Journal of Biochemistry and Molecular Biology, Vol. 32, No. 5, September 1999, pp. 445–450

JOURNAL OF
Biochemistry
and
Molecular Biology

Purification of Bovine Pregnancy-Associated Proteins by Two-Dimensional Gel Electrophoresis

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Received 13 April 1999, Accepted 26 May 1999

We purified and characterized a bovine pregnancy-associated protein in pregnant cow urine using two-dimensional gel electrophoresis. Urine from cows was collected according to their status of pregnancy and non-pregnancy. Proteins in the cow urine were fractionated with 50% ammonium sulfate prior to two-dimensional gel electrophoresis. Proteins separated on the gels were compared in terms of expression level and new expression by molecular mass and isoelectric point. We localized two pregnancy-associated protein spots on the gels at molecular masses of 24 kDa and 20 kDa and isoelectric points of 5.5 and 5.7, respectively. Likewise, two non-pregnancy specific proteins were localized at 27 kDa and 28 kDa with isoelectric points of 5.7 and 5.9, respectively. To rule out the possibility that environmental or genetic factors might influence the expression of the proteins, we demonstrated the pregnancy-associated expression of the proteins in two-dimensional gels with pregnant urine taken from cows raised in a different institute. The pregnancy-associated protein with molecular mass of 20 kDa and isoelectric point of 5.7, namely spot 2, was microsequenced and found to be highly homologous to the bovine collagen alpha 1 chain.

Keywords: 2D gel electrophoresis, Fractionation, Pregnancy-associated proteins, Urine.

Introduction

On conception, numerous signals are produced during bovine early pregnancy. These include steroids, prostaglandins, and proteins (Bartol *et al.*, 1984; Knickerbocker *et al.*, 1986). Many of these hormones and proteins are of fetal-placental origin rather than of maternal origin (Chard and Grudzinskas, 1987). They are required for the successful establishment of pregnancy and the proliferation of normal and neoplastic cells. However, this early pregnancy factor (EPF) was found to be not confined to pregnancy (Morton *et al.*, 1987; 1992). It could be detected in serum of patients and animals bearing a variety of tumors. Thus, although there have been many attempts to develop diagnostic methods for the detection of cow pregnancy, none have been successful.

In domestic animals, several pregnancy proteins have also been reported. Equine chorionic gonadotropin (eCG) is currently used for pregnancy diagnosis in mares (Cole and Hart, 1942). In cows, placental lactogen (bPL) (Wooding and Beckers, 1987), a placental glycoprotein called pregnancy-specific protein B (PSPB) (Sasser *et al.*, 1989) and a bovine pregnancy-associated glycoprotein (bPAG) (Zoli *et al.*, 1991) are the reported pregnancy-specific factors used for the diagnosis of bovine pregnancy. In addition, progesterone concentration in cow milk is also used for the detection of pregnancy. However, the overall accuracy of the tests is low because of the high number of false-negative and false-positive diagnoses. The inaccuracy might be due, in part, to embryonic death and variations among individuals (Humbolt *et al.*, 1988; Zoli *et al.*, 1992a).

Purification of proteins from complex biological fluids, such as plasma and urine, requires good means of resolution and identification. A technique first developed in the mid-'70s by Patrick O'Farrel, two-dimensional (2-D) gel electrophoresis (O'Farrel, 1975), may be the key to meet these requirements. In this method, proteins from biological fluids are separated on polyacrylamide gels, first by charge and then by size. The resulting gel patterns, representing up to 10,000 proteins, reveal not only the amount of each protein that is being expressed but also allow one to determine if they have been newly expressed (Klose and Kobalz, 1995). In disease states or other physiological changes, the protein profile in body fluid is altered.

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Furthermore, the protein content of these body fluids is not independent. Proteins in urine are mainly the products of filtration of the plasma. Thus, scanned images of 2-D gels of body fluids from different physiological states can be analyzed and the changes quantified (James, 1997).

