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Preparation of protein samples for gel electrophoresis by sequential extraction

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Abstract: Since preparation and solubilization of protein samples are crucial factors in proteome research, the authors established a sequential extraction technique to prepare protein samples from the body wall of the 5th instar larvae of silkworm, *Bombyx mori*. Two kinds of protein samples were obtained from the body wall using the method. Between the two types of samples only about 15% proteins were identical; the majority were different, indicating that more species of proteins could be obtained with the sequential extraction method; which will be useful for preparation of protein samples for proteome study.

Key words: Proteome, Sequential extraction, 2D-electrophoresis, Protein spot match, Amino acid sequence
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INTRODUCTION

With the progress of genome study, more and more gene information was obtained for our improved understanding of the profoundly mysterious aspects of life. However, it was also realized that many questions such as functions of genes cannot be explained with genomic sequences information, because functions of genes are carried out by their coding proteins. The new field of proteome research was born in 1995 to elucidate the functions of genes (Wasinger et al., 1995). In proteomic research, the structures and functions of all proteins in cells or tissues are studied to gain understanding of the splicing style, post-translational modifications, biochemical and physical properties, and biological functions of the gene. All these start with the isolation of protein samples from cells or tissues.

The composition of genes in the genome is relatively stable while the expression of genes varies with the developmental stages of the organism and its physiological conditions. Correspondingly, the protein species vary in different tissues or different stages, indicating the variability of proteomes. In the process of protein separation, it is important to get as many species as possible and maximum amounts of proteins expressed in a specific stage. This paper presents the protein profiles of the silkworm body walls using sequential extraction with phosphate buffer.

MATERIALS AND METHODS

1. Materials

Commercial silkworm varieties (Feng1 × 54A and Xinhang × Keming) were reared to the 2nd or 5th day of the 5th instar with fresh mulberry leaves.

2. Preparation of protein samples by sequential extraction

A chitin-free body wall of silkworm (1 g) was homogenized with 10 ml of phosphate buffer (PB) pH 7.6, containing K_2HPO_4 (32.5 mmol/L), KH_2PO_4 (2.6 mmol/L) and NaCl (400 mmol/L) at 4 ℃. The homogenate was mixed for 20 min and sonicated for 2 min, then centrifuged at 12000 ×g for 10 min at 4 ℃. The supernatants were collected for further centrifugation at 15000 ×g for 10 min at 4 ℃, the resulting supernatants were stored for TCA (trichloroacetic acid) treatment.

The pellets from two centrifugations were combined, resuspended with 0.5 ml of lysis buffer containing 8 mol/L urea, 2% V/V NP-

40, 2% Ampholine pH 3.5-10 and 5% V/V β-Mercaptoethanol and sonicated for 2 min. After centrifugation at 12000 ×g for 10 min at 4 ℃, the supernatants were saved as PB-insoluble protein samples of body wall and stored at −20 ℃ for further use.

TCA (100%) was added to the above supernatant samples to a final concentration of 10%. The mixtures were kept at 4 ℃ for 20 min and then centrifuged at 12000 ×g for 10 min at 4 ℃. The pellets were collected for further centrifugation at 12000 ×g for 1 min at 4 ℃. The resulting pellets were resuspended with 0.5 ml of the above lysis buffer and sonicated for 2 min, then adjusted to pH 7.0 with 2 mol/L NaOH. The solutions served as the PB-soluble protein samples of body wall and stored at −20 ℃ for further use.

3. Gel electrophoresis

The protein samples were subjected to 2D-PAGE as previously described (Zhong 1997a). Non-linear gradient gel electrophoresis (O'Farrell system, pH 3.5-10) was used in the first dimension. Sodium dodecyl sulfate-polyacrylamid gel electrophoresis (SDS-PAGE) in the second dimension was performed with 5% stacking and 15% separating gels. The protein samples were diluted 10 times with lysis buffer and 0.1 ml of the solutions was subjected to 2D-PAGE. The gels were stained with Coomassie Brilliant Blue R250 after electrophoresis.

