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Comparative proteomic analysis of *Bombyx mori* hemolymph and fat body after calorie restriction

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Calorie restriction (CR) is known to extend life span from yeast to mammals. To gain an insight into the effects of CR on growth and development of the silkworm *Bombyx mori* at protein level, we employed comparative proteomic approach to investigate proteomic differences of hemolymph and fat body of the silkworm larvae subjected to CR. Thirty-nine differentially expressed proteins were identified by MALDI TOF/TOF MS. Among them, 19 were from the hemolymph and 20 from the fat body. The hemolymph of the CR group contained two down-regulated and 17 up-regulated proteins, whereas the fat body contained 15 down-regulated and five up-regulated ones. These proteins belonged to those functioning in immune system, in signal transduction and apoptosis, in regulation of growth and development, and in energy metabolism. Our results suggest that CR can alter the expression of proteins related to the above four aspects, implying that these proteins may regulate life span of the silkworm through CR.

Keywords: *Bombyx mori*, calorie restriction, MALDI-TOF/TOF MS, proteomic analysis, two-dimensional gel electrophoresis

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INTRODUCTION

Calorie restriction (CR) was first described in the 1930s by McCay *et al.* (1935) and was later shown to extend life span in different species from yeast to mammals (Masoro, 2000; Stern *et al.*, 2001; Heilbronn & Ravussin, 2003; Hursting *et al.*, 2003). CR is also known to slow down many physiological aging processes and prevent age-associated pathologies. The mechanisms by which CR acts to slow down aging in laboratory animals is unknown. It may involve modulating the activity of evolutionarily conserved nutrient response pathways, such as those mediated by insulin/IGF-1-like signaling and the target of rapamycin kinase (Sutphin & Kaeberlein, 2008). In insects, bombyxin, also named prothoracicotropic hormone, is the first discovered insect insulin-like peptide. It is secreted by the silkworm brain and has similar structure and the same function as insulin and IGFs in mammals.

Recently, microarrays have been used to measure differences in gene expression after CR applied to organisms, particularly mammals. Cao *et al.* (2001) utilized genome-wide microarrays to examine the life- and health-span-extending effects of CR on gene expression among young and old mice and between long-term CR and short-term CR. Swindell (2008) conducted a comparative analysis of microarray data to collectively examine the effects of CR in 10 different tissues in mouse and identified 28 genes that had common responses to CR. Sharov *et al.* (2008) used DNA microarrays to examine global changes of gene expression patterns in ovaries and testes between a group of mice on *ad libitum* feeding and a group of those on life-span-extending 40% CR. They found that in both ovary and testis, CR caused small and mostly gonad specific effects: suppression of ovulation in the ovary and activation of testis-specific genes in testis.

The above studies reported effects of CR at the mRNA level and many genes were found to be related to life span extention, such as *sir-2*, *daf-2*, *pit-1*, *amp-1*, *clk-1*, *p66Shc*, *hst2* and *pha-4* (Kaeberlein *et al.*, 2006; Wang *et al.*, 2006; Chen & Guarente, 2007). However, there have been few related reports on changes caused by CR at the proteomic level. Proteomic approach provides direct measurement of protein expression levels and insights into the activity of all relevant proteins (Naaby-Hansen *et al.*, 2001). In combination with mass spectrometry (MS) and bioinformatic approaches, two-dimensional gel electrophoresis (2-DE) has been developed into an efficient method to analyze quantitatively complex protein mixtures, offering a powerful tool for understanding complex physiological processes at the protein level.

Up to now, CR has been studied in many species ranging from yeast to mammals, but not in Lepidoptera. The silkworm *Bombyx mori* is a good model organism in Lepidoptera. It is an ideal insect for CR studies owing to their uniformity of growth and development, ease of handling and maintaining, relatively short life cycle and consistency in food and environmental control. The fifth instar is a transition period for silkworm metamorphosis from larva to pupa, and for biosynthesizing and spinning silk, during which larvae take in almost the entire nutrition for the whole life span. Day 3 of the fifth instar has been found to be a boundary for larval development. Most biological processes may be similar before this time point, but after that, the larvae begin to synthesize silk proteins in mass. Thus, the study of this time point will be helpful to elucidate the growth and development of silkworm.

In the present study, we used 2-DE combining with MS to explore the differences in protein expression after CR was applied to silkworm larvae by feeding them at various frequencies.