In this study, we developed a method for the localization of pregnancy-specific proteins from cow urine on 2-D gel. The proteins were transferred to membrane and microsequenced. One of the proteins was identified and found to be a good candidate for developing a cow pregnancy detection assay.

Materials and Methods

Materials All reagents used in this experiment were of ACS grade or higher. Gel electrophoresis reagents were purchased from Bio-Rad (Hercules, USA). All other chemicals were obtained from Sigma Chemical Co. (St. Louis, USA).

Preparation of urine samples Urine samples from Holstein dairy cows (Table 1) were collected at the affiliated ranches of Kyungpook University in Chilgog, Kyungpook province and Seoul National University in Suwon, Kyung-gi province. Precautions for the collected urine were taken as indicated (Rasmussen *et al.*, 1996). In brief, urine was chilled in a glass flask with an ice bath immediately after collection. To remove insoluble material such as hair, dirt, and fecal debris, the urine was filtered through 3 MM Whatman filter paper and further filtered through a 0.45 μm membrane. To adjust the pH and salt concentration, urine was dialyzed against phosphate-buffered saline (PBS) at 4°C overnight with several buffer changes. If necessary, the urine samples were stored frozen before they were utilized (Park *et al.*, 1997). Ammonium sulfate fractionation was done by slowly adding ammonium sulfate crystals to a final concentration of 30% or 50%. The supernatant and pellets were dissolved in PBS and dialyzed against PBS. Appropriate volumes of samples containing 0.4 mg of protein in 1.5 ml tubes were treated with 10% trichloroacetic acid (TCA) to precipitate proteins (Sambrook *et al.*, 1989). Each sample was dissolved in 30 μl of lysis solution containing 9.8 M urea, 2% (w/v) NP-40, 2% ampholyte, pH 3–10, and 100 mM dithiothreitol (DTT). Samples were cleared by centrifugation at 10,000 × *g* for 10 min at room temperature. Protein concentrations were determined by the Bradford method (Bradford, 1976) using bovine serum albumin (BSA) as a standard.

Two-dimensional gel electrophoresis Up to 0.4 mg of protein was used for initial isoelectric focusing (IEF) and subsequent sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) IEF tube gels 1-cm long and 1-mm thick, with pH ranging from 3 to 10, were polymerized for 2 h at 25°C. For the first-dimension focusing, samples were run at 4°C for 2 h at 300 V, 2 h at 500 V, and 18 h at 800 V. Thereafter, the run gels were extruded from the tubes and laid on top of the second dimension SDS-polyacrylamide vertical slab gels. Second-dimension separations were run at 6 mA per gel overnight at 4°C. At the end of each run, the separating gel was subjected to silver staining or Coomassie Brilliant Blue staining. The molecular mass and the isoelectric points (pIs) were determined from calibration curves using 2-D SDS-PAGE Standards (Bio-Rad, USA) (Cho *et al.*, 1999). The standard proteins used were hen egg white conalbumin type I (76 kDa, pI 6.0–6.6), bovine serum albumin (66.2 kDa, pI 5.0–5.18), bovine muscle actin (43 kDa, pI 5.47–5.53), rabbit muscle glyceraldehyde 3-phosphate dehydrogenase (36 kDa, pI 8.3–8.5), bovine carbonic anhydrase (31 kDa, pI 5.9–6.0), soybean trypsin inhibitor (21.5 kDa, pI 4.5), and equine myoglobin (17.5 kDa, pI 7.0).

Silver staining To visualize proteins, SDS-PAGE gels were stained by the silver staining method. A gel was prefixed for 20 min in fixing solution (50% methanol, 10% acetic acid). The prefixed gel was quickly rinsed with distilled water before being fixed in 10% glutaraldehyde for 30 min, washed with plenty of distilled water, and then reduced with 5 mg/l DTT for 15 min. Silver staining was then followed with 0.1% silver nitrate for 15 min. The stained gel was rinsed again with distilled water and developed with 0.019% formaldehyde in 3% sodium carbonate until the desired level of stain was obtained. The addition of 5 ml of 2.5 M citric acid stopped the reaction.

