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Isolation, Purification, and Characterization of Pregnancy-Specific Protein B from Elk and Moose Placenta[1]

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ABSTRACT

Pregnancy-specific protein B (PSPB) was isolated, purified, and partially characterized from elk and moose placenta. The procedure, which was monitored by bovine PSPB (bPSPB) RIA, included homogenization and extraction in aqueous solution, acidic and ammonium sulfate precipitation, and ion exchange, gel filtration, and affinity chromatographies. The estimated molecular sizes of moose PSPB (mPSPB) were 58 kDa and 31 kDa, and of elk PSPB (ePSPB) were 57 kDa, 45 kDa, and 31 kDa by SDS-PAGE. The isoelectric points of mPSPB were 4.8, 6.6, and 6.7, and of ePSPB were 4.8, 4.9, 6.1, and 6.2 as determined by isoelectric focusing and two-dimensional gel electrophoresis. The carbohydrate contents of mPSPB and ePSPB were approximately 3.15% and 4.98%, respectively. Although ePSPB and mPSPB were recognized by anti-bPSPB in an Ouchterlony double immunodiffusion test, they were found to share identical epitopes and partial identities compared to bPSPB. After treatment at different temperatures (20–60°C) for 1 h, the immunoreactivities of ePSPB and mPSPB in serum were very stable. Only ePSPB in serum treated at 60°C lost some immunoreactivity. After alteration of serum pH (pH 3–11) for 2 h, the immunoreactivities of ePSPB and mPSPB became lower at pH 3 and 4, and remained stable from pH 5 to 11. These data show that moose and elk PSPB have properties similar to those of bovine and ovine PSPB.

INTRODUCTION

Proteins produced by the placenta have been reported for many years in several species. Examples are hCG [1], eCG [2], and interferon-τ [3–6].

Pregnancy-specific protein B (PSPB) is a protein that was first isolated from bovine extra-embryonic membranes [7] and was found in the serum of pregnant cattle [8]. This protein was found after immunizing rabbits with homogenates of whole bovine placenta and adsorbing the antisera with somatic tissues to remove antibodies to proteins not specific to the placenta. Estimates of the molecular size of bovine PSPB (bPSPB) varied considerably, with values ranging from 37 kDa to 78 kDa, and isoelectric points (pI) ranged from 4.0 to 4.4. The protein with a molecular mass of 64 kDa contained approximately 5% hexose sugars and 3% sialic acid [9]. Ovine PSPB (oPSPB) from sheep placenta was also isolated. The molecular masses of three variants were 66 kDa, 59 kDa, and 43 kDa, and isoelectric points ranged from 4.06 to 4.65 [10].

The gene for PSPB was cloned, and PSPB is known to be in the aspartic acid proteinase family of proteins [11, 12]. It shares a significant homology with several aspartic proteinases, such as pepsinogens, pepsins, and cathepsin D. Bovine PSPB is antigenically unrelated to alpha-fetoprotein, fetuin, bovine placental lactogen [7], BSA, and human pregnancy-specific beta 1 glycoprotein [13].

The same protein was isolated by Zoli et al. [13] and named bovine pregnancy-associated glycoprotein-1 (bPAG-1). The PAGs were also isolated from sheep [14] and goat placenta [15], and cDNA sequences for PAGs were found in horse [16] and pig placenta [17].

PSPB appears to be produced in the trophoblastic binucleate giant cells of the ruminant placenta [8, 18–20]. Although PSPB is quite similar to other aspartic proteinases, it is an inactive proteinase because of a base substitution in the active site [11, 21]. PSPB is a major secretory product of the placenta in ruminants once placentation has begun. It is initially produced as a large protein and then undergoes posttranslational modification to form smaller products [22]. The exact biofunction of PSPB is still not clear. It may play an important role in maintenance of pregnancy.