4. Protein analysis

The isoelectric point, relative molecular weight and amount of the protein at each spot were evaluated automatically with Phoretix 2D gel analysis software version 5.01 (Nonlinear Dynamics Ltd) using isoelectric point (pI) and relative molecular weight marker protein kits (Pharmacia Biotech), with which the protein spots were matched automatically. The N-terminal amino acid sequences of proteins were determined by an automatic sequencer (Zhong et al., 1997b).

RESULTS

1. Protein spots match analysis

A total of 92 protein spots from the PB-soluble protein samples and 124 protein spots from the PB-insoluble protein samples of the 2nd day of 5th instar larvae body walls of Feng1 × 54A, were automatically identified on 2D-PAGE gels (Fig. 1). The patterns of the PB-soluble and PB-insoluble protein samples were different, and the results were reproducible. The protein spots match analysis based on isoelectric point (pI) and relative molecular weight showed that only fifteen pairs of protein spots had similarity (Table 1), accounting for 16.3% (15/92) of the spots from PB-soluble protein samples and 12.1% (15/124) from the PB-insoluble protein samples, with average of 14.2%. The majority of the proteins (85.8%) were different between the two types of samples.

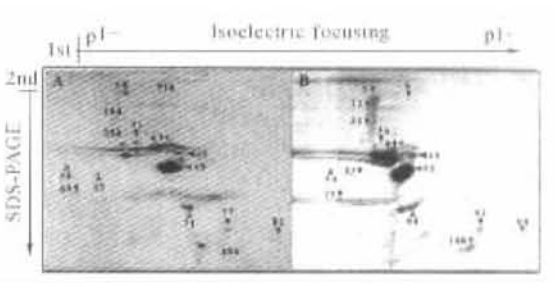

Fig. 1 2D-PAGE pattern of protein from body wall of 2nd day of 5th instar larvae of Feng1 × 54A
A: PB-soluble protein sample; B: PB-insoluble protein sample.

In the fifteen pairs of similar proteins, the protein amount of the coupled spots was different, with some protein amounts being over 10 times those of other couples (such as the couple of PB-soluble protein №9 and PB-insoluble protein №5, and the couple of PB-soluble protein №29 and PB-insoluble protein №22). Only three couples of proteins, №10, №18 and №82 from the PB-soluble protein samples and №6, №12 and №99 from the PB-insoluble protein samples had similar amount. The spots with similar amount was about 3.3% (3/92) of the protein spots from PB-soluble sample and 2.4% (3/124) from PB-insoluble protein samples.

2. N-terminal amino acid sequential analysis

The proteins from the samples of 5th day of 5th instar larvae body wall of Xinhang × Keming were separated by 2D-PAGE. Fourteen dense spots were selected for N-terminal amino acid sequence analysis of proteins (Fig. 2A, cir-

Table 1 Protein spots match analysis of samples of 2nd day of 5th instar larvae body walls of silkworm (Feng1 × 54A)

PB-soluble protein				PB-insoluble protein			
Match No.	pI	M_r(kDa)	Amount	Match No.	pI	M_r(kDa)	Amount
9	5.57	65.4	186	5	5.54	68.2	1984
10	5.34	64.3	31	6	5.24	67.3	39
18	5.61	55.0	56	12	5.60	57.8	64
29	5.62	46.2	543	22	5.62	47.4	47
41	5.52	36.7	1153	36	5.55	38.7	350
44	5.39	35.6	2715	44	5.46	35.9	11921
45	5.19	34.0	946	45	5.30	34.0	157
49	5.30	30.5	7950	52	5.29	30.8	21846
50	5.96	30.5	91	50	5.96	31.9	51
57	5.75	28.5	175	53	5.68	29.7	35
69	5.89	24.9	532	75	5.86	25.4	287
71	5.08	23.7	5375	80	5.19	24.3	6391
77	4.49	21.9	2194	92	4.48	22.0	2591
82	3.72	21.3	466	99	3.98	21.5	466
86	4.57	20.9	733	108	4.57	20.6	3076