Microsequencing We slightly modified the conditions utilized by Lee *et al.* (1996). Proteins separated on 2-D gel were electrotransferred to polyvinylidene difluoride (PVDF) membrane (Bio-Rad, USA) at 120 V for 1.5 h in a transfer buffer containing 20% methanol. The membrane was stained with 0.1% Coomassie Brilliant Blue in 40% methanol and 10% acetic acid. The interested protein spots were cut with razor blades and their N-terminus regions microsequenced by the Edman degradation method. The sequence obtained from the N-terminal sequencing was used to search the SWISS-PROT protein sequence database (release 37.0) using an algorithm developed by Altschul *et al.* (1990).

Image analysis The silver-stained gels were scanned with an Epson GT-9500 scanner. Scanned image files were exported to the GeneTools analysis program (Syngene, Cambridge, UK) and the intensity of spots on the gel were compared in terms of molecular mass and isoelectric point (pI).

Results

The protein profiles of cow urine on two-dimensional gels Total proteins from pregnant and non-pregnant cow urine were obtained with the addition of trichloroacetic acid. Approximately 0.35 mg protein of each sample was loaded onto each first dimension isoelectric focusing gel. Generally, the protein concentration of 60–90 mg/l in pregnant urine was about three-folds higher than that of non-pregnant urine. Interestingly though, the total proteins in the four urine samples from the different cows in terms of age and status of pregnancy (Table 1) showed similar migration patterns on 2-D gels (Fig. 1). A majority of the proteins migrated to the acidic side. This 2-D gel pattern is quite different from that of human urine (Rasmussen *et al.*, 1996). While the majority of proteins in human urine are distributed within the pI range of 4.0 to 7.0, the bovine urine proteins were distributed in a narrower range of pI 3.5 to 5.5.

Table 1. Sampling of urine from the pregnant and non-pregnant cows.

Identification number	Date of birth	Duration at the sampling (month)	
		Pregnancy*	Non-Pregnancy
19	1991.7.20	2 and 5	
30	1993.1.31		2
46	1994.12.10	2, 4, and 5	
48	1995.3.22		1
A	1997.9.14	4	
B	1997.9.10		18

* after artificial insemination.

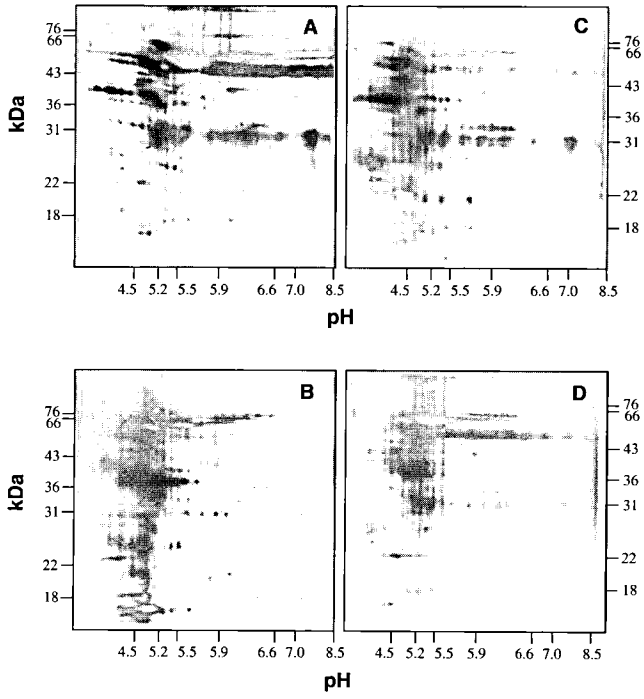

Fig. 1. Comparison of the total urine proteins from pregnant and non-pregnant Holstein dairy cows. Cows #46 (A) and #19 (B) were pregnant for 2 months and 5 months, respectively, at the time of sampling. Non-pregnant cows were #48 (C) and #30 (D).