Immunoreactive PSPB has been detected in sera of some other pregnant ruminant animals, such as mountain goats [23], mule and white-tailed deer [24], red deer [25], muskoxen [26], goats [27], wood bison [28], moose [29, 30], fallow deer [31–33], elk [34, 35], sika deer [36, 37], and caribou [38]. But PSPB has never been isolated and characterized in any species other than the bovine, ovine, and caprine; and the characteristics of cervid PSPB are not known. Therefore, the aim of this study was to isolate, purify, and characterize PSPB from the placenta of elk and moose.

MATERIALS AND METHODS

Isolation and Purification

Assays for PSPB and protein concentration. During the isolation procedure, the presence of PSPB in each step was determined by a bovine PSPB RIA [8]. Pure bovine PSPB (R-37) was used as tracer and standard, and antibody (RGS 38–1) against bPSPB from a rabbit was used for binding at a final dilution of 1:145 000. Protein concentration was determined by a micro-BCA protein assay using BSA as a standard (Pierce, Rockford, IL).

Collection of cotyledons. Elk cotyledons were collected from hunter-killed pregnant elk at the Starkey Experimental Forest and Range, LaGrande, Oregon, and moose cotyledons were collected from animals at the Alaska Department of Fish and Game, Moose Research Center, Soldotna, Alaska. The cotyledons were collected and stored on ice within 2 (elk) or 1 (moose) h of death and were stored at −20°C

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within 3 h of death. They were shipped to the University of Idaho on dry ice and were stored at −20°C until use.

Extraction. Three elk fetal cotyledons and two moose fetal cotyledons were used. The wet weights were 470 g and 320 g, respectively. They were thawed and frozen three times and cut into approximately 2-cm^2 pieces. Then the tissue was stirred with sand in 900 ml Tris buffer (0.01 M Tris-HCl, pH 7.8) at 4°C for 2 h. The homogenate was gently adjusted to pH 3.6 with 1 N HCl and stirred for 10 min. It was then centrifuged at 3000 rpm for 30 min, the pellets were discarded, and the supernatant solutions were readjusted to pH 7.5 using 1 N NaOH.

Ammonium sulfate precipitations. Dry ammonium sulfate was slowly added to the stirred supernatant to obtain a 40%-saturated solution. After stirring for 30 min, centrifugation was applied at 3000 rpm for 30 min. The pellets were discarded, additional ammonium sulfate was similarly added to the stirred supernatant to achieve a 75%-saturated solution, and the precipitate was retained. During this procedure, the solution was kept at pH 7.5 by adding 1 N NaOH. There were 6.7 g and 15.7 g precipitate for elk and moose, respectively. These were dissolved in 12 ml and 20 ml, respectively, of 0.01 M Tris-HCl buffer (pH 7.5) and dialyzed against the same buffer at 4°C for 36 h.

DEAE-cellulose ion exchange chromatography. The ammonium sulfate precipitates from elk and moose placenta were chromatographed separately on a 2 × 30-cm DEAE-cellulose (DE 52; Whatman, Clifton, NJ) column that had been previously equilibrated with 0.01 M Tris-HCl buffer (pH 7.5). Eighteen milliliters (approximately 300 mg) of elk proteins and 37 ml (approximately 1000 mg) of moose proteins were loaded onto the column. The unbound proteins were washed out with 0.01 M Tris-HCl buffer (pH 7.5). The column was eluted with the same buffer in four steps containing increasing ionic strengths of NaCl (0.04 M, 0.06 M, 0.08 M, and 0.1 M). Protein concentration was monitored by UV absorption at 280 nm. Fractions with high antigenic activity, monitored by RIA, were collected, pooled, and lyophilized.

Sephadex G-75 gel filtration chromatography. The lyophilized proteins were dissolved in small amounts of 0.01 M Tris-HCl buffer (pH 7.5) and submitted to gel filtration on a Sephadex G-75 (Pharmacia, Kalamazoo, MI) column (1 × 20 cm), which was equilibrated with the same buffer. Three milliliters (approximately 64 mg) of elk protein and 3 ml (approximately 110 mg) of moose protein with the highest antigenic activity were loaded. The column was then eluted with 0.01 M Tris-HCl buffer (pH 7.5). Protein concentration of fractions was monitored by UV absorption at 280 nm. The PSPB-positive fractions were collected.