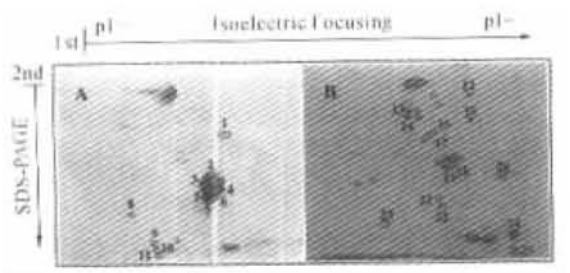

Fig. 2 2D-PAGE pattern of protein from body wall of 5th day of 5th instar larvae of Xinhang × Keming

A: PB-soluble protein sample; B: PB-insoluble protein sample

cled spots) from the PB-soluble protein samples, and yielded eleven sequences (Fig. 2A, numbered spots). Analysis of the PB-insoluble protein samples, 28 dense spots' N-terminal sequences (Fig. 2B, circle spots) revealed the amino acid sequences of fourteen proteins (Fig. 2B, numbered spots). Comparative analysis of amino acid sequences showed that №1 protein from the PB-soluble protein samples was identical to №15 protein from the PB-insoluble protein samples. №2, 3 and 4 proteins from the PB-soluble samples were identical to №18 and 19 from the PB-insoluble samples (Table 2 (see next pape)). Other proteins had no similarity in amino acid sequence between two types of protein samples. Only 4 spots from the PB-soluble protein samples and 3 spots from the PB-insoluble protein samples were identical, accounting for 36.4% (4/11) of the analyzed proteins from the PB-soluble protein samples and 21.4% (3/14) from the PB-insoluble protein samples respectively, with the average of 28.9%.

Table 2 Structural characterization of the proteins from 5th day of 5th instar silkworm larvae body walls (Xinhang × Keming)

No	pI	M_r (kDa)	N-terminal sequence	No	pI	M_r (kDa)	N-terminal sequence
PB-soluble proteins				PB-insoluble proteins			
1	5.08	51.9	DVFFEEKFPDDSXESNKVYSEAPGK DFEXK	12	4.98	71.8	SPVISYDKT
2	5.25	34.3	KDNAMDKADTCEQQARDANL	13	5.60	55.3	ALGKFKN
3	5.36	31.6	KDNAMDKADTCEQQARDANL	14	5.61	51.5	SKYESEGVARSEEL
4	5.22	29.0	KDNAMDKADTCEQQARDANL	15	4.93	50.2	DVFFEEKFPDDSXES

continued in the next page

PB-soluble proteins				PB-insoluble proteins			
5	5.34	26.5	RAALCEQQAKDANLRAEKAE	16	5.37	42.0	QEVXQAFADAGRKPI
6	5.20	26.5	DAMMNQAVXE	17	5.40	40.8	PEVHQAFADAGQKPG
7	5.27	25.8	AEKAEEQARQLQKKIQTVEN	18	5.15	31.5	KDNAMDKADTCEQQARDANL
8	6.48	25.1	GRKFVVGGNXKMNGDKNXIIEIVV	19	5.21	30.2	KDNAMDKADTCEQQARDANLRAE KVNEXVXXL
9	6.23	25.1	PAKAVCVLRGDVSGT	20	4.61	28.6	VVXXQTLXDXRGIYGDQQGSIGPXXI XGLQGDRDAD
10	6.21	20.5	PARNKDQEQEVLTXI	21	5.34	25.0	IVLDSGDGVSXTV
11	6.20	19.0	SKSLFYQKQYDNINE	22	5.34	24.6	IVLDSGDGVSXTVPI
				23	6.24	22.9	VQDGNPPDDNVFXAFCNALYNTVD
				24	4.49	21.9	GSNVFSMFSQKQVAEFKEAFQL MDADKDGI
				25	4.48	21.0	KNDLRAIFDSLGRLASXKXL