Due to the large number of proteins within the narrow pI range and the low spot intensity on the 2-D gels, we could not distinguish any pregnancy-associated proteins even after careful comparisons between the total pregnant and non-pregnant urine proteins (Figs. 1A and 1B vs. 1C and 1D for pregnant and non-pregnant urine, respectively). Although there were several suspicious spots detected, it was not possible to pin-point any particular protein at this level of resolution and reproducibility in terms of molecular mass and pI.

Localization of pregnancy-associated proteins on two-dimensional gel after ammonium sulfate fractionation For better separation and improved spot intensity on the gel, urine samples were fractionated with ammonium sulfate. When the proteins were separated on 2-D gels, proteins in the 30% ammonium sulfate fraction did not show any significant difference between the pregnant and non-pregnant urine (data not shown). However, in the 50% ammonium sulfate fraction, two pregnancy-associated and two non-pregnancy specific spots were found on the gels (Fig. 2). In particular, spot 2 was isolated very well at 20 kDa and pI 5.7, and expressed at a significant level in pregnancy-associated urine. Considering the spot intensity after silver staining, the amount of the protein in the spot was believed to be in the tens of nanograms range. The spot 1 protein was localized at 24 kDa and pI 5.5, and was also found in pregnant urine, but expressed at a lower level. As the expression level of spot 1 protein was low, its reproducibility needs to be re-evaluated at an elevated resolution and protein concentration (Figs. 2A and 2B). Spots 3 and 4 were found only in non-pregnant urine (Figs. 2C and 2D). Throughout this experiment, we focused on proteins whose expression was pregnancy-associated.

Pregnancy-associated protein expression To determine a correlation between the expression level of the proteins

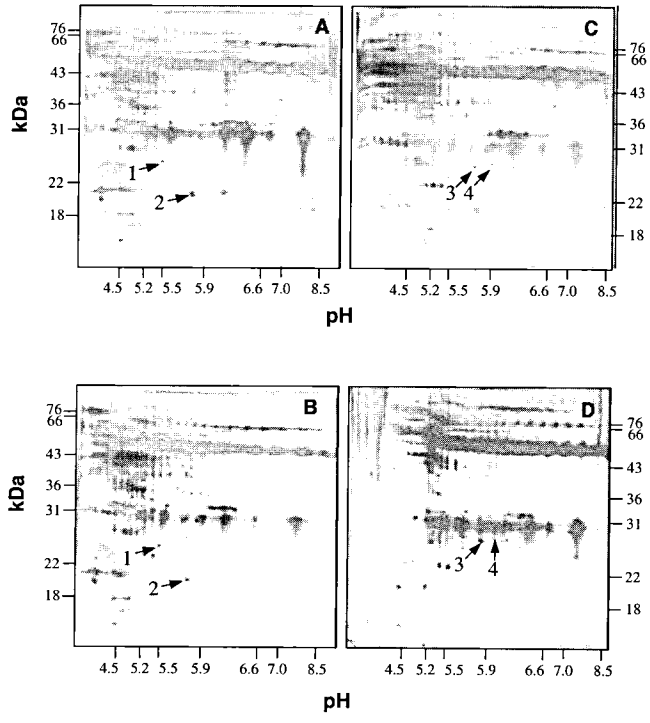

Fig. 2. Comparison of the pelleted proteins of pregnant (#46, A, and #19, B) and non-pregnant (#48, C and #30, D) urine at 50% ammonium sulfate saturation. The #46 and #19 cows were pregnant for 5 months at the time of sampling.