Affinity column chromatography. The AminoLink Immobilization Kit (Pierce) was used for affinity chromatography. The column was equilibrated with coupling buffer (0.1 M sodium phosphate, 0.05% sodium azide, pH 7.0), and 2 ml of anti-bovine PSPB (B5, 5 mg IgG) was coupled to the gel over a 6-h period. The antibody-coupled column was then equilibrated with 0.01 M Tris-HCl buffer (pH 7.5). One milliliter elk protein (1 mg) or 1 ml moose protein (8 mg) were allowed to enter the gel bed and were incubated for 1 h at room temperature. After the column was washed with 14 ml 0.01 M Tris-HCl buffer (pH 7.5), the protein was eluted by the ImmunoPure elution buffer (Pierce) and collected in five 1-ml fractions.

Characterization

SDS-PAGE. One-dimensional SDS-PAGE analysis was carried out with mercaptoethanol (2.5%). Slab gels (10% acrylamide) were run at 50 mA for 3 h on a Mini-PROTEAN II vertical cell system (Bio-Rad, Cambridge, MA). Broad-range molecular weight standards (Bio-Rad) were run simultaneously. The gels were stained with Coomassie Brilliant Blue R 250 dye and destained with 45% methanol, 10% acetic acid.

Isoelectric focusing (IEF). Isoelectric point determination was carried out by processing IEF Ready Gels (pH 3–10, 5% acrylamide gel, native conditions; Bio-Rad) at 200 V for 1.5 h and 400 V for 1.5 h on a Mini-PROTEAN II vertical cell system (Bio-Rad). After electrophoresis, the proteins were visualized by Coomassie Brilliant Blue R 250 staining (0.1% w:v in methanol, water, and acetic acid 45: 45:10) and destaining (methanol, water, and acetic acid 45: 45:10).

Two-dimensional gel electrophoresis. This gel electrophoresis was performed by using IEF Ready Gels (pH 3–10, 5% acrylamide gel, native conditions; Bio-Rad) for the first dimension followed by SDS-PAGE (10% acrylamide) in the second dimension. Focusing was carried out at 200 V for 1.5 h and 400 V for 1.5 h, and SDS-PAGE was run at 50 mA for 3 h.

Western blotting. Immediately after SDS-PAGE, the slab gel was equilibrated in transfer buffer (25 mM Tris, 192 mM glycine, and 20% methanol, pH 8.3) for 30 min and then transferred onto a nitrocellulose membrane (Bio-Rad) using a constant voltage of 30 V overnight or 100 V for 1 h in a Mini Trans-Blot cell system (Bio-Rad). After transfer, the nitrocellulose membrane was allowed to dry at 4°C for 12 h and was then rinsed in 500 mM NaCl, 20 mM Tris-HCl, pH 7.2 (TBS) for 10 min and incubated with blocking buffer (TBS containing 1% BSA, and 0.05% Tween 20) for 2 h at 25°C. Then the membrane was incubated with 10 ml of rabbit anti-bPSPB (RGS 38–1, 1:300) for 2 h at 25°C and then with goat anti-rabbit IgG conjugated to horseradish peroxidase (1:1000; Sigma, St. Louis, MO) for 1 h. After being washed, the membrane was incubated for 30 min with 100 ml chloronapthol (4CN) substrate (Pierce).

Determination of carbohydrate content. The carbohydrate content of elk and moose PSPB was analyzed by using a glycoprotein carbohydrate estimation kit (Pierce). After placement of 50 μl proteins (2.5 mg/ml) in the wells, 25 μl of 10 mM sodium meta-periodate and 150 μl of 0.5% aldehyde detection reagent (Pierce) were added to the wells and incubated for 1 h at room temperature. Then the optical density was read at 550 nm. Lysozyme and BSA were used as negative controls, while ovalbumin, human apotransferrin, fetuin, and α_1-acid glycoprotein were used as positive controls.

