帝塚山大学現代生活学部紀要　第2号　1～9（2006）

An Approach for Proteomic Analysis of *Bombyx* Humoral Lectin Related Proteins using Two-Dimensional Gel Electrophoresis

Yasuo KATO

INTRODUCTION

Major advancements are being made in the field of proteomics with mass spectrometric identification of proteins separated by two-dimensional gel electrophoresis (2-DE), currently (FURUTA *et al.,* 2002 ; OHISHI, 2002 ; KONDO, 2004). 2-DE can reveal virtually all proteins present in various cells and tissue at any given time, and prepare respective proteins for protein identification and/or protein structural analysis. High-resolution 2-DE should be capable of preparing sufficient amounts of each protein for such structural analysis methods as amino acid sequencing or mass spectrometry (OHISHI, *et al.,* 2000).

To date, I try the novel technology to fractionate lectin related proteins in the hemolymph of the domesticated silkworm, *Bombyx mori,* by two-dimensional polyacrylamide gel electrophoresis (2-D PAGE), in relation to the investigation of the activation of the *Bombyx* lectin (KATO, 2004, 2005).

Generally, biological activities elicited by lectins include cell agglutination, mitosis, apoptosis and so on. For these activities, animal lectins seem to play an important role in the defense mechanism of the living body, as removal of a foreign matter or a biopolymer which has lost the normal function in the living body (KOTANI *et al.,* 1995 ; FUJITA *et al.,* 1998 ; KAWABATA, 2000 ; FURUTA *et al.,* 2001 ; KAWASAKI, 2001 ; WAGO, 2001 ; KOYAMA *et al.,* 2002 ; UJITA *et al.,* 2002 ; HIRABAYASHI, 2004,).

I showed that a humoral lectin (130 K-glycoprotein) of the *B. mori* played a physiological role on cellular communication throughout the metamorphosis of the *B. mori* (KATO *et al.,* 1994). I also emphasized the possibility that the humoral lectin was produced and activated in the fat body of the *B. mori*, and that it was secreted into the hemolymph (KATO *et al.,* 1998). Moreover, I reported on neuraminidase as the *Bombyx* humoral lectin activating factor (KATO and NAKAMURA, 2000). Meanwhile, I tried to make a study on galactosidase because the active *Bombyx* humoral lectin halted the activity, when it was treated with galactosidase *in vitro* (KATO *et al.,* 1988). The result showed

that both α-galactosidase activity and β-galactosidase activity were present in the hemolymph and the fat body of the *B. mori* (KATO and NAKAMURA, 2001 ; KATO, 2002, 2003).

My purpose in this paper is to approach proteomic analysis of *Bombyx* humoral lectin related proteins by 2-D PAGE using the 2-D minislab system, i.e., to continue with my study to obtain perfect profiles of 2-D PAGE by indicating the electric point and the molecular weight.

This investigation provides useful information for understanding of the activation mechanism and the original role of the lectin protein *in vivo*.

MATERIALS and METHODS

(1) Two-dimensional polyacrylamide gel electrophoresis

Two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) was performed using the 2-D minislab system (Atto Corp.). The proteins were subjected to agar gel for isoelectric focusing (the first dimension) with a linear immobilized pH gradient. Electrophoresis was carried out for 210 min with a constant voltage of 300 V per gel tube. The proteins were then separated by SDS-PAGE (10%) in the second dimension, and electrophoresis was carried out for 90 min with a constant electric current of 20 mA per gel.

After electrophoresis, the slab gel was stained with Rapid Stain CBB (Coomassie Brilliant Blue) Kit (NAKALAI Tesque Inc.) and then destained with distilled water. The Coomassie stained 2-D PAGE gel was interposed between two wet cellophane sheets to be dried in a Rapid Dry Mini type AE-3711 (Atto Corp.).

(2) Preparation of samples

A hybrid race, Shunrei×Shougetu, of the domesticated silkworm, *Bombyx mori,* was used in this experiment. In preparing the samples for this research, hemolymph and fat body were collected. The hemolymph was collected by cutting the pleopods and abdominal legs of the larvae. After centrifuging them at 3,500 rpm for 15 min at 4℃ to remove hemocytes, the resulting supernatant was lyophilized. Meanwhile, the fat body was collected from the dissected larvae, washed carefully in a cold 0.7% NaCl solution to remove hemocytes and homogenized in a glass homogenizer with a Teflon pestle. After centrifuging them at 3,500 rpm for 15 min at 4℃, the resulting supernatant was lyophilized.