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Abbreviations: CR, calorie restriction; 2-DE, two-dimensional gel electrophoresis; DTT, 1,4-dithiothreitol; GST, glutathione-*S*-transferase; Hsp, heat shock proteins; IEF, isoelectric focusing; IGF, insulin-like growth factor; MS, mass spectrometry; Ley I-L, Leydig insulin-like protein; Prxs, peroxiredoxins.

MATERIALS AND METHODS

Silkworm larvae. The experimental silkworm strain Qiufeng was maintained in our laboratory. Newly hatched larvae were selected at random and separated into two groups: the caloric restriction group (CR group) and the control group. Feeding was carried out using fresh mulberry leaves for 6 and 24 h per day (hours/day) for the CR group and the control group, respectively (CR group was fed at 8:00–11:00 a.m. and 8:00–11:00 p.m. and the control group was fed day and night). All larvae were kept in a chamber with a controlled temperature of 25±1 °C, relative humidity of 80±3% and a 12-hour light/dark cycle.

Protein sample preparation. On day three of the fifth instar, ten female and ten male larvae were sampled from the two groups. Their hemolymph was collected and kept on ice for about 1 min and sonicated for 5 s with a 10-s breaks to eliminate air bubbles, and then centrifuged 15 min at 12000 × *g* at 4 °C. The supernatant was transferred into another Eppendorf (EP) tube and kept at –80 °C for later use. Fat body was collected by scraping off the epidermis, washed with ice-cold PBS (0.14 M NaCl, 0.003 M KCl, 0.01 M Na_2HPO_4, 0.002 M $KHPO_4$, pH 7.4) twice, blotted with filter paper, and then flash-frozen in liquid nitrogen and kept at –80 °C for later use.

Proteins of the fat body were homogenized in a lysis buffer containing 5 M urea, 2 M thiourea, 4% Chaps, 0.2% Bio-lyte (pH 4–6 and pH 5–7), 40 mM Tris-base, 50 mM DTT, 2 mM TBP, and EDTA-free proteinase inhibitor cocktail. The homogenate was kept for 10 min at room temperature, sonicated in an ice bath for 2 min, and centrifuged at 12000 × *g* for 30 min at 4 °C. The supernatant was transferred to another EP tube.

Protein concentration was determined according to the instruction of Bio-Rad protein assay kit (Bio-Rad). Bovine serum albumin (0, 0.3, 0.6, 0.9, 1.2, 1.5 mg/ml) was used to prepare the standard curve.

Two-dimensional gel electrophoresis. IPG strips (7-cm nonlinear pH 4–7, Bio-Rad) containing 300 μg of proteins were rehydrated in a buffer consisting of 7 M urea, 2 M thiourea, 4% Chaps, 65 mM DTT, 0.2% Bio-Lyte (pH 4–6 and pH 5–7) and 0.001% bromophenol blue, then subjected to isoelectric focusing (IEF) using a Protean IEF Cell (Bio-Rad). Focusing was performed as follows: 50 V for 12 h, 250 V for 25 min, 500 V for 30 min, 1000 V for 60 min, 4000 V for 3 h and then up to 18000 Vh for approx. 4 h. The electric current was controlled not to exceed 50 mA per strip.

After IEF, the IPG strips were equilibrated for 15 min in equilibration buffer I (6 M urea, 2% SDS, 0.375 M Tris/HCl (pH 8.8), 20% glycerol and 2% (w/v) 1,4-dithiothreitol (DTT) followed by 15 min in buffer II (same as buffer I but containing 2.5% iodacetamide instead of DTT).

For second dimension electrophoresis, the strips were transferred onto a 15% polyacrylamide gel (SDS/PAGE) and overlaid with 0.5% agarose. Electrophoresis was performed at 4 °C under 70 V for 30 min and then under 120 V for 1.5 h in Tris/glycine buffer (25 mM Tris, 192 mM glycine, 0.1% SDS).

Protein visualization and image analysis. Gels were fixed overnight in a solution of 45.4% methanol and 9.2% acetic acid and stained with Coomassie Blue G-250 (0.01% w/v) or silver staining. The resulting 2-DE protein patterns were scanned with ScanMaker 9700XL (600 ppi) and analyzed with the PDQuest software 16.0.0.0.

