

## Phosphorylation of 30K Proteins in Silkworm Hemolymph under Heat Shock Conditions: Post-print

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### Abstract

This study aimed to investigate the expression changes of phosphorylated proteins in silkworm hemolymph under heat shock conditions, providing a reference for in-depth functional studies. The experiment consisted of a control group and a heat shock group, with 30 silkworm larvae at the 3rd day of the 5th instar selected for each group (half male and half female), subjected to heat shock treatment at 40 °C for 0 and 10 min, respectively. Differentially expressed phosphorylated proteins were then analyzed using two-dimensional electrophoresis, Pro-Q Diamond staining, and mass spectrometry. The results showed: 1) A large number of proteins accumulated in the 30 ku region of the silver-stained gel, and Pro-Q Diamond staining revealed that they were phosphorylated proteins. 2) After multiple sampling and mass spectrometry identification, three 30K proteins were ultimately obtained, namely PBMHPC-6, PBMHPC-19, and PBMHPC-12. 3) After 10 min of heat shock treatment, the expression levels of the three 30K proteins were significantly upregulated compared with those at 0 min of heat shock treatment ( $P < 0.05$ ). It was concluded that the hemolymph of 5th instar silkworms is rich in 30K proteins, which not only play important roles in lipid transport but are also likely involved in heat shock response.

### Full Text

## Study on 30K Protein Phosphorylation in Silkworm Haemolymph under Heat Shock

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## Abstract

This study investigated the expression changes of phosphorylated proteins in silkworm haemolymph under heat shock to provide a foundation for further functional analysis. Fifth-instar silkworms (30 individuals per group, half male and half female) were randomly divided into two groups and subjected to heat shock at 40°C for 0 min (control) or 10 min (heat shock group). Differentially expressed phosphoproteins were analyzed using two-dimensional electrophoresis, Pro-Q Diamond staining, and mass spectrometry. The results demonstrated that: (1) protein spots concentrated in the 30 ku region of silver-stained gels were identified as phosphorylated proteins by Pro-Q Diamond staining; (2) three 30K proteins were identified as PBMHPC-6, PBMHPC-19, and PBMHPC-12 through multiple sampling and mass spectrometry; and (3) compared with the control, the expression levels of all three 30K proteins were significantly up-regulated after 10 min heat shock ( $P < 0.05$ ). These findings indicate that fifth-instar silkworm haemolymph is rich in 30K proteins, which not only play crucial roles in lipid transport but may also participate in heat shock responses.

**Keywords:** heat shock; silkworm; haemolymph; 30K proteins; phosphorylation; two-dimensional electrophoresis

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## 1. Materials and Methods

**1.1 Experimental Materials** The silkworm strain Qiufeng was reared on fresh mulberry leaves under standard conditions. Fifth-instar larvae were randomly divided into control and heat shock groups (30 individuals each, with equal sex ratio). The heat shock group was exposed to 40°C for 10 minutes, while the control group remained at room temperature. Haemolymph was collected by cutting the abdominal legs, immediately mixed with 1/10 volume of 10 mM phenylthiourea solution to prevent melanization, and stored at -80°C until use.

**1.2 Protein Extraction** Haemolymph proteins were extracted using a lysis buffer containing 8 mol/L urea, 2 mol/L thiourea, 4% (v/v) CHAPS, 2% (v/v) IPG buffer (pH 3-10), and 30 mmol/L DTT. Samples were vortexed for 30 minutes at 25°C, then centrifuged at 15,000 r/min for 15 minutes at 4°C. Protein concentration was determined using the Bradford method by measuring absorbance at 595 nm, with BSA as the standard. Purified protein samples were stored at -20°C.

**1.3 Two-Dimensional Electrophoresis** 2-DE was performed according to the method of Zhou et al. using the Ettan IPGphor3 IEF system and Ettan DaltSix electrophoresis unit (Amersham Biosciences). First-dimension isoelectric focusing was conducted on 24 cm IPG strips with a linear pH gradient of

3-10. After IEF, strips were equilibrated and subjected to second-dimension SDS-PAGE on 12.5% gels.

**1.4 Image Acquisition and Analysis** Gels were stained with Pro-Q Diamond phosphoprotein stain followed by silver staining. Images were acquired using an Image Scanner 2D (Amersham Bioscience) and analyzed with Image Master 2D Platinum software for spot detection, quantification, and comparative analysis. Protein spot volumes were normalized and statistically evaluated.

**1.5 Mass Spectrometry Identification** Protein spots of interest were excised from gels, destained, and digested with trypsin according to the protocol of Gharahdaghi et al. Peptide mass fingerprinting was performed using an ABI 4700 Proteomics Analyzer. Proteins were identified by searching the NCBI database with GPS Explorer<sup>TM</sup> software.

**1.6 Prediction of Phosphorylation Sites** Fasta sequences of identified proteins were retrieved from NCBI. Phosphorylation sites were predicted using the KinasePhos web server (<http://kinasephos.mbc.nctu.edu.tw/>) with 100% specificity threshold. Potential kinase-specific phosphorylation sites for serine (S), threonine (T), and tyrosine (Y) residues were analyzed according to the method described by Huang et al.

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## 2. Results

**2.1 2-DE Map Analysis of Phosphorylated Proteins** Pro-Q Diamond staining revealed approximately five phosphorylated protein spots in the 30 ku region (designated h1-h5). Based on molecular weight and isoelectric point analysis, spots h4 and h5 exhibited molecular weights of approximately 45 ku and pI values of 4.6, consistent with the characteristic properties of 30K proteins. Quantitative analysis showed that spots h1, h4, and h5 displayed significantly higher intensities than h2 and h3, indicating differential phosphorylation levels among these proteins.

**Fig. 1** Two-dimensional electrophoresis map of phosphorylated proteins from silkworm haemolymph

**Fig. 2** Silver stain map (left) and Pro-Q Diamond stain map (right) for 30K proteins

**2.2 Identification of 30K Proteins** Through repeated sampling and mass spectrometry analysis, spots h1, h2, and h3 were identified as PBMHPC-6, PBMHPC-19, and PBMHPC-12, respectively (Table 1). These proteins showed sequence coverage of 42.5% with 80 matched peptides and high confidence identification scores.

**Table 1** MS identification of 30K proteins in silkworm haemolymph

**2.3 Phosphorylation Analysis of 30K Proteins** Image analysis revealed that all three 30K protein spots showed significant up-regulation after 10 min heat shock compared to the control group ( $P < 0.05$ ). The phosphorylation signal intensities increased by 2.15-, 1.86-, and 1.81-fold for PBMHPC-6, PBMHPC-19, and PBMHPC-12, respectively. According to the criterion that a phosphorylation level change greater than 1.8-fold with a coefficient of variation less than 0.55 indicates differential phosphorylation, these results demonstrate that heat shock induces significant changes in 30K protein phosphorylation.

KinasePhos prediction identified six potential phosphorylation sites in both PBMHPC-6 and PBMHPC-12, and four sites in PBMHPC-19 (Table 2). For PBMHPC-6, six phosphorylation sites were predicted at positions 34, 76, 140, 169, 217, and 239, with five serine/threonine kinase recognition motifs identified (Table 3).

**Table 2** Predicted phosphorylated sites of three 30K proteins**Table 3** Predicted phosphorylated sites of PBMHPC-6

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