Gel filtration was performed using a Superdex 200 column (2.6×60 cm, Amershan Pharmacia Biotech Ltd.) equilibrated with 0.1 M Tris-HCl buffer (pH 8.0) containing 0.1 M NaCl, at a constant flow rate of 150 m*l*/hr. The effluent was collected in 5 m*l* fractions and measured at 280 nm with a Shimadzu spectrophotometer type UV 1200. Each frac-

tion was obtained from the hemolymph or the fat body by this method.

RESULTS and DISCUSSION

Initially, the comparative study was performed on pH range for isoelectric focusing (the first dimension). Isoelectric point Kit proteins were subjected to agar gel for isoelectric focusing (the first dimension) with a linear immobilized pH gradient. Namely, the comparison of the profiles using gel of pH range 5-8 and gel of pH range 3-10 showed that clearer profile was obtained using the gel of pH range 5-8 rather than the gel of pH range 3-10 (data not shown). Accordingly, I tried to estimate the isoelectric point of each sample protein using the gel of pH range 5-8. Table 1 summarizes the relationship of molecular weight and Rf (Rate of flow) value of six marker proteins on SDS-PAGE (the second dimension). Accordingly, I tried to estimate the molecular weight of each sample using the relationship of molecular weight and Rf value as shown in Table 1.

Figure 1 shows two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) pattern of standard β-galactosidase, grade III from bovine liver by Sigma Corp. using the 2-D minislab system. Four mg of the standard β-galactosidase was solubilized with 1 ml of the first dimension sample buffer containing 0.1 M Tris, 6 M urea, 1 M thiourea, complete mini EDTA-free (a grain 10 ml), 1% CHAPS, 1% Triton X-100 and 1% DTT, and then 25 μl of the solution was subjected to agar gel (pH range 5-8) for isoelectric focusing (the first dimension). After the first dimension run was complete, the tube gel was loaded on the slab gel for 2-D PAGE with SDS-PAGE buffer containing 0.5 M Tris-HCl (pH 6.8), glycerol, 10% SDS, 5% bromophenol blue. The horizontal way of Fig. 1 showed the first dimension (IEF) electrophoresis and the vertical way showed 2-D PAGE. The right lane in the gel of Fig. 1 shows SDS-PAGE profile of Low molecular weight Kit protein. The molecular weights ($\times 10^{-3}$) of marker proteins were indicated to the right of Fig. 1. The

Table 1 The relationship of molecular weight and Rf value of SDS-PAGE molecular mass marker proteins

Protein	Molecular Weight	Rf (Rate of flow) Value
Phosphorylase b	97,000	0.32
Bovine Serum Albumin	66,000	0.457
Ovalbumin	45,000	0.543
Carbonic Anhydrase	30,000	0.657
Trypsin Inhibitor	20,100	0.714
α-Lactalbumin	14,400	0.814