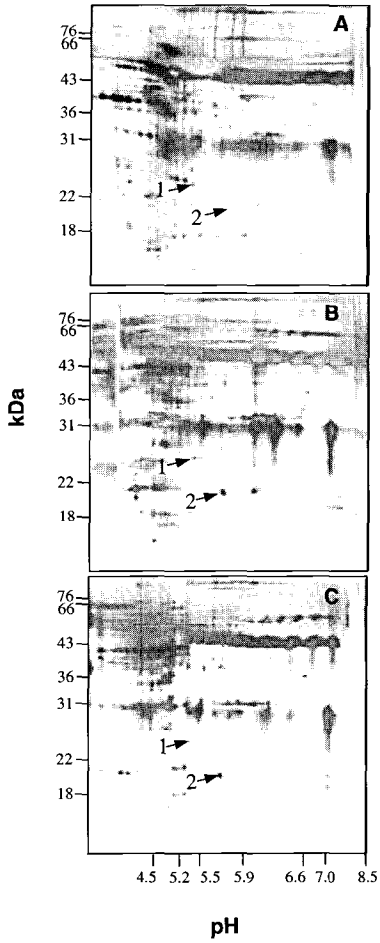

Fig. 3. The time-course expression of pregnant-associated proteins in #46 urine at 2 months (A), 4 months (B), and 5 months (C).

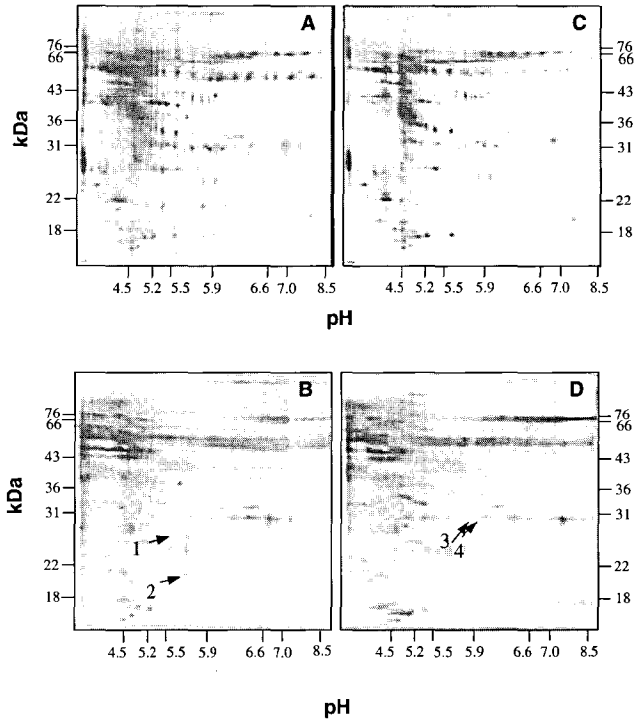

Fig. 4. The protein profiles of pregnant (A and B) and non-pregnant (C and D) urine obtained from cows raised at a different ranch. Total protein (A and C) and proteins from 50% ammonium sulfate saturation (B and D) were separated on 2-D gel.

and the duration of pregnancy, the relative expression levels of spots 1 and 2 in the urine of cow #46 was compared at 2, 4, and 5 months of pregnancy. In this period, the expression of the spot 2 protein increased significantly while that of the spot 1 protein remained constant as the pregnant state proceeded (Fig. 3).

The possibility of the influence of feeding conditions or other genetic differences on the expression of the pregnancy-associated proteins localized on the 2-D gel was ruled out by analyzing pregnant and non-pregnant cow urine from another institute housing a different lineage of cows and receiving different feed. Interestingly, the profiles of the total urine protein of the cows from two different institutes were highly similar on 2-D gels (compare Fig. 1 and Figs. 4A and 4C). When the proteins were fractionated with 50% ammonium sulfate and separated on 2-D gels, the pregnancy-associated protein spots 1 and 2 could be detected only in the pregnant urine (Fig. 4B). Again, spot 2 was clearly stained and spot 1 appeared as a weak smear. As the pregnant cow was artificially inseminated four months before the urine sampling (Table 1) and the protein was expressed at a significant level (Figs. 3 and 4B), it is possible to speculate that this protein is expressed much earlier than the four-month stage, and perhaps even earlier than two months (Fig. 3A) after artificial insemination.

