5928.0			
2905	"apalbumin5"	66.49	6.30		2220.8		
2907	"apalbumin5"	66.65	6.19		1143.4		
2909	"glucose oxidase"	72.33	6.40	2711.2			
3907	"glucose oxidase"	71.64	6.55	1455.7			
3909	"glucose oxidase"	71.38	6.64	828.1			

Note: SSP represents protein spot number that is automatically produced by the Pdquest software; Mr, pI and RT represent molecular weight, isoelectric point, room temperature, respectively. Identical letters at top right of the figures indicate no significant difference; different letters indicate significant differences. PPM, giving units of parts per million in normalizing the spots volume, is a common scaling factor is 106 produced by the Pdquest software.

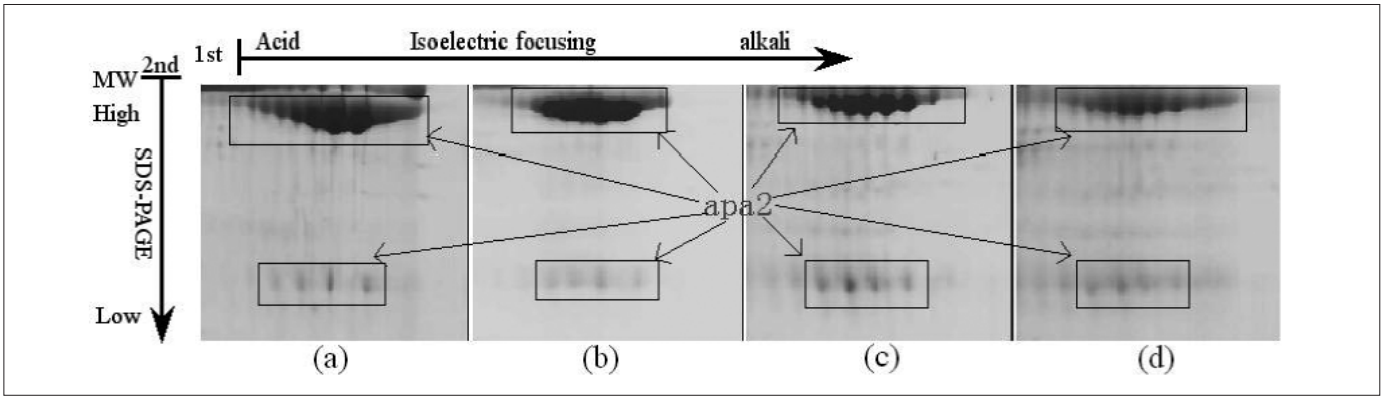

Fig. 3. Apalbumin 2 in all gels (cropped from the corresponding whole image in Fig. 1). 310 μg of each sample was subjected to 2-DE and stained by CCB G-250. (a), (b), (c) and (d) represent the image of apalbumin 2 in RJ proteome stored at -20°C for 80 days, at 4°C for 80 days, at room temperature for 30 days, at room temperature for 80 days, respectively. Apa2 represents apalbumin 2

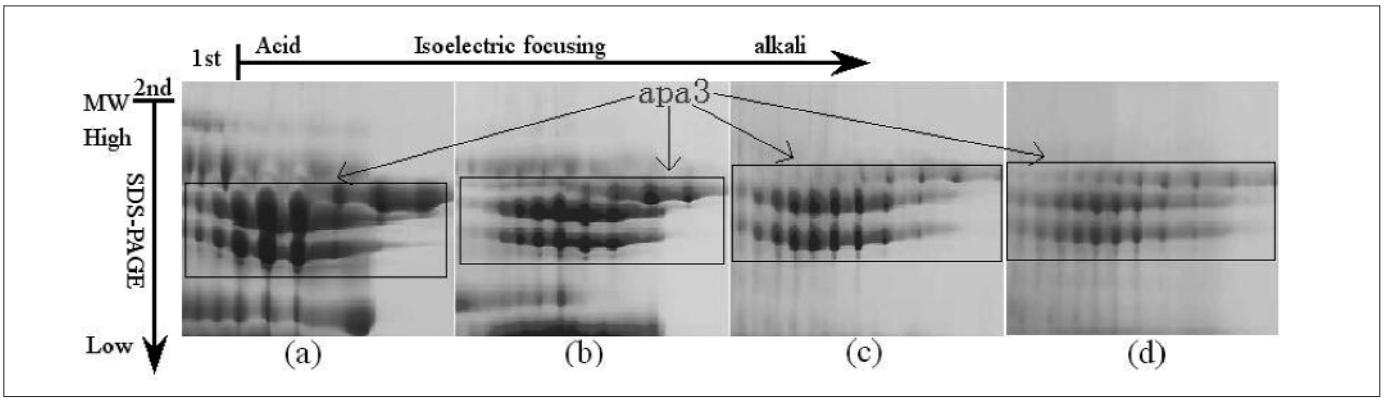

Fig. 4. Apalbumin 3 in all gels (cropped from the corresponding whole image in Fig. 1). 310 μg of each sample was subjected to 2-DE and stained by CCB G-250. (a), (b), (c) and (d) represent the image of apalbumin 3 in RJ proteome stored at -20°C for 80 days, at 4°C for 80 days, at room temperature for 30 days, and at room temperature for 80 days, respectively. Apa3 represents apalbumin 3.