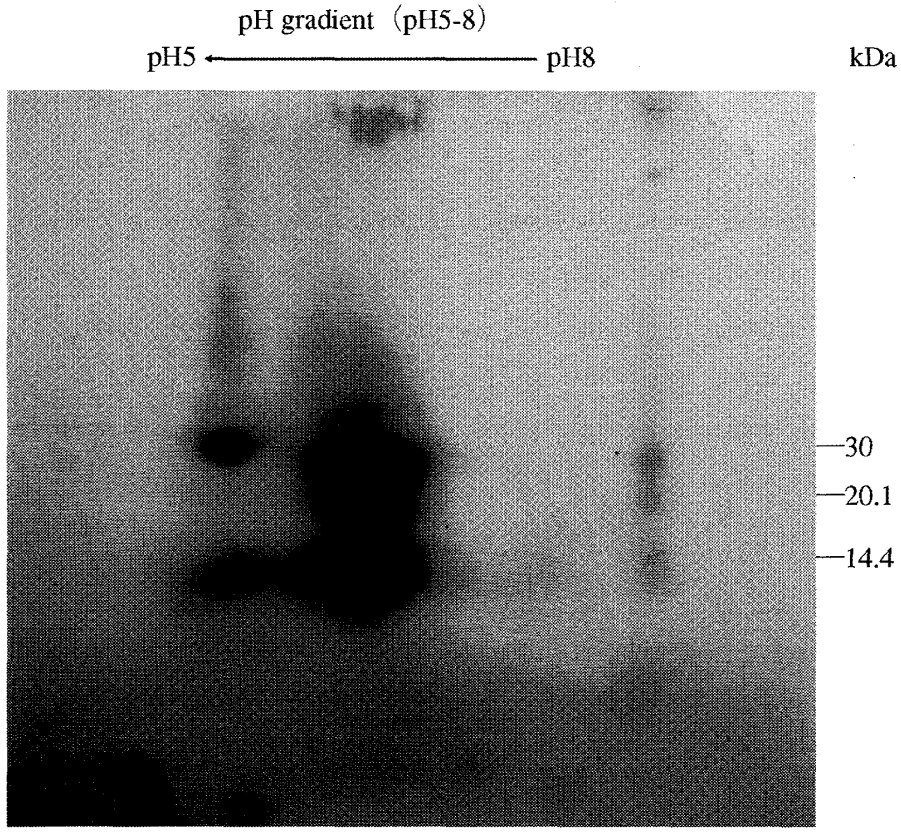

Fig. 1 2-D PAGE pattern of standard β-galactosidase, grade III from bovine liver by Sigma Corp.

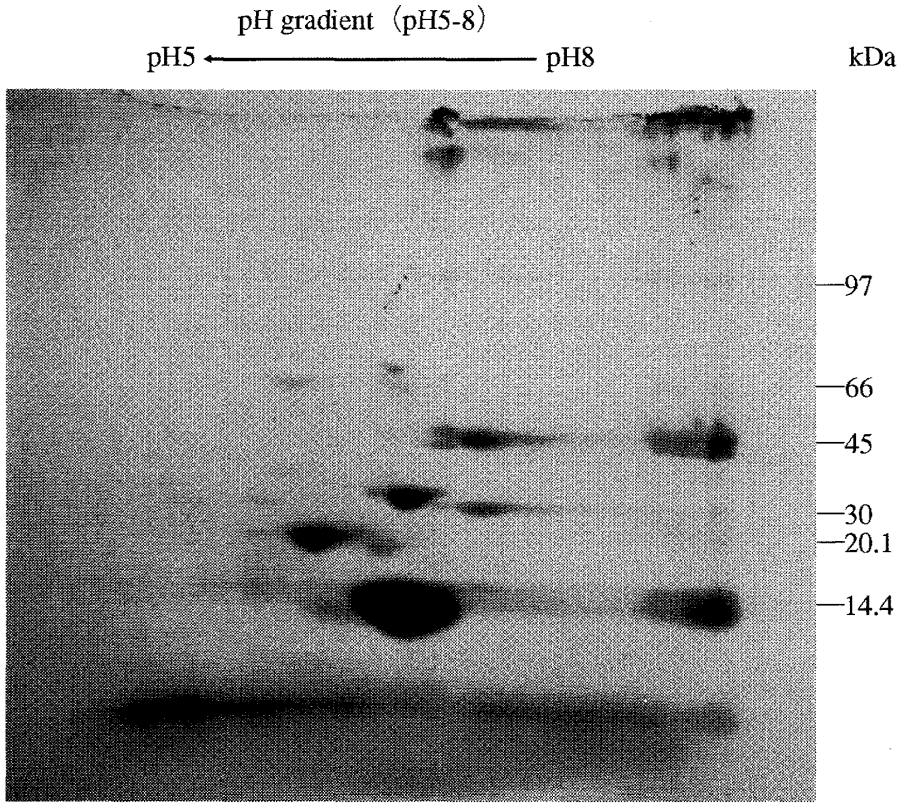

Fig. 2 2-D PAGE pattern of the larval hemolymph in *Bombyx mori.*

result showed that the standard β-galactosidase gave some clear spots in the gel, and that isoelectric point (pI) and molecular weight (MW) of main protein spots were estimated at pH 6.0～6.5 and 14.4 kDa～45 kDa, respectively.