Microsequencing of the pregnancy-associated protein

Urine from four-months pregnant cow A (Table 1) was fractionated with 50% ammonium sulfate and the pellet protein was separated on IEF gel and 12% SDS-PAGE. A part of the gel area including spots 1 and 2 was cut and the proteins in the area were electrotransferred to PVDF membrane. Proteins on the membrane were visualized by Coomassie Blue staining. Unfortunately, the sensitivity of the staining was not good enough to detect spot 1. However, spot 2 was clearly visible and so was used for N-terminal microsequencing. The sequence of the final eleven amino acid residues from the N-terminus (Fig. 5B) was used to search the SWISS-PROT protein database. Although there was no 100% matched sequence, the highest match was found to be with the bovine collagen alpha 1 chain (Rauterber *et al.*, 1972; Fietzek and Kuehn, 1975) (Fig. 5B).

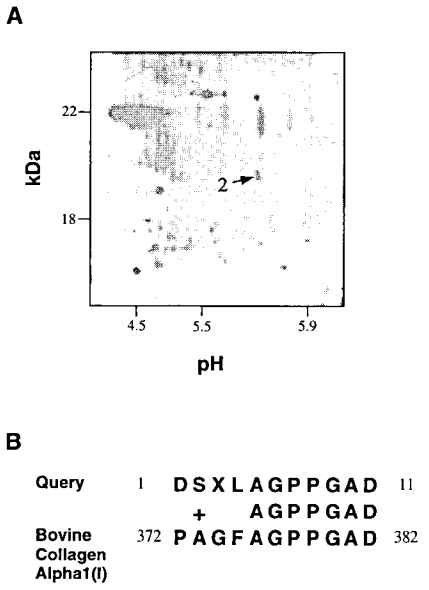

Fig. 5. Proteins separated on 2-D gel were electrotransferred to PVDF membrane and stained with Coomassie Brilliant Blue (A). Spot 2 protein was localized on the membrane by comparing the protein profile on the silver-stained gel. Coomassie Blue staining could not detect the spot 1 protein on the membrane. The N-terminus of the spot 2 protein was microsequenced by the Edman degradation method (B) and used to search the SWISS-PROT database. The X denotes ambiguity of the sequence. The agreement between the sequences is designated in the middle and the + represents the same amino acid group.

Discussion

Using a 2-D gel electrophoresis system, we could localize four possible pregnancy- and non-pregnancy-associated proteins on 2-D gel (Table 2). Due to the resolution and the protein loading capacity of the gel, it was necessary to locate proteins whose expression is obviously different between non-pregnant and pregnant states. The results from the 2-D gel separation demonstrate that spots 1 and 2 proteins were most possibly pregnancy-associated proteins. In particular, the expression level of the protein of spot 2 significantly increased as pregnancy continued over a 5-month period (Fig. 3). Fortunately, these proteins are well isolated from other proteins on 2-D gel so that they could be easily purified and characterized.

Table 2. Pregnancy-associated proteins identified on 2-D gels.

Protein spot	Molecular mass (kDa)	pI	Protein expression during pregnancy
1	24	5.5	Constant
2	20	5.7	Increased
3	27	5.7	Disappeared
4	28	5.9	Disappeare

To obtain good isolation of the protein on 2-D gel, it was critical to remove salts from the urine samples by trichloroacetic acid (TCA) precipitation. Critical steps in this separation also included the fractionation of the samples with 30% and 50% ammonium sulfate and reproducible IEF procedures. Using tube gels for IEF, it was possible to load a maximum of 400 μg of protein. After SDS-PAGE, some spots could be seen by Coomassie Blue staining, but silver staining better revealed most spots. This suggests that the amount of protein in most of the spots is in nanogram quantities, i.e., not enough amount for microsequencing. However, with Immobilized pH Gradient (IPG) gels (Pharmacia, USA) for first-dimension IEF, we should be able to achieve better reproducibility and higher loading capacity in terms of protein amounts as well as sample volume, which generally facilitates microsequencing and amino acid analysis. With better resolution and reproducibility using higher amounts of protein, the comparison between pregnant and non-pregnant urine samples should be easier and more accurate.