Cross-reactivity of PSPB. The cross-reactivity of elk and moose PSPB from placenta with anti-bovine PSPB was tested by Ouchterlony double immunodiffusion (Pierce). Twenty microliters each of cotyledonary homogenate supernatant and antisera were loaded into agar wells and incubated at room temperature overnight to allow the visible line of precipitation to form.

Temperature and pH effect on PSPB immunoreactivity. Samples of sera that contain a high level of elk or moose PSPB were treated at different temperatures (20–60°C) for 1 h or at different pH (3–11) for 2 h. A specific heterologous RIA that can quantify elk and moose PSPB in serum was then applied to test the immunoreactivity of samples after treatments.

NH_2-Terminal microsequence analysis of PSPB. After SDS-PAGE, the proteins were transferred from the gel onto a polyvinylidene difluoride (PVDF) membrane (Millipore,

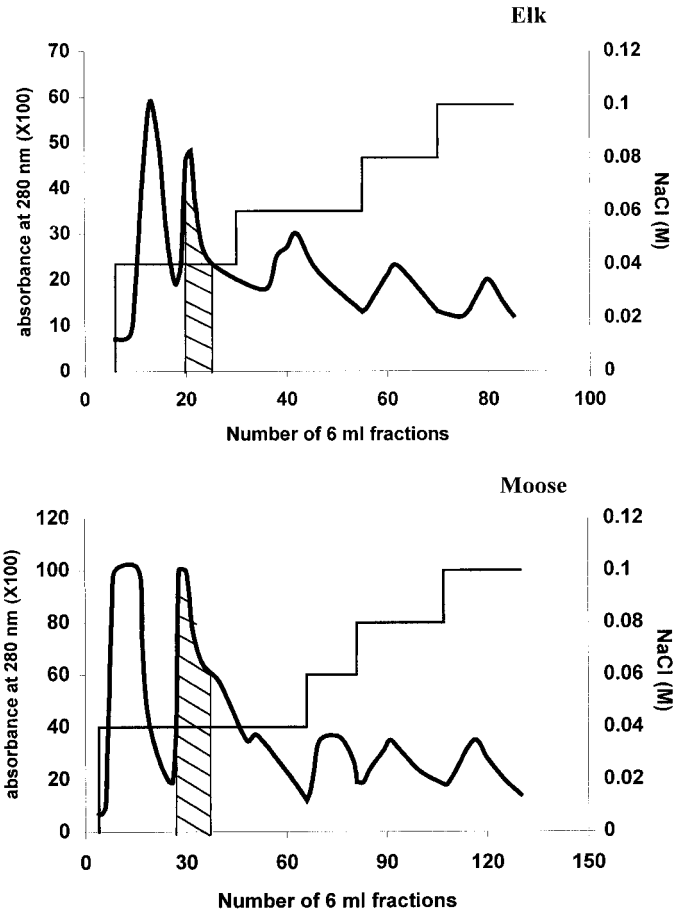

FIG. 1. DEAE-cellulose chromatographic elution profile of elk and moose proteins. The straight lines indicate the salt concentration of 0.01 M Tris-HCl buffer (pH 7.5), and the hatched areas indicate the fractions with the highest immunoreactivity.

Bedford, MA). Transfer was carried out at a constant voltage (30 V) overnight or at 100 V for 1 h on the Mini Trans-Blot cell system (Bio-Rad). After being washed, the membrane was stained by Coomassie Brilliant Blue R 250. Visible bands of interest were cut and subjected to amino acid microsequence analysis, which was performed by automated Edman degradation of approximately 300 pmol protein on an ABI 470 protein sequencer (ABI Analytical, Ramsey, NJ).

RESULTS

Isolation and Purification

Most immunoreactive proteins remained in the extraction supernatant after homogenization and pH treatment,

TABLE 1. Quantity of elk and moose tissue or protein during isolation.