In-gel digestion and protein identification. Protein spots that had interesting patterns of differential expression between the CR and control groups were subjected to identification of proteins using peptide mass fingerprinting (PMF). Protein spots were excised with circular bores 2–3 mm in diameter, and transferred to 1.5 ml EP tubes. Coomassie Blue-stained gel pieces were first destained with 50 μl of 50% 50 mM NH_4HCO_3 and 50% acetonitrile for 10 min, followed by three washes with 50 μl of MilliQ water. The gel pieces were then dehydrated with 100% acetonitrile for 5 min, and dried in a SpeedVac (Thermo Savant, USA) for 30 min. The dried gel particles were rehydrated at 4 °C for 30 min with 2.5 μl/well trypsin (sequencing grade; Promega, Madison, WI, USA) in 50 mM NH_4HCO_3 (20 μg/ml), and then incubated at 37 °C overnight. Peptide mixture (1 μl) was mixed with 1 μl of 10 mg/ml α-Cyano-4-hydroxycinnamic acid (Sigma), spotted on an MTP Anchor Chip (Bruker, Germany) and analyzed by matrix-assisted laser desorption/ionization-time of flight mass spectrometry (ultraflex tof/tof, Bruker, Germany). The mass spectra were interpreted using the Mascot peptide mass fingerprint engine available on the web site (http://www.matrixscience.com) for protein identification.

RESULTS

2-DE patterns of proteins from hemolymph and fat body after CR

There were prominent differences between 2-DE patterns of proteins from the hemolymph and fat body after CR was applied to larvae (Fig. 1). Approximately 180 individual spots with a pI from 5–7 were detected in the hemolymph of the CR and control groups with 2-DE separation using Coomassie Blue G-250 (Fig. 1A, B). Approximately 580 individual spots with a pI from 5–7 were detected in the fat body using silver stain (Fig. 1C, D). The general distribution pattern of protein spots in these images was almost the same.

Qualitative and quantitative comparisons were made between proteins from the CR and control groups using the PDQuest software. Twenty-two and thirty-two spots were differentially expressed in the hemolymph and fat body, respectively. In the hemolymph, samples of the CR larvae contained 18 up-regulated and four down-regulated spots, and in the fat body, the CR group contained seven up-regulated and 25 down-regulated spots.

Mass-spectrometry identification of differentially expressed proteins

The above 54 spots were excised from 2-DE gels and subjected to in-gel trypsin digestion and subsequent MALDI-TOF/TOF identification. Finally, 39 proteins were successfully identified as shown in Table 1 and Fig. 1, among which 19 spots were of the hemolymph and 20 of the fat body. Spot volume comparisons of the regulated spots (Fig. 2) indicated pronounced difference in amounts of specific proteins present in both hemolymph and fat body between the CR and control groups.

Samples of the hemolymph from the CR group contained two down-regulated proteins, namely arylphorin and 30K lipoprotein, and 17 up-regulated proteins, including imaginal disk growth factor, transferrin, prophenoloxidase, β-1,3-glucan recognition protein, diapause bioclock protein, heat shock cog-

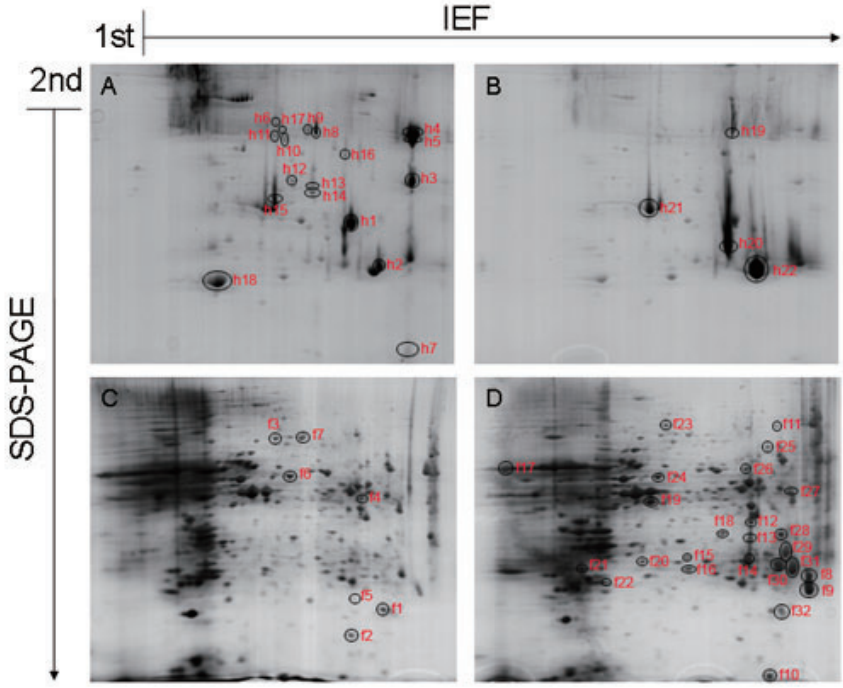

Figure 1. 2-DE pattern of proteins in hemolymph and fat body
(A) represent hemolymph of CR group, **(B)** hemolymph of control group, **(C)** fat body of CR group and **(D)** fat body of control group, respectively. Proteins were applied to IPG strip (7 cm, pH 4–7, NL) and 12 % SDS/PAGE was carried out for separation in the second dimension. The differentially expressed protein spots are marked with a small letter 'h' and 'f' representing hemolymph and fat body, respectively.