Figure 2 shows a 2-D PAGE pattern of the larval hemolymph in the domesticated silkworm, *Bombyx mori,* on day 10 in the fifth instar. The result indicated that the larval hemolymph proteins were separated clearly, i.e., some distinct spots were obtained in the gel. I estimated pI and MW of main spots at pH 6.0～6.5 and 14.4 kDa～45 kDa, respectively. On the other hand, the 2-D PAGE profile of the larval fat body in the *B. mori* on day 10 in the fifth instar obtained by this method was not good as shown in Fig. 3, i.e., no clear spot was obtained in the gel. Judging from Fig. 2 and Fig. 3, more distinct 2-D PAGE profile was obtained from the larval hemolymph than the larval fat body.

Figure 4 shows a 2-D PAGE profile of the pupal hemolymph in the *B. mori* on day 3 after pupation. The result showed that the pupal hemolymph proteins were separated clearly, i.e., some distinct spots were obtained in the gel. I estimated pI and MW of main spots at pH 6.0～6.5 and 14.4 kDa～45 kDa, respectively. The distinct 2-D PAGE pattern of the larval hemolymph was obtained as good as that of the pupal hemolymph as shown in Fig. 2 and Fig. 4. However, I obtained a poor 2-D PAGE pattern of the pupal fat body in the *B. mori* by this method, i.e., no clear spot was obtained in the gel (data not