DISCUSSION

In proteome study, one important work is to improve protein solubility to get more protein spots. Thiourea solution can improve protein solubility (Fialka et al., 1997; Pasquali et al., 1997; Rabilloud et al., 1997). Traditional buffers use surfactant such as Triton X-100 or Nonidet P-40 (Rabilloud 1996), but CHAPS was more commonly used recently (Rabilloud et al., 1997). Some experiments showed that SB 3-10 (N-decyl-N, N-dimethyl-3-ammonio-1-propanesulfonate) was better than CHAPS in improving sample solubility (Gianazza et al., 1987; Rabilloud et al., 1990). Chevallet (1998) designed several kinds of ionic surfactant such as ASB-n, BisASBn, TriSBn and CnΦ to improve the solubility of membrane proteins (Chevallet et al., 1998).

Sequential extraction was proved to be an effective method for sample preparation. Molloy (1998) used Tris-base buffer to solubilize proteins, the resultant pellet was then subjected to conventional solubilizing solutions containing CHAPS and DTT (dithiothreitol), and then the pellet was treated again with a solution containing CHAPS, TBP (tributyl phosphine), SB 3-10 and thiourea. Five new proteins were obtained with this method (Molloy et al., 1998). In other experiments, good separation was also achieved by using the sequential extraction technique with solubilizing solution containing thiourea, CHAPS, SB 3-10, TBP and thiourea, ASB-14, TBP respectively (Molloy et al., 1999). Use of a solubilizing solution containing Triton X-100, DTT and CHAPS as the first buffer, and solution containing Triton X-100, thiourea, C8Φ as the second buffer also resulted in good separation (Santoni et al., 1999).

Phosphate buffer is commonly used in protein extraction. In general, proteins were extracted with phosphate buffer, subjected to TCA treatment and then solubilized with lysis buffer (Zhong 1997a; Zhong et al., 1997b; Zhong, 1999). In this study, we used the general protocol to get PB-soluble proteins from the supernatant samples. The PB-insoluble proteins were extracted from the pellets with a lysis buffer. Many protein spots were obtained from the two types of samples.

In the process of protein spots match analysis, №2, 3 and 4 from the PB-soluble or №18 and №19 from the PB-insoluble protein samples were analyzed as one protein spot. But in the process of N-terminal amino acid sequence analysis, the big spots were divided into two or three parts. The results of N-terminal amino acid sequence analysis indicated that all of those parts were identical. The identity rate in the two types of protein samples, the PB-soluble and PB-insoluble, would be only 22.2% (2/9) and 15.2% (2/13) respectively, with average of 18.8%, if the identical proteins were calculated as one protein. These results were similar to the results of protein spots match analysis. On average, about

85% of proteins were different between the two types of samples. In the 15% identical proteins, only 2.9% were similar in amount. It indicated that the solubility of proteins differs greatly in phosphate buffer. Some protein samples were very soluble in phosphate buffer while others were not. Proteins with medium solubility in phosphate buffer could be found in both types of protein samples. This mean that only parts of protein spots could be obtained from the supernatant; and that other protein spots were from the PB-insoluble protein samples.

The sequential extraction method established in this study can improve the recovery of more protein spots. Two types of protein samples were obtained with sequential extraction and overlap of protein spots in electrophoretic patterns will be reduced, which would make the sequential work a lot easier. Protein spots match analysis showed that similar species and amount of proteins can be obtained using both the Molloy's method (Molloy et al., 1998) (data not shown) and our technique. Protein extraction from the body wall of silkworm is more difficult than from other organs. This method could extract protein samples from silkworm body wall more completely. Thus, good protein extraction can be anticipated when it is applied on other organs.

In summary, the method established in this work has the advantage of fewer steps and easier operation in sample handling. It could be a good protocol for protein sample preparation for proteome study, and will be useful for analysis of the patterns of gene expression.

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