nate protein, serine proteinase-like protein, proteinase inhibitors, aminoacylase, leukotriene A4 hydrolase, arylphorin and mature 30K lipoprotein. Samples of the fat body from the CR group contained five up-regulated proteins, including thiol peroxiredoxin and annexin, and 15 down-regulated proteins, including glutathione *S*-transferase, Leydig insulin-like protein, 30K lipoprotein, heat shock protein hsp21.4, calreticulin, glycerol-3-phosphate dehydrogenase-1, antichymotrypsin precursor and 14-3-3zeta.

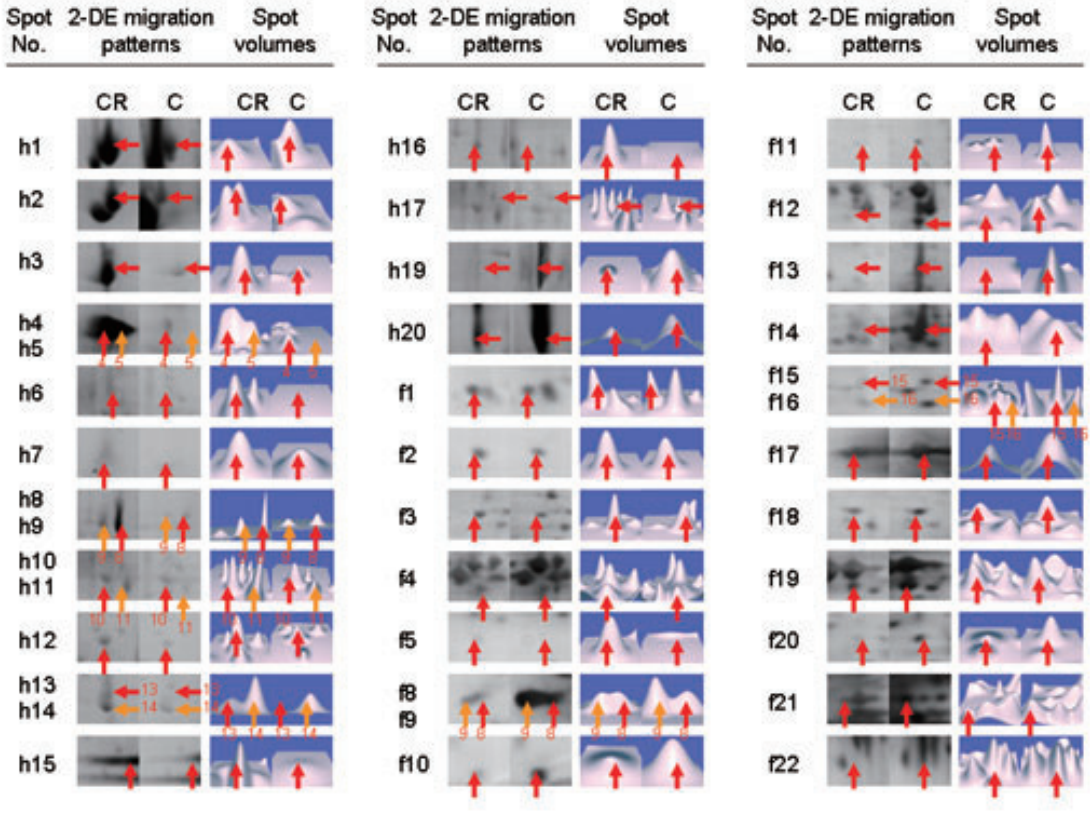

Figure 2. Selected regions of two-dimensional gels and corresponding spot volumes for selected spots in hemolymph and fat body of CR and control group
Image pairs and detection of protein spots with relative spot volumes were achieved using PDQuest software (BioRad).