Isolation steps	Elk	Moose
Cotyledons (wet weight)	470 g	320 g
Homogenation extract	3163 mg	5339 mg
Supernatant after 40%-saturated ammonium sulfate	1212 mg	2652 mg
Precipitate from 40–75%-saturated ammonium sulfate	468 mg	1404 mg
Samples applied to DEAE-cellulose chromatography	300 mg	1000 mg
Samples applied to Sephadex G-75 chromatography	64 mg	110 mg
Protein peak eluted from Sephadex G-75 column	26 mg	75 mg

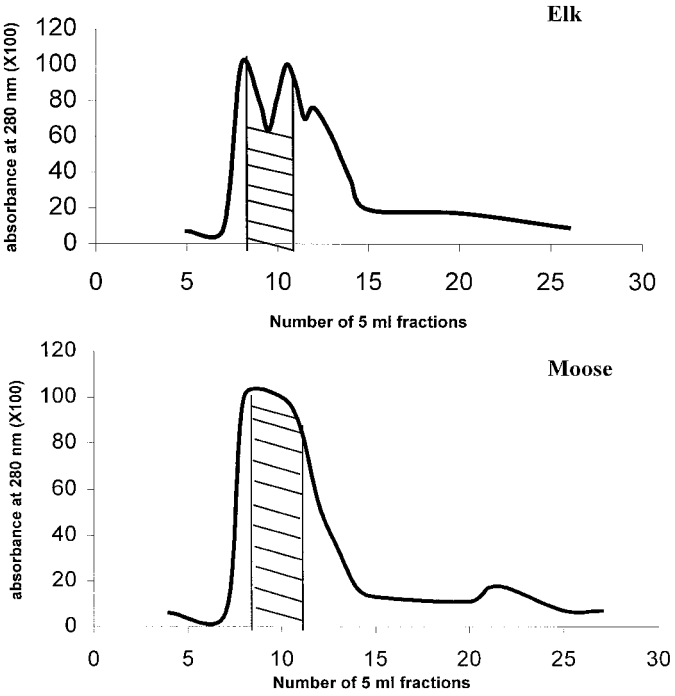

FIG. 2. Sephadex G-75 gel filtration chromatographic elution profile of elk and moose proteins. The hatched areas indicate the location of the fractions with the highest immunoreactivity.

and were precipitated between 40%- and 75%-saturated ammonium sulfate. When applied to the DEAE-cellulose column, the majority of the immunoreactive proteins were eluted in the second peak at 0.01 M Tris-HCl (pH 7.5) with 0.04 M NaCl (Fig. 1). The proteins that had PSPB immunoreactivity were eluted from the Sephadex G-75 column using 0.01 M Tris-HCl (pH 7.5; Fig. 2). Proteins after Sephadex G-75 purification could bind to the affinity column containing antibodies to bPSPB and were eluted by the ImmunoPure elution buffer (Pierce). Most immunoreactive protein from moose and elk placenta was eluted in the second 1-ml fraction. Quantities of elk and moose protein obtained during isolation are shown in Table 1.

Characterization

After Sephadex G-75 purification, there were two bands of moose PSPB (mPSPB) and three bands of elk PSPB (ePSPB) on SDS-PAGE (10% acrylamide) stained with Coomassie Blue (Fig. 3). The estimated molecular weights of mPSPB were 58 kDa and 31 kDa, and of ePSPB were 57 kDa, 45 kDa, and 31 kDa. All bands were visible after Western blotting using antibodies to bPSPB (Fig. 4). After affinity column chromatography, only the major protein of

TABLE 2. Estimated molecular weights (MW), isoelectric points, and carbohydrate content for bovine, moose and elk PSPB.

PSPB	Estimated MW (kD[a])	Isoelectric variants (pI)	Carbohydrate content
bPSPB (21-A)	64	5.0	
mPSPB	58	4.8, 6.6, 6.7	3.15%*
	31	6.6, 6.7	
ePSPB	57	4.8, 6.1, 6.2	4.98%*
	45	4.9	
	31	6.1, 6.2	

* Content for combined bands for each species.