Apparently, there were some additional proteins whose expression fluctuated with pregnancy (for instance, compare the spot at 20 kDa and pI 6.1 on panels A and B in Fig. 2). However, the expression was inconsistent between individuals or was present at a certain basal level. For developing a bovine pregnancy diagnostic assay, we need a newly-expressed protein upon the onset of pregnancy. Certainly, apart from the four proteins we mentioned in this paper, there should be numerous other proteins whose expressions are highly correlated to pregnancy. Considering that the detection limit by silver staining is at the low femtomole level, the unrecognized proteins could be present at lower than the detection limit by this method. Another possibility is that the protein spots are overlaid by other proteins with similar molecular mass and pI values. To confirm that the proteins summarized in Table 2 are true pregnancy-associated proteins, it is necessary to clone the genes for the proteins (Cavanagh, 1996). The proteins can then be expressed in a high amount and easily purified in order to raise antibodies for testing. When antibodies against the pregnancy-associated proteins are available, they will be invaluable reagents for developing diagnostic assays (Zoli *et al.*, 1992b). Figure 3 indicates that the protein concentration of spot 2 was already higher than nanogram quantity, only two months after artificial insemination. The amount of protein loaded on a gel was about 0.4 mg, representing 5 to 8 ml of urine. If we assume that spot 2 contains 10 ng of protein, the concentration of the protein is about 0.1 nM. As good antibodies can detect 0.01 to 1 nM quantity of protein in urine, it should be possible to detect pregnancy much earlier than two months after artificial insemination. Furthermore, it could be possible to identify the physiological roles of the proteins by monitoring protein concentration in the urine or serum in conjunction with fetal development.

With a perfect match of 7 of the last 11 residues of the protein, it is possible that the spot 2 protein belongs to the collagen alpha chain family. It is also possible that this protein is totally different from the collagen family, and only happens to have homology to this family. To better identify the protein and clone the gene responsible, we are currently pursuing peptide mapping and C-terminal sequencing.

Although the separation and identification of pregnancy-associated proteins is at an initial stage at this time, it has already been proven to be helpful in studies of protein variations in pregnant cows.

Acknowledgments We are especially grateful to Mr. Doo-Kyung Sung at the affiliated ranch of Kyungpook University for helping us to collect urine samples and for providing information on the cows.

References

Altschul, S. F., Gish, W., Miller, W., Myers, E.W. and Lipman, D. J. (1990) Basic local alignment search tool. *J. Mol. Biol.* **215**, 403–410.

Bartol, F. F., Robert, R. M., Bazer, F. W., Lewis, G. S., Godkin, J. D. and Thatcher, W. W. (1984) Characterization of proteins produced *in vitro* by peri-attachment bovine conceptuses. *Biol. Reprod.* **32**, 681–694.

Bradford, M. M. (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72,** 248–254.

Cavanagh, A. C. (1996) Identification of early pregnancy factor as chaperonin 10: implications for understanding its role. *Rev Reprod.* **1**, 28–32.

Chard, T. and Grudzinskas, J. G. (1987) Early pregnancy factor. *Biol. Res. Pregnancy Perinatol.* **8**, 53–56.

Cho, Y. W., Park, E. H. and Lim, C. J. (1999) A second thioltransferase from Chinese cabbage: Purification and characterization. *J. Biochem. Mol. Biol.* (formerly *Korean Biochem. J.*) **32**, 133–139.

Cole, H. H. and Hart, G. H. (1942) Diagnosis of pregnancy in the mare by hormonal means. *J. Am. Vet. Med. Assoc.* **101**, 124–128.

Fietzek, P. P. and Kuehn K. (1975) The covalent structure of collagen: amino-acid sequence of the cyanogen-bromide peptides alpha-1-CB2, alpha-1-CB4 and alpha-1-CB5 from calf-skin collagen. *Eur. J. Biochem.* **52**, 77–82.