Table 1. Differentially expressed protein spots identified by MALDI-TOF/TOF

Spot no.	Protein name	Source	Gene no.	pI(cal)	M_r(cal)	Sequence coverage (%)	Score
h1	ribonuclease, Rne/Rng family protein	*Alcanivorax sp. DG881*	gi|196195980	8.04	119194	19	104
h2	hemolymph protein	*Bombyx mori*	gi|187281703	6.23	29776	30	80
h3	imaginal disk growth factor	*Bombyx mori*	gi|152061158	7.64	48362	33	149
h4	transferrin	*Bombyx mori*	gi|112983240	6.89	75674	18	109
h5	prophenoloxidase subunit 1	*Bombyx mori*	gi|112983667	6.25	79305	20	89
h6	β-1,3-glucan recognition protein	*Bombyx mori*	gi|112983972	5.19	56109	24	88
h7	diapause bioclock protein	*Bombyx mandarina*	gi|73671225	6.12	18340	40	71
h8	prophenoloxidase-2	*Bombyx mori*	gi|9957279	5.75	80514	29	177
h9	prophenoloxidase-2	*Bombyx mori*	gi|163838668	5.67	80644	20	106
h10	promoting protein	*Bombyx mori*	gi|112983946	8.37	17698	37	58
h11	heat shock cognate protein	*Bombyx mori*	gi|112982828	5.33	71359	19	90
h12	serine proteinase-like protein	*Bombyx mori*	gi|114052256	5.58	44116	12	41
h13	hemocyte protease	*Bombyx mori*	gi|114050919	5.73	44432	24	84
h14	aminoacylase	*Bombyx mori*	gi|114052174	6.03	46557	33	96
h15	antitrypsin	*Bombyx mori*	gi|112983770	5.41	43528	20	99
h16	masquerade-like serine proteinase homolog	*Bombyx mori*	gi|112983100	4.96	46764	29	84
h17	leukotriene A4 hydrolase	*Bombyx mori*	gi|114051598	5.34	69669	16	90
h19	arylphorin	*Bombyx mori*	gi|124430725	5.70	83569	42	200
h20	mature 30K lipoprotein	*Bombyx mori*	gi|1335608	6.37	28555	50	147
f1	thiol peroxiredoxin	*Bombyx mori*	gi|112982996	6.09	22073	34	81
f2	hypothetical protein BpseP_03000570	*Burkholderia pseudomallei* Pasteur	gi|100063999	5.97	27058	37	85
f3	hypothetical protein Wendof_01000927	*Wolbachia endosymbiont of Drosophila willistoni TSC#14030-0811.24*	gi|99034205	6.44	22395	31	81
f4	argininosuccinate lyase	*Aedes aegypti*	gi|157135840	6.05	53687	16	57
f5	annexin	*Bombyx mori*	gi|10801568	4.89	36105	19	55
f8	glutathione *S*-transferase 2	*Bombyx mori*	gi|112983028	5.98	23596	39	85
f9	glutathione *S*-transferase 2	*Bombyx mori*	gi|112983028	5.98	23596	56	116
f10	NAD-dependent epimerase/dehydratase	*Beijerinckia indica subsp. indica ATCC 9039*	gi|182679463	6.37	39808	19	72
f11	Leydig insulin-like protein	*Mus musculus*	gi|1771292	8.60	13522	20	51
f12	ribonucleic acid binding protein S1	*Bombyx mori*	gi|114050961	11.78	32787	11	39
f13	mature 30K lipoprotein	*Bombyx mori*	gi|1335608	6.37	28555	25	86
f14	mature 30K lipoprotein	*Bombyx mori*	gi|1335608	6.37	28555	29	58
f15	Adenine phosphoribosyltransferase (APRT)	*Drosophila pseudoobscura*	gi|1703349	5.55	20054	41	86
f16	heat shock protein hsp21.4	*Bombyx mori*	gi|112983414	5.79	21391	20	46
f17	calreticulin	*Bombyx mori*	gi|28804517	4.49	46082	24	113
f18	glycerol-3-phosphate dehydrogenase-1	*Bombyx mori*	gi|112983418	5.89	40326	24	59
	glycerol-3-phosphate dehydrogenase-2	*Bombyx mori*	gi|51555848	5.62	39287	24	60
f19	antichymotrypsin precursor	*Bombyx mori*	gi|112983471	5.21	44715	24	84
	chymotrypsin inhibitor CI-8A	*Bombyx mori*	gi|14028769	5.20	43915	20	53

f20	ABC transporter related	*Trichodesmium erythraeum IMS101*	gi|113476749	9.54	27922	29	77
	TPA: TPA_inf: HDC12758	*Drosophila melanogaster*	gi|41618660	4.58	7790	34	48
f21	14-3-3zeta	*Bombyx mori*	gi|124365237	4.90	28193	27	71
f22	alkaline nuclease	*Bombyx mori*	gi|148298778	9.48	51448	17	56