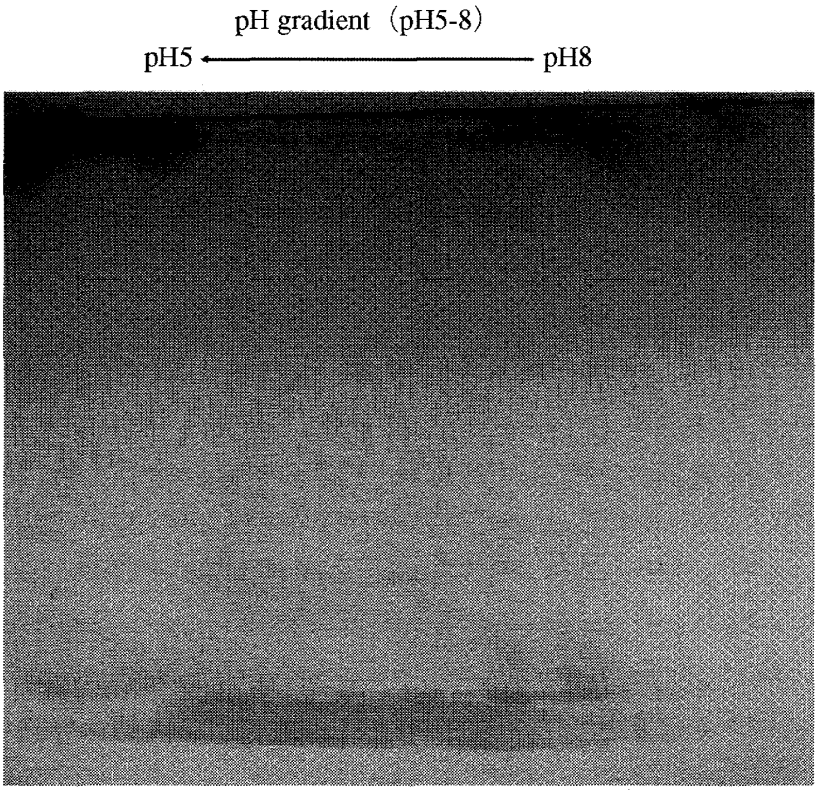

Fig. 3 2-D PAGE pattern of the larval fat body in *Bombyx mori.*

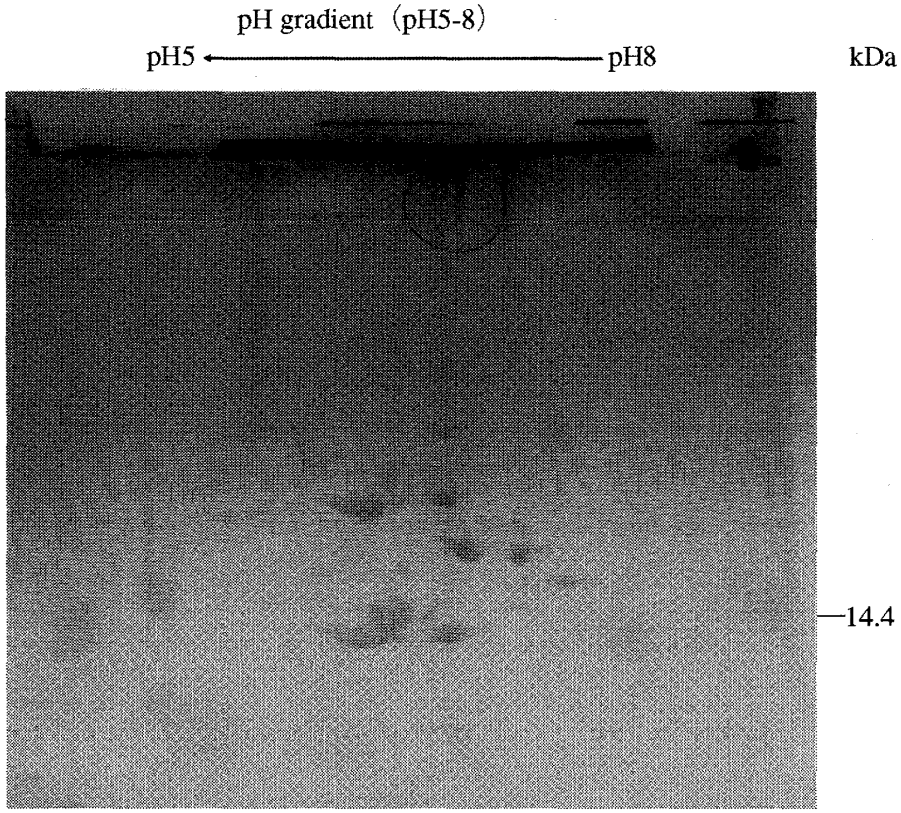

Fig. 4 2-D PAGE pattern of the pupal hemolymph in *Bombyx mori*.

shown). These observations indicate that more distinct 2-D PAGE profile was obtained from the pupal hemolymph than the pupal fat body. Namely, more distinct 2-D PAGE profile was always obtained from the hemolymph than the fat body as described above.

Next, the gel filtration of the larval hemolymph of *B. mori* on the 10 th day in the fifth instar was performed. A Superdex 200 column was equilibrated with 0.1 M Tris-HCl buffer containing 0.1 M NaCl (pH 8.0) and eluted with the same buffer. Three significant fractions, Fraction I, Fraction II and Fraction III were obtained by gel filtration and Fraction II was rich in both of α-galactosidase and β-galactosidase as described in my former report (KATO, 2005). Accordingly, I tried to study the possibility about further separation of Fraction II using the 2-D PAGE method. Figure 5 shows the result of 2-D PAGE on Fraction II obtained by gel filtration from the larval hemolymph of the *B. mori*. The result showed that Fraction II proteins gave two clear spots in the gel, and that pI and MW of them were estimated at pH 6.4～6.5 and 30 kDa～45 kDa, respectively as shown in Fig. 5. But the result showed that Fraction II proteins obtained by gel filtration from the larval fat body of the *B. mori* were separated faintly by this method (data not shown). These observations showed that clearer 2-D PAGE profile of Fraction II was obtained from the larval hemolymph than the larval fat body.

I conclude from these observations described above that the hemolymph protein, the