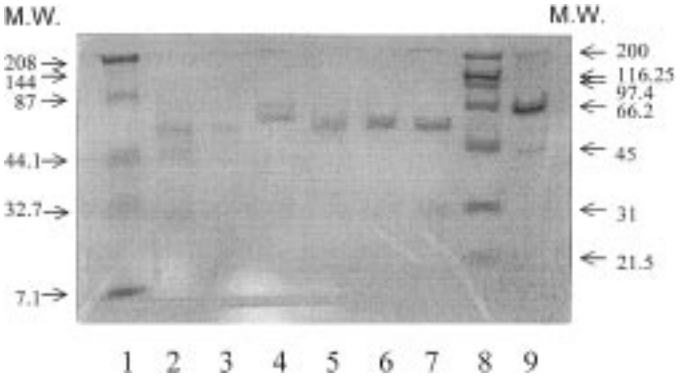

FIG. 3. Coomassie Blue-stained SDS-PAGE (10%) run with reducing agent. Lane 1, prestained molecular weight standards; lane 2, ePSPB after Sephadex G-75; lane 3, ePSPB after affinity column; lane 4, bPSPB (21-A, same as R-37); lane 5, mPSPB after affinity column; lane 6, mPSPB designate preparation m18 used for RIA standard; lane 7, mPSPB after Sephadex G-75; lane 8, broad-range molecular weight standards; lane 9, BSA. Molecular weights ($\times 10^{-3}$) are on the left and right.

each, 58 kDa for mPSPB and 57 kDa for ePSPB, was present on SDS-PAGE (10% acrylamide) stained with Coomassie Blue (Fig. 3). The IEF and two-dimensional gel electrophoresis showed that mPSPB and ePSPB had isoelectric variants from 4.8 to 6.7 and 4.8 to 6.2, respectively. The estimated carbohydrate content of mPSPB was 3.15% and of ePSPB was 4.98%. Table 2 summarizes the estimated molecular weights, isoelectric points, and carbohydrate content of elk and moose PSPB.

Ouchterlony double immunodiffusion tests showed that there was a smooth continuous precipitate line between ePSPB and mPSPB whereas bPSPB had a precipitation spur with ePSPB or mPSPB. This indicated that, when recognized by anti-bPSPB, ePSPB and mPSPB shared identical epitopes and both had partial identities compared to bPSPB.

After being treated at different temperatures (20–60°C) for 1 h, the immunoreactivities of ePSPB and mPSPB in

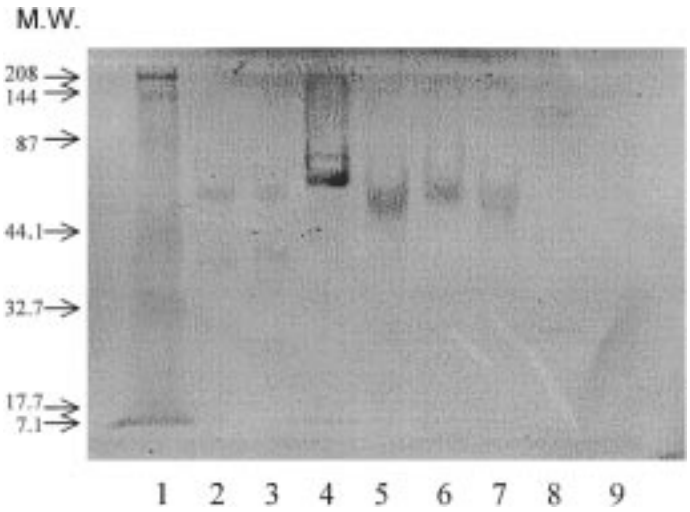

FIG. 4. Same as Figure 3, Western blotting with anti-bPSPB. Lane 1, prestained molecular weight standards; lane 2, ePSPB after Sephadex G-75; lane 3, ePSPB after affinity column; lane 4, bPSPB (21-A, same as R-37); lane 5, mPSPB after affinity column; lane 6, mPSPB designate preparation m18 used for RIA standard; lane 7, mPSPB after Sephadex G-75; lane 8, broad-range molecular weight standards; lane 9, BSA. Molecular weights ($\times 10^{-3}$) are on the left.