The small letters 'h' and 'f' represent hemolymph and fat body, respectively

DISCUSSION

We studied the growth and development processes of CR group and control group in our previous studies and found that moderate CR extended the life span of silkworm (Li *et al.*, 2009). The amount of ingested leaves by larvae in the control group was 3.18 times that in the CR group. The body mass of larvae increased when the food intake increased, while the larval period shortened. The larval period in the CR group was 1.45 times that in the control group. Compared with the control group, when the food intake was decreased by 69%, the larval stage was extended by 45%.

In this paper, we chose hemolymph and fat body, two important tissues related to the development and metabolism in the silkworm, to further explore the differences in protein expression when CR was applied to larvae. The results showed that the changes of protein abundance caused by CR in the hemolymph and fat body were different. Most of the proteins from the hemolymph of the CR group were up-regulated, but most proteins from the fat body were down-regulated. It indicated that these tissues had different responses to CR, which might be related to different functions of these tissues.

We successfully identified 39 protein spots which represented 33 proteins and 18 of them were considered to be important. These proteins can be classified into five categories, i.e., proteins related to the immune system, to signal transduction and apoptosis, to growth and development, to energy metabolism and to anti-toxicity.

The proteins related to the immune system included β-1,3-glucan recognition protein (spot h6), prophenoloxidase (spots h5, h8 and h9), serine proteinase (spots h12 and h16), proteinase inhibitors (spots h13 and h14) and transferrin (spot h4). β-1,3-Glucan recognition protein, prophenoloxidase, serine proteinase and proteinase inhibitors constitutes the prophenoloxidase (proPO)-activating system (proPO-AS), which is an efficient non-self recognition system in *Bombyx mori*, that can recognize and respond to picograms per litre of lipopolysaccharides or peptidoglycans from bacteria and β-l,3-glucans from fungi. As a result of activation of the proPO-AS, the parasite is blackened in the host hemolymph by deposition of melanin due to the action of phenoloxidase. This response is called the melanization reaction and is easily observed around parasites in the hemolymph (Soderhall & Cerenius, 1998). The proPO-associated proteins in the hemolymph of the CR group were up-regulated, which indicated that proPO-AS had a higher activity under starvation condition and could strengthen the defense against various kinds of micro-organisms. proPO-AS may participate in the regulation of the life span, and the innate immune mechanisms may have inherent relationship with the life span of the silkworm.

In our experiment, transferrin was up-regulated in the hemolymph of the CR group, implying that the up-regulation of transferrin had a close relationship with extension of the silkworm's life span. Transferrin binds iron and transports it in the serum in vertebrates. But recently, transferrin has been hypothesized to participate in insect innate immune response to microbial infection by sequestering iron (Valles & Pereira, 2005). do Nascimento *et al.* (2004) discovered different mechanisms related to iron metabolism in invertebrates. They found that insect ferritin was predominantly a secreted rather than cytosolic protein. A number of other observations have led to the hypothesis holding that the genuine hemolymph iron transporter in insects may be ferritin, leaving transferrin to serve primarily as an antibiotic factor in the defense response. This functional switch from a transporter protein to a component of the insect immune response system makes transferrin one of the candidate determinants of longevity (do Nascimento *et al.*, 2004).