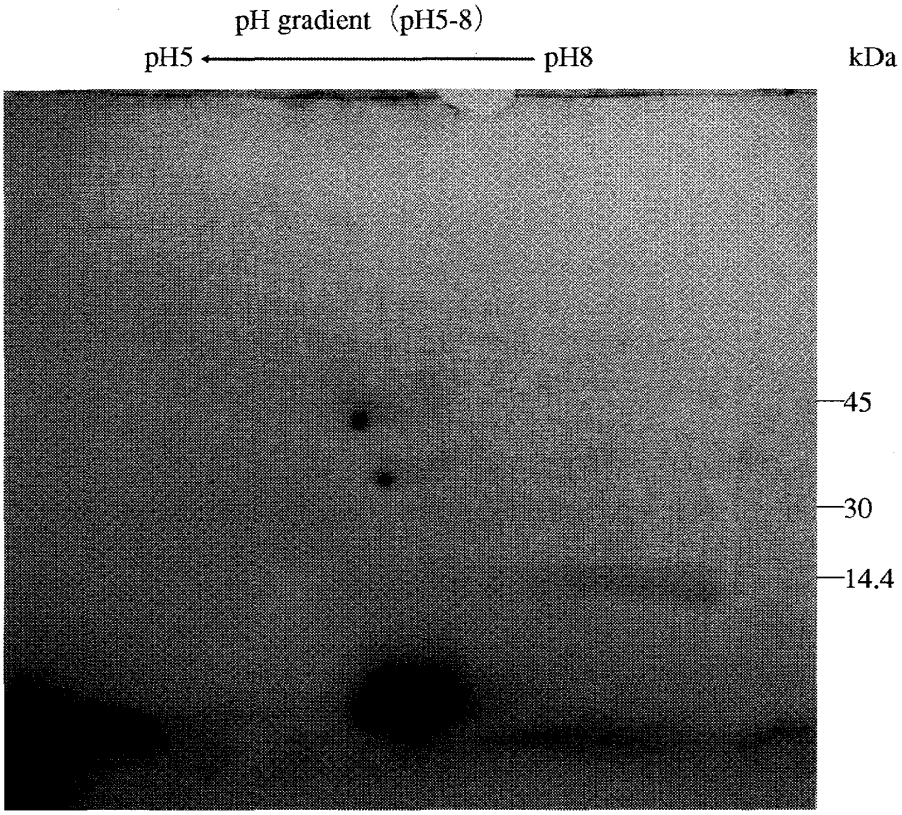

Fig. 5 2-D PAGE pattern of the Fraction II obtained from the hemolymph by gel filtration.

significant fraction, Fraction II, obtained by gel filtration from the larval hemolymph and the standard β-galactosidase seemed to be separated clearly by the 2-D PAGE method, and that the method would be particularly suitable in studying the proteomic analysis of the *Bombyx* humoral lectin related proteins. However, further study is necessary for obtaining distinct profile of 2 D-PAGE from the fat body of the *B. mori.* Moreover, further study is needed from various points of view about the *Bombyx* humoral lectin and the enzymes relating to the lectin activity, such as neuraminidase and galactosidase, in view of their significance to the living body.

SUMMARY

Comparative study of two-dimensional polyacrylamide gel electrophoresis (2 D-PAGE) was performed on the hemolymph and the fat body of the domesticated silkworm, *Bombyx mori.* The result showed that both the larval hemolymph on day 10 in the fifth instar and the pupal hemolymph on day 3 after pupation were obtained distinct 2 D-PAGE profiles. However, faint 2 D-PAGE profiles were obtained for both the larval fat body and the pupal fat body. Similar results were obtained on the Fraction II from the larval hemolymph and from the larval fat body, i.e., clearer 2-D PAGE profile of Fraction

II was obtained from the larval hemolymph than the larval fat body.

This research was partly supported by a grant from Tezukayama Gakuen.

REFERENCES

FUJITA, Y., KURATA, S., HOMMA, K. and NATORI, S. (1998) A novel lectin from *Sarcophaga*—its purification, characterization and cDNA cloning. *J. Biol. Chem.*, **273**, 9667–9672.

FURUTA, E., YAMAGUCHI, K. and SORIMACHI, K. (2001) Internal defense factors in terrestrial slug. *PROTEIN, NUCLEIC ACID AND ENZYME* **46**, 401–407.

FURUTA, M., ITO, T., EGUCHI, C., TANAKA, T., WAKABAYASHI-TAKAI, E. and KANEKO, K. (2002) Two-Dimensional Electrophoresis/Phage Panning (2 D-PP): A Novel Technology for Direct Antibody Selection on 2-D Blots. *J. Biochem.* **132**, 245–251.

HIRABAYASHI, J. (2004) Glycan recognition by galectins and comparative glycomics. *SEIKAGAKU* **76**, 256–268.