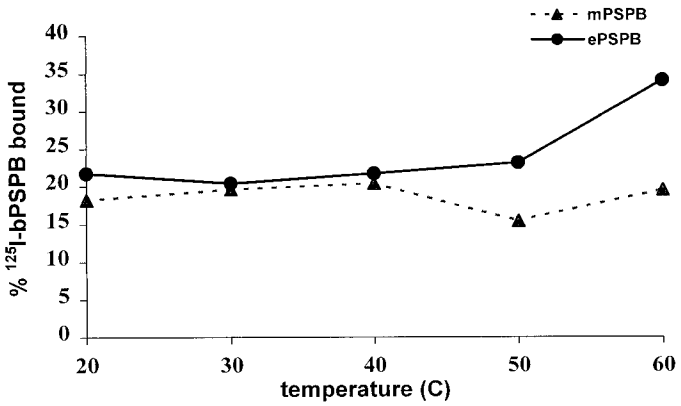

FIG. 5. The effect of temperature on serum PSPB immunoreactivity. Sera containing PSPB were treated at different temperatures for 1 h, and the immunoreactivities were tested for inhibition of binding using sheep anti-moose PSPB IgG. Nonpregnant moose serum as a reference had 100% binding.

serum tested by RIA were very stable. Only ePSPB treated at 60°C lost some immunoreactivity (Fig. 5). After alteration of serum pH (pH 3–11) for 2 h, the immunoreactivities of ePSPB and mPSPB became lower at pH 3 and 4, and remained stable from pH 5 to 11 (Fig. 6).

DISCUSSION

The PSPB has been found in sera of pregnant elk [34, 35] and moose [29, 30] and other cervids. It has been considered as a marker of pregnancy for ruminant animals. This study describes the first isolation, purification, and characterization of PSPB from elk and moose placenta. The isolation procedure, which included extraction, acidic and ammonium sulfate precipitation, and ion exchange, gel filtration, and affinity chromatographies, was based on that first described by Butler and colleagues [7] for bPSPB.

The unique steps of extraction were thawing and freezing cotyledons three times and stirring with sand to abrade the surface binucleate cells. This limited the isolation of unwanted sub-epithelial proteins.

Acidic precipitation was used in PAG isolations [13–15]. In our study, we found that although most immunoreactive proteins remained in the supernatant after pH treatment, the acidic precipitation may be not necessary. Because a protein is least soluble when in a solution at a pH equal to the isoelectric point for the protein, the acidic precipitation might have resulted in loss of some PSPB isoforms that

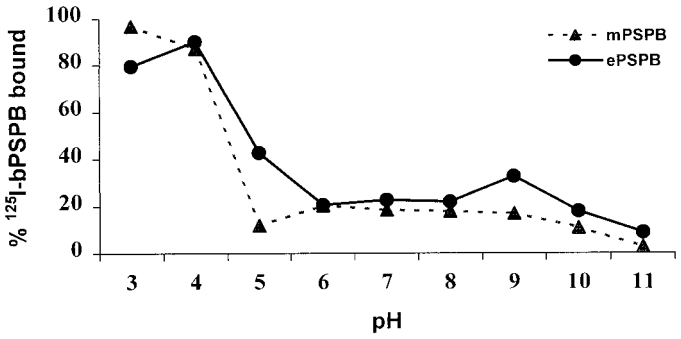

FIG. 6. Effect of change in pH on serum PSPB immunoreactivity. Sera containing PSPB were treated at different pH for 2 h, and the immunoreactivities were tested for inhibition of binding using sheep anti-moose PSPB IgG. Nonpregnant moose serum as a reference had 100% binding.

had a low isoelectric point. This could be one reason why the isoelectric point of elk and moose PSPB was higher than that reported for bovine [7] and ovine [10] PSPB.