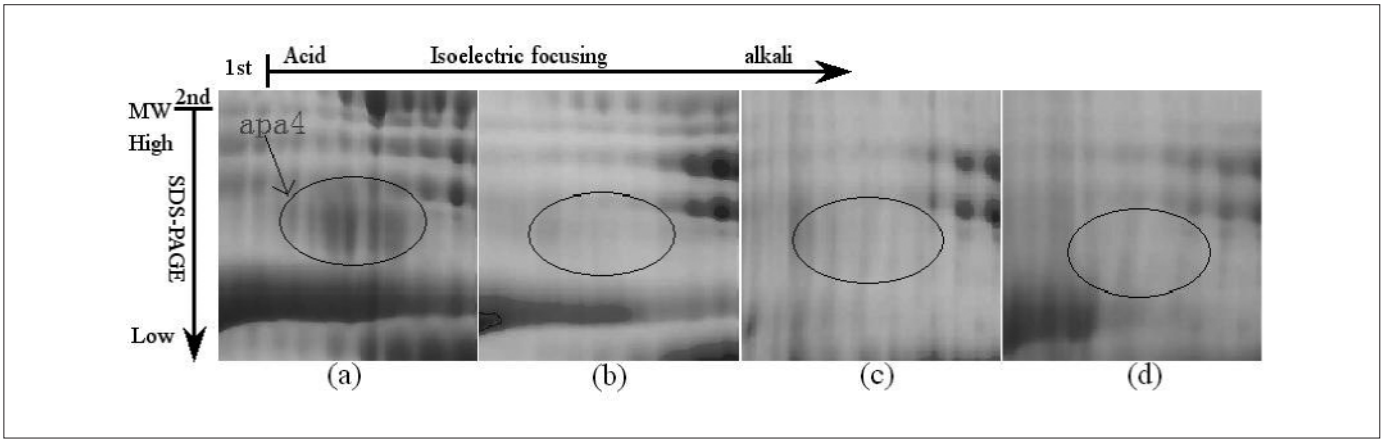

Fig. 5. Apalbumin 4 in all gels (cropped from the corresponding whole image in Fig. 1). 310 μg of each sample was subjected to 2-DE and stained by CCB G-250. (a), (b), (c) and (d) represent the image of apalbumin 4 in RJ proteome stored at -20°C for 80 days, at 4°C for 80 days, at room temperature for 30 days, and at room temperature for 80 days, respectively. Apa 4 represents apalbumin 4.

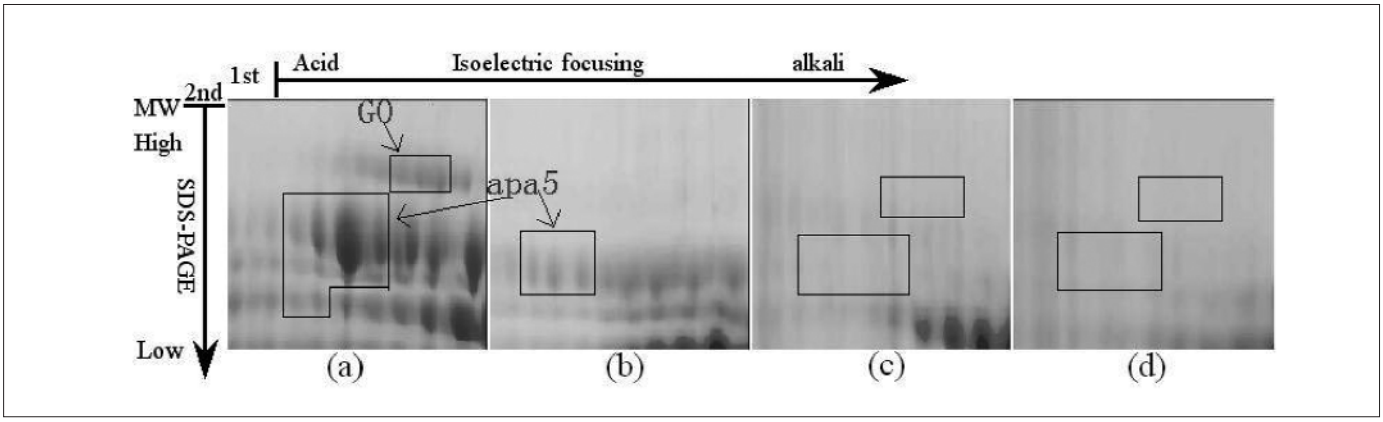

Fig. 6. Apalbumin 5 and glucose oxidase in all gels (cropped from the corresponding whole image in Fig. 1) 310 μg of each sample was subjected to 2-DE and stained by CCB G-250. (a), (b), (c) and (d) represent the image of apalbumin 4 and glucose oxidase in RJ proteome stored at -20°C for 80 days, at 4°C for 80 days, at room temperature for 30 days, and at room temperature for 80 days, respectively. Apa 5 represents apalbumin 5; GO represents glucose oxidase.