KATO, Y. (2002) Study of Galactosidase in Hemolymph of *Bombyx mori* by FPLC. *J. of Tezukayama College* **39**, 88–94.

KATO, Y. (2003) Fractionation of Galactosidase Relating to the Disappearance of *Bombyx* Humoral Lectin Activity. *J. of Tezukayama College* **40**, 85–92.

KATO, Y. (2004) Two Dimensional Electrophoresis of Galactosidase Relating to the Disappearance of *Bombyx* Lectin Activity. *J. of Tezukayama College,* **41**, 93–99.

KATO, Y. (2005) Comparative Studies of Two-Dimensional Electrophoresis of Galactosidase Relating to *Bombyx* Lectin Activity. *J. of Contemporary Human Life Science, Tezukayama University* **1**, 3–12.

KATO, Y. and NAKAMURA, T. (2000) Effect of Triton X 100 on isolation of a *Bombyx* humoral lectin activating enzyme. *J. of Tezukayama College* **37**, 148–157.

KATO, Y. and NAKAMURA, T. (2001) A Study on Galactosidase Relating to a *Bombyx* Humoral Lectin Activity. *J. of Tezukayama College* **38**, 95–102.

KATO, Y., NAKAMURA, T. and TAKEUVHI, T. (1988) Relationship between lectin activity and terminal sialic acid of glycoprotein in hemolymph of *Bombyx mori. The abstracts of 58th academic meeting of Japanese Society of Sericultural Science,* p. 78.

KATO, Y., NAKAMURA, T. and TAKEUCHI, T. (1994): Haemagglutination activity of hemolymph of *Bombyx mori* treated with a juvenile hormone analogue. *J. Seric. Sci. Jpn.* **63**, 221–228.

KATO, Y., NAKAMURA, T. and TAKEUCHI, T. (1998): Production and activation of humoral lectin protein in *Bombyx mori. J. Seric. Sci. Jpn.* **67**, 319–326.

KAWABATA, S. (2000) Nonself-recognizing lectins in invertebrate innate immunity. *PROTEIN, NUCLEIC ACID AND ENZYME* **45**, 679–689.

KAWASAKI, T. (2001) Mammalian lectins in the innate immune system. *SEIKAGAKU* **73**, 167–178.

KONDO, T. (2004) Proteomics using two-dimensional polyacrylamide gel electrophoresis and fluorescence dye labeling technology. *SEIKAGAKU* **76**, 385–390.

KOTANI, E., YAMAKAWA M., IWAMOTO, S., TASHIRO, M., MORI, H., SUMIDA, M., MATSUBARA, F., TANIAI K., KADONO-OKUDA, K., KATO, Y. and MORI, H. (1995) Cloning and expression of the gene of hemocytin, an insect humoral lectin which is homologous with the mammalian von Willebrand factor. *Biochim. Biophys. Acta.* **1260**, 245–258.

KOYAMA, Y., KATSUNO, Y., MIYOSHI, N., HAYAKAWA, S., MITA, T., MUTO, H., ISEMURA, S., AOYAGI,

Y. and ISEMURA, M. (2002) Apoptosis Induction by Lectin Isolated from the Mushroom *Boletopsis leucomelas* in U 937 Cells. *Biosci. Biotechnol. Biochem.* **66**, 784–789.

OHISHI, M. (2002) Two-dimensional gel electrophoresis with agarose gels in the first dimension with wide applicability in analyzing various tissues and cell. *SEIKAGAKU,* **74**, 413–417.

OHISHI, M. SATOH, M. and MAEDA, T. (2000) Preparative two-dimensional gel electrophoresis with agarose gels in the first dimension for high molecular mass proteins. *Electrophoresis* **21**, 1653–1669.

UJITA, M., KIMURA, A., NISHINO, D., YOKOYAMA, E., BANNO, Y., FUJII, H. and HARA, A. (2002) Specific Binding of Silkworm *Bombyx* 30-KDa Lipoproteins to Carbohydrates Containing Glucose. *Biosci. Biotechnol. Biochem.* **66**, 2264–2266.

WAGO, H. (2001) Host defensive protein of planaria. *PROTEIN, NUCLEIC ACID AND ENZYME* **46**, 408–413.