Proteins related to signal transduction and apoptosis included annexin (spot f5), Leydig insulin-like protein (Ley I-L) (spot f11), calreticulin (spot f17), 14-3-3zeta (spot f21), leukotriene A4 hydrolase (spot h17), arylphorin (spot h19), 30 kDa lipoprotein (spots h20, f13 and f14) and heat shock protein (spots h11 and f16). Annexins are a family of Ca^{2+}-binding proteins which bind to acidic phospholipids. A variety of other functions have been found for annexins and these include endocytosis, exocytosis, transmembrane ion channels, inhibition of phospholipase A2, inhibition of blood coagulation, transduction of signals for differentiation or mitogenesis, and regulation of apoptosis, cell–matrix interactions and cell–cell adhesion (Waisman, 1995). Ley I-L is a novel member of the insulin-like hormone superfamily, which comprises insulin, relaxin, and insulin-like growth factors I and II (IGF I and II) (Zimmermann *et al.*, 1997). Calreticulin is an important calcium-binding protein with multiple functions as a lectin-like chaperone in the endoplasmic reticulum, modulating calcium storage and homeostasis, influencing cell adhesion, affecting steroid-sensitive gene expression, mediating immunity between host and parasite and transporting calcium ions during dentin mineralization (Michalak *et al.*, 1999). A number of studies have demonstrated that annexin, Ley I-L and calreticulin have a close relationship with the insulin signal pathway, offering one of the most important hypotheses to explain how CR works (Walker *et al.*, 2005). Biener *et al.* (1996) also found that annexin II had tyrosine phosphorylation activity and was involved in insulin signal transduction. Jalali *et al.* (2008) found that, in mouse embryonic fibroblast cells, the insulin receptor density and activity were up-regulated in the absence of calreticulin function. In our study, annexin was up-regulated in the fat body of the CR group, and calreticulin and Ley I-L were both down-regulated. It seemed to contradict the suggestion that the decrease of insulin signal pathway activity will extend an organisms' life span. This discrepancy can be explained from three aspects**.** First, the exact function of annexin and calreticulin in the insulin signal pathway are still unknown. They may have differ-

ent response in different physiological conditions. Second, most of the studies about these two proteins were carried out in mammals. They may have different physiological function in insects compared with mammals. Last, they may work through another pathway that is still not so clear. However, it can be seen that these two proteins are related to the insulin signal pathway, while whether the traditional insulin signal pathway could explain the life span extension caused by CR needs further research.

14-3-3 protein belongs to a highly conserved family of molecules that regulate intracellular signal transduction and the cell cycle and prevent apoptosis (Fu *et al.*, 2000; Brunet *et al.*, 2002). Arylphorin, an arylphorin-type storage protein rich in phenylalanine and tyrosine (Fujii *et al.*, 1989), is presumed to be used as an amino acid depot required for the development of adult tissues at later stages and the formation of eggs in females (Song *et al.*, 1997; Hwang *et al.*, 2001; Telang *et al.*, 2002; Tungjitwitayakul *et al.*, 2008). The 30 kDa lipoprotein is a specific type of plasma lipoproteins, synthesized in the larval fat body and then released into the hemolymph (Kim & Park, 2003). Leukotriene A4 hydrolase is a soluble enzyme catalyzing hydrolysis of allylic epoxide leukotriene A4 to dihydroxy acid leukotriene B4 (leukotriene A4 hydrolase in human leukocytes), participates in host defense response under pathophysiological conditions such as immediate hypersensitivity and inflammation. Increased leukotriene B4 can induce the expression of TNF2α and IL21β which are two important apoptosis factors, whereas arylphorin and 30 kDa lipoprotein can suppress apoptosis (Rhee *et al.*, 2007). 14-3-3 protein was down-regulated in the fat body of the CR group. Arylphorin and 30 kDa lipoprotein were down-regulated in the hemolymph of the CR group, and the leukotriene A4 hydrolase up-regulated. Our findings suggested that CR enhanced the rates of apoptosis. It was consistent with the report of Hursting *et al.* (2003), which suggested that CR enhanced the rates of apoptosis concomitant with decreases in DNA synthesis, markedly reducing the number and volume of preneoplastic lesions. We conjecture that CR led to the synthesis of 14-3-3 protein, a decrease of arylphorin and 30 kDa lipoprotein, and an increase of leukotriene B4 in the hemolymph, which may weaken the anti-apoptosis effect and accelerate the rate of apoptosis. Thus, the mechanism employed by organisms to clear redundant cells could work well, which may decrease the risk of some lethal diseases.

Heat shock proteins (Hsp) were first discovered as a set of highly conserved proteins whose expression was induced by heat shock. They were found to play an essential role in intracellular "house-keeping" by assisting the correct folding of nascent and stress-accumulated misfolded proteins and preventing their aggregation (Parcellier *et al.*, 2003). There are four major classes of Hsp, namely Hsp90, Hsp70, Hsp60 and small Hsp. Hsp27 (a kind of small Hsp) and Hsp70 can prevent apoptosis, but the effects of Hsp90 and Hsp60 on apoptosis are still ambiguous (Parcellier *et al.*, 2003; Takayama *et al.*, 2003). In this study, we found two kinds of Hsps, Hsp70 in the hemolymph and Hsp27 in the fat body. These two Hsps are both anti-apoptosis proteins. Hsp70 was up-regulated in the hemolymph of the CR group, whereas Hsp27 down-regulated in the fat body. This further indicated that CR can regulate apoptosis and modulate the life span. We have discussed the relationship between apoptosis and CR above. But here we found that this kind of regulation seemed different in various silkworm tissues.