Discussion

Most of apalbumin 1-5 in this experiment were within range of molecular weight of 43.80-68.32 kDa (*Table 1*), which was almost consistent to the 49-87kDa reported previously, but there were four spots of apalbumin 2 with lower molecular weight (24.05-24.28 kDa), and their location in images matched to that of Sano *et al* (2004) without pI and Mr information available. The number of total protein spots and some of the spot volumes did not follow a regular trend, showing increases and decreases that were not related to the different storage conditions, suggesting that the present method may need further improvement.

Apalbumin 1 in all of the images was saturated, with no significant differences in volume, indicating it is not sensitive to both storage temperature and time period. The volume of apalbumin 2 and apalbumin 3 detected in all images did not always follow a regular trend. Some of them showed a significant decrease in a regular trend (*Table 1, Fig. 3, Fig, 4*), whilst some were significantly higher at higher temperature (*Table 1, Fig. 3, Fig, 4*) possibly because the proteins polymerized during storage (Chen & Chen, 1995), indicating that these two proteins are also sensitive to storage conditions. Moreover, the spots of apalbumin 2 and apalbumin 3 unique in each of image might be isoforms because of the heterogeneity in both Mr and pH of apalbumins 2 and 3. Apalbumin 1 is likely to promote liver regeneration and may have a cytoprotective action on hepatocytes (Simuth, 2001). Apalbumins 2 and 3 seem to function as a store of a processable form of nitrogen, and apalbumin 3 can exhibit potent immunoregulatory (Albert *et al*., 1999).

In contrast, the spots of apalbumin 4 and glucose oxidase were detected only at -20°C for 80 days (*Table 1, Fig. 5, Fig. 6*), but not under all other conditions, indicating that apalbumin 4 and glucose oxidase are sensitive to storage temperature. Apalbumin 4 is almost consistent to a 57kDa glycoprotein suggested as a freshness marker of RJ (Kamakura & Fukushima, 2002), in terms of its sensitivity to temperature and molecular weight range of 54.57-59.10kDa (*Table 1*). Apalbumin 4 was not detected by 2-DE until 2004 (Sano *et al*., 2004). In the 2D gel of Scarselli *et al* (2005), some spots of which the identity is not reported seem to match those of apalbumin 4 found in this work, possibly because the volume was not sufficient to identify since the IPG strip they used (11cm) was shorter than ours (17cm) even if the RJ was fresh. The RJ used in our experiment was from the apiary of our laboratory, collected from queen cell cups and immediately frozen at -20°C, refrigerated at 4°C and stored at room temperature, which guaranteed the RJ quality. The spots of apalbumin 5 were detected at -20°C and 4°C for 80 days, but not at room temperature for 30 days and 80 days. Furthermore, the volume of apalbumin 5 at 4°C was significantly lower than that at -20°C, indicating that apalbumin 5 is also sensitive to storage temperature, although less than apalbumin 4 and glucose oxidase, so apalbumin 4, apalbumin 5 and glucose oxidase might be used as a reference in the assessment of RJ freshness. Both apalbumin 4 and apalbumin 5 supply nutritive components as essential amino acids (Schmitzova *et al*., 1998; Albert *et al*.,1999), and their activity can be maintained by storing at low temperature due to their sensitivity to temperature.

The results indicate that apalbumin 1 is not sensitive to storage temperature, whereas apalbumin 2, apalbumin 3, apalbumin 4, apalbumin 5 and glucose oxidase are. Apalbumin 2 and apalbumin 3, are, however, not suitable as markers for the freshness of RJ because they can be detected under every condition even with changes in quantity. In contrast, apalbumin 4, apalbumin 5 and glucose oxidase are clearly absent at room temperature, even quantitatively less represented than apalbumin 2 and apalbumin 3. Therefore, apalbumin 4, apalbumin 5 and glucose oxidase could be used as references in the assessment of RJ freshness. Samples of RJ frozen at -20°C represent the best way to maintain quality. The present work should be considered as a preliminary study since the storage period needs to be extended for much longer. In order to further examine the effects of different storage conditions on changes in RJ proteins, 2-DE will be performed immediately after RJ is collected and the total proteins are extracted, and the effect of longer storage times should be investigated. Thus, comparison can be made among the 2-DE images of the RJ proteome over different storage durations, and the sensitivity of RJ proteins not only to storage temperature but also to storage duration can be estimated.

Acknowledgements

This work is supported by the project of research and demontration of bee products security and application, a key project of the national scientific supporting plan of the 11th Five-Year Development (2006BAD06B04), and initiation project found for talent person of Chinese Academy of Agricultural Science.

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