The estimated carbohydrate content of elk and moose PSPB was 4.98% and 3.15%, respectively, which was close to that of bPSPB (5%) [9] and less than that of bPAG-1 (10.02 ± 1.09%) [13]. Because PSPB or PAGs are glycoproteins, varied content of sialic acid could have caused a difference in isoelectric point. The isoelectric points of bPSPB ranged from 4.0 to 4.4 [9] and those of oPSPB varied from 4.06 to 4.65 [10]. The range for PAG was wider, from 4.4 to 5.4 for bPAG-1 and from 4.8 to 6.2 for goat PAG. This study showed that elk and moose PSPB had isoelectric points from 4.8 to 6.2 and 4.8 to 6.7, respectively. In both species, the major band of PSPB, 57 kDa for elk and 58 kDa for moose, had three isoelectric points that all included a 4.8 and the isoelectric point of 31-kDa protein. The isoelectric point of elk and moose PSPB was higher than expected for aspartic acid proteinase proteins. This may be caused by the content of sialic acid and possibly by the acidic precipitation step (pH 3.6) during purification.

The proteins from elk and moose placenta had similar chromatographic profiles. When applied to the DEAE-cellulose column, the Sephadex G-75 column, or the affinity column, the majority of the immunoreactive proteins from elk and moose placenta were eluted at similar conditions. This indicated that the characteristics of elk and moose PSPB are similar.

The molecular masses of mPSPB were 58 kDa and 31 kDa, and those of ePSPB were 57 kDa, 45 kDa, and 31 kDa. The major band was at 58 kDa and 57 kDa, respectively. These molecular sizes are in the range of other PSPB or PAGs. Bovine PSPB had molecular masses of 37 kDa to 78 kDa with a major band at 64 kDa [9], and molecular masses of ovine PSPB were 66, 59, and 43 kDa [10]. Bovine PAG-1 isolated by Zoli and coworkers [13] had a molecular mass of 67 kDa, and goat PAGs had molecular masses of 62, 59, and 55 kDa [15]. Xie and Nagel [22] suggested that PSPB or PAGs were initially produced as large proteins and then became smaller by posttranslational modification.

Although all bands of elk and moose PSPB were visible after Western blotting using antibodies to bPSPB, only the major proteins, 58 kDa for mPSPB and 57 kDa for ePSPB, were present on SDS-PAGE (10% acrylamide) stained with Coomassie Blue after affinity column chromatography. One reason may have been that a low amount of protein was present. The 31-kDa mPSPB and the 31-kDa and 45-kDa ePSPB were only a small portion of the total PSPB. Another possible reason may have been the immunoreactivity of proteins. Zoli and coworkers [13] found that the immunoreactivity decreased from the most basic bPAG-1 to the most acid bPAG-1, and there was good correlation among sialic acid content, isoelectric points, and immunoreactivity. This might also be true for elk and moose PSPB.

The isolation procedure was monitored using the bovine PSPB RIA. Thus, only the proteins that were recognized by anti-bPSPB antibody were chosen for subsequent isolation steps. The Ouchterlony double immunodiffusion test showed that there was a smooth precipitate line between ePSPB and mPSPB, whereas bPSPB had a precipitate spur with ePSPB or mPSPB. This indicated that ePSPB and mPSPB had some similar epitopes to that of bPSPB, but there were also differences. It is possible that some elk or moose PSPB with unique epitopes might have been lost during purification.

Both elk and moose PSPB are very stable. Because they might be inactive proteinases, only the immunoreactivity can be tested. When stored at 4°C, or even at room temperature, the immunoreactivity of elk and moose PSPB did not change over a long period of time (data not shown). When the PSPBs were treated at different pHs, they denatured at acid conditions and were stable at neutral pH. The results after basic pH treatment were unexpected. Because we did not adjust the pH back to pH 7.5 before the RIA, we do not know whether the change in binding rate was caused by the change in immunoreactivity or the change in the RIA buffer pH.

An attempt was made to determine the N-terminal amino acid microsequence of elk and moose PSPB. The bands tested were 57, 45, and 31 kDa for elk and 58 and 31 kDa for moose protein. Because the N-terminals of the proteins were blocked at natural conditions, the sequence was not obtainable.

In summary, the ePSPB and mPSPB were isolated, purified, and partially characterized from elk and moose placenta, respectively. Although some characteristics are not known and the biofunction of PSPB is not clear, ePSPB and mPSPB can be used as an antigen to establish a specific RIA to quantify elk and moose PSPB in serum.

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