Proteins related to the growth and development included diapause bioclock protein (spot h7) and imaginal disk growth factor (spot h3). Diapause bioclock protein, a metalloglycoprotein, also named protein esterase A4, is a crucial element in the measurement of duration of cold exposure. It has been suggested to serve as a cold-duration clock because its characteristic ATPase activity is transiently elevated at the end of the necessary cold period (Hiraki *et al.*, 2008). Imaginal disk growth factor is a soluble polypeptide growth factor first identified in *Drosophila melanogaster*. Imaginal disk growth factor can promote cell proliferation in imaginal discs and cooperate with insulin in stimulating the growth of imaginal disc cells, which suggests that imaginal disk growth factor might function as a cofactor of *Drosophila* insulin or insulin like peptide (Varela *et al.*, 2002; Zhang *et al.*, 2006). Protein esterase A4 and imaginal disk growth factor were up-regulated in the hemolymph of the CR group, which seemed to indicate that the insulin signal pathway was activated and the life span should be shortened. Actually, it may not be like this. At present, there are still many disputes about the mechanism whereby CR can prolong an organism's life span. The effect of CR is too complex to be described with any single hypothesis ever proposed. We conjecture that these two proteins work together to regulate the life span of silkworms through another mechanism that we are still not very clear about.

Proteins related to energy metabolism included glycerol-3-phosphate dehydrogenase (spots f18). A glycerol phosphate shuttle moves the cytosolic redox carrier NADH against its gradient and across the mitochondrial membrane for the respiration, ensuring productivity of the respiratory electron transport chain. It requires the oxidoreductase activity of glycerol-3-phosphate dehydrogenase, which catalyzes the conversion of glycerone phosphate and NADH into *sn*-glycerol 3-phosphate and NAD^+. The up-regulation of glycerol-3-phosphate dehydrogenase in the fat body of *ad libitum*-fed silkworms compared with the CR group suggested that the exuberant metabolism and increased energy requirement in the control group promoted the efficiency of oxidative phosphorylation and NADH transportation. So far, it has been suggested that glycerol-3-phosphate dehydrogenase, as a critical oxidireductase in the central junction of respiration, glycolysis and energy metabolism, functions as a regulator of CR-mediated life span extension in the silkworm, but the underlying mechanism remains to be investigated.

Proteins related to anti-toxicity comprised thiol peroxiredoxin (spot f1), glutathione-*S*-transferase (spots f8 and f9) and aminoacylase (spot h14). Peroxiredoxins (Prxs) constitute a large family of antioxidant proteins identified in a variety of prokaryotic and eukaryotic species. It can eliminate hydroperoxide with thioredoxin as an immediate hydrogen donor. A number of reports showed that Prxs play a particularly central role in the enzymatic removal of reactive oxygen species (ROS). Prx was up-regulated in the fat body of the CR group, which indicates that Prx may play important roles in the life span extension caused by CR. When CR was applied to larvae, the abundance and probably also activity of Prx was increased, more hydroperoxides were eliminated, and the effect of ROS was decreased, all of which are favorable to extension of the silkworm's life span.

Glutathione-*S*-transferase (GST) was down-regulated in the fat body of the CR group. GSTs are a family of phase II detoxification enzymes that catalyze the conjugation of glutathione to a wide variety of endogenous and exogenous electrophilic compounds. GSTs

have been implicated in the development of resistance toward chemotherapy agents, insecticides, herbicides, and microbial antibiotics. Many studies have demonstrated a regulatory role for the π and μ classes of GSTs in the mitogen-activated protein kinase pathway that participates in cellular survival and death signaling (Townsend & Tew, 2003). We conjecture that GST participates in the regulation of silkworms' life span through an apoptosis pathway or by modulating the cell defense against numerous harmful chemicals produced endogenously and in the environment.

Aminoacylases (*N*-acyl amino acid amidohydrolases) catalyze the hydrolysis of *N*-acyl amino acids to yield the corresponding organic acids and amino acids. In mammals, aminoacylases are thought to function in the detoxification of xenobiotic-derived amino-acid derivatives (Story *et al.*, 2001). But untill now, the role of aminoacylases in insects is ill defined at best. We found that aminoacylase was up-regulated in the hemolymph of the CR group. We conjecture that aminoacylase participates in the mechanism of CR extending silkworms' life span *via* its anti-toxicity.

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