Humbolt, P., Camous, S., Martal, J., Charlery, J., Jeanguyot, N., Thibier, M. and Sasser, R. G. (1988) Pregnancy-Specific Protein B, progesterone concentrations and embryonic mortality during early pregnancy in dairy cows. *J. Reprod. Fert.* **83**, 215–223.

James, P. (1997) Breakthroughs and views of genomes and proteomes. *Biochem. Biophys. Res. Com.* **231**, 1–6.

Klose, J. and Kobalz, U. (1995) Two-dimensional electrophoresis of proteins: an updated protocol and implications for a functional analysis of the genome. *Electrophoresis* **16**, 1034–1059.

Knickerbocker, J. J., Thatcher, W. W., Bazer, F. W., Drost, M., Baron, D. H., Fincher, K. B. and Roberts, R. M. (1986) Proteins secreted by day 16 and 18 bovine conceptuses extend *corpus luteum* function in cows. *J. Reprod. Fert.* **77**, 381–391.

Lee, D. G., Liu, M. Z., Kim, K. L. and Hahm, K. S. (1996) N-terminal amino acid sequence of receptor-like proteins that bind to preS1 of HBV in HepG2 cells. *J. Biochem. Mol. Biol.* (formerly *Korean Biochem. J.*) **29,** 180–182.

Morton, H., Rolfe, B. E. and Cavanagh, A. C. (1987) Ovum factor and early pregnancy factor. *Current Topics Dev. Biol.* **23**, 73–92.

Morton, H., Rolfe, B. E. and Cavanagh, A. C. (1992) Early pregnancy factor. *Seminars Reprod. Endocrinol.* **10**, 72–82.

O'Farrel, P. H. (1975) High resolution two-dimensional electrophoresis of proteins. *J. Biol. Chem.* **250**, 4007–4021.

Park, J. H., Lee, J. H., Baek, S. H., Moon, T. C., Lee, J. M., Kim, N. S., Nam, K. S. and Chang, H. W. (1997) Detection and characterization of novel extracellular phospholipase A_2 in urine of patients with acute pyelonephritis. *J. Biochem. Mol. Biol.* (formerly *Korean Biochem. J.*) **30,** 101–105.

Rasmussen, H. H., Orntoft, T. F., Wolf, H. and Celis, J. E. (1996) Towards a comprehensive database of proteins from the urine of patients with bladder cancer. *J. Urol.* **155**, 2113–2119.

Rauterberg, J., Timpl, R. and Furthmayr, H. (1972) Structural characterization of N-terminal antigenic determinants in calf and human collagen. *Eur. J. Biochem.* **27**, 231–237.

Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual,* 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Sasser, R. G., Crock, J. and Ruder-Montgomery, C. A. (1989) Characteristics of pregnancy-specific protein B in cattle. *J. Reprod. Fertil. Suppl.* **37**, 109–113.

Wooding, F. B. P. and Beckers, J. F. (1987). Trinucleate cells and the ultrastructural localization of bovine placental lactogen. *Cell Tissue Res.* **247**, 667–673.

Zoli, A. P., Beckers, J.-F., Closset, J., Ballman-Wouters, P., Falmagne, P. and Ectors, F. (1991) Purification and characterization of a bovine pregnancy-associated glycoprotein. *Biol. Reprod.* **45**, 1–10.

Zoli, A. P., Guilbault, L. A., Delahaut, P., Ortiz, W. B. and Beckers, J.-F. (1992a) Radioimmunoassay of a bovine pregnancy-associated glycoprotein in serum: its application for pregnancy diagnosis. *Biol. Reprod.* **46**, 83–92.

Zoli, A. P., Demez, P., Beckers, J.-F., Reznik, M. and Beckers, A. (1992b). Light and electron microscopic immunolocalization of bovine pregnancy-associated glycoprotein in the bovine placentome. *Biol. Reprod.* **46**, 623–629.