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DETECTION OF INSULIN-LIKE GROWTH FACTOR BINDING PROTEINS (IGFBPs) IN PORCINE SERUM

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Multiple insulin-like growth factor binding proteins (IGFBPs) are found in sera of various species. This study was conducted to investigate whether IGFBPs in porcine serum could be detected using antibodies and antisera raised against human IGFBP-1, -2, -3 and -4. Western ligand blot procedure was used for the identification of IGFBPs in porcine serum. The results presented in this work showed that the heterologous antibodies can be used to identify IGFBP-1, IGFBP-2 and IGFBP-3, but not IGFBP-4 in the porcine serum. We recommend an affinity-purified goat polyclonal antibody (Cat No DSL-R00337) from Diagnostic System Laboratories Inc. (DSL, Webster, USA) for the detection of porcine IGFBP-1, an affinity-purified goat polyclonal antibody from DSL (Cat No DSL-R00437) or a goat polyclonal antibody from Santa Cruz Biotechnology, Inc., Santa Cruz, USA (Cat No sc-6002) for the detection of porcine IGFBP-2 and, finally, an affinity-purified goat polyclonal antibody from DSL (Cat No DSL-R00536) for the detection of porcine IGFBP-3, all following a reducing SDS PAGE.

Key words: IGFBP-1, IGFBP-2, IGFBP-3, IGFBP-4, Western blotting, pig

INTRODUCTION

Insulin-like growth factors (IGFs) are very potent mitogenic growth factors that have been shown to affect proliferation and differentiation of a wide variety of cell types (Sara and Hall, 1990). *In vivo*, IGFs are bound to one of six different IGF binding proteins (IGFBPs), which bind to IGFs with high affinity and specificity (Clemmons, 1997; Firth and Baxter, 2002). Five of these have been described in pigs (McCusker *et al*., 1985; Coleman *et al*., 1991). IGFs circulate in plasma tightly bound to specific IGFBPs (Humbel, 1990). Binding of IGFs to IGFBPs extends the half-life of IGFs and regulates their biological activity (Clemmons, 1997). The predominant IGFBP in porcine blood is IGFBP-3, other circulating IGFBPs include IGFBP-1, -2 and -4 (Coleman *et al*., 1991; Dauncey *et al*., 1993). The concentration of IGF-I in sera of newborn piglets may be influenced by different factors (Stojić *et al*., 2003).

In parallel with humans, IGFs and their binding proteins are expected to play an important role in the process of domestic animal growth. Changes in circulating levels of IGFBPs may impact the rate and efficiency of growth. A number of groups have studied the effects of various factors on pig growth, but few have tried to identify the molecular mechanisms involved. Guan and co-workers investigated how the growth of pigs was influenced by dietary protein content and growth hormone administration and found that growth was related to IGF-I expression and alterations in circulating IGFBPs concentrations (Guan *et al*., 1997).

Taking into account the importance of porcine growth in meat production, we considered the pattern of circulating IGFBPs in porcine circulation an important issue of research. A general lack of commercially available antibodies directed against porcine IGFBPs together with the reasonable sequence homology that exists among IGFBPs of different mammalian species made a ground for this work. The present study was undertaken to examine the usefulness of antibodies and antisera directed against human IGFBPs for the detection and identification of porcine circulating IGFBPs.

MATERIALS AND METHODS

Samples

Sera of healthy swine were provided by dr Marko Kirovski, from the Veterinary Institute, Belgrade, Serbia. After separation from the coagulum, sera were stored at -20 ^{o}C until use. The study was approved by the local institutional ethical committee.

Electrophoresis and detection of IGFBPs

The porcine sera were analysed both by reducing and by non-reducing SDS PAGE using 4% stacking gel and 12% resolving gel, following the procedure given by Hossenlopp *et al*., 1986. The sera were diluted (1:10 or 1:20) in PBS buffer (0.05 M sodium phosphate, 0.15 M NaCl, pH 7.5), mixed with equal volumes of the sample buffer (0.125 M Tris-HCl, containing 4% (w/v) SDS, 20% (v/v) glycerol, 0.01% (w/v) bromphenolblue, pH 6.8), boiled for 7 min and then loaded onto the gels (50 μL). The sample buffer for the reducing SDS PAGE also contained 10% (v/v) 2-mercaptoethanol. Low range molecular mass markers (Bio-Rad Laboratories) were run in parallel. The sera were electrophoresed in a Mini-Protean 3 Cell (Bio-Rad Laboratories) at a constant voltage of 150 V. After SDS PAGE, the proteins from a gel were transferred to a nitrocellulose (NC) membrane (0.45 μm, Whatman Protran, PerkinElmer Life and Analytical Sciences, Boston, USA), which was used for blotting essentially as described (Baričević *et al*., 2006). Transfer was carried out at a constant voltage of 25 V during 1 h using a Trans-Blot SD Semi-Dry Transfer Cell (Bio-Rad) and a transfer buffer (0.025 M Tris-HCl, 0.19 M glycine, pH 8.3, 20% (v/v) methanol). Electrotransfer was confirmed by reversible staining of the membrane with 5% Ponceau S solution (1 min) and the positions of the mass markers and of serum albumin were labelled on the membrane. Transferred proteins were analysed by Western immunoblotting

(WIB). Non-specific binding on NC membrane for WIB was prevented by immersing it in TBST buffer containing 5% non-fat dry milk, for 1 h at room temperature. Membranes were shaken in TBST containing 1% non-fat dry milk and primary antibody (or antiserum), overnight at 4°C. Thirteen different polyclonal antibodies and antisera raised against IGFBPs were used in this experiment. The goat polyclonal anti-IGFBP-1 (Cat No sc-6072), anti-IGFBP-2 (sc-6002), anti-IGFBP-3 (sc-6004) and anti-IGFBP-4 (sc-6005) antibodies were purchased from Santa Cruz Biotechnology, Inc, Santa Cruz, USA. The affinity purified goat polyclonal anti-IGFBP-1 (Cat No DSL-R00337), anti-IGFBP-2 (DSL-R00437) and anti-IGFBP-3 (DSL-R00536) antibodies and goat anti-IGFBP-1 antiserum (DSL-R00336) were from Diagnostic Systems Laboratories (DSL) Inc, Webster, USA. Rabbit antisera against IGFBP-1 (Cat No PAP1), against IGFBP-2 (PAQ1), against IGFBP-3 (PAV1) and against IGFBP-4 (PAR1) were from GroPep, Adelaide, Australia. A rabbit polyclonal anti-IGFBP-2 antibody (Cat No A682/R7H) was bought from Biogenesis, Poole, UK. Different dilutions of primary antibodies and antisera were tested in WIB (from 1:50 to 1:10000).

The membranes were thoroughly washed and further incubated with secondary antibodies for 1 h at room temperature. Two different horseradish peroxidase (HRP)-conjugated secondary antibodies were used for detection of the primary antibodies bound to NC membranes: swine anti-goat (SAG) IgG (Biosource, Camarillo, USA) and donkey anti-rabbit (DAR) IgG (GE Healthcare, Amersham, Little Chalfont, UK). SAG IgG was used in 1:10000 dilution, whereas DAR IgG was used in 1:7000 dilution. After extensive washing of the membranes, IGFBPs were detected using the enhanced chemiluminescence (ECL) kit (GE Healthcare, Amersham, Little Chalfont, UK). Visualisation of IGFBPs bands was by autoradiography. The X-ray films and autoradiography reagents were from KODAK, Paris, France. All other chemicals were of reagent grade (Sigma-Aldrich, Steinheim, Germany).

RESULTS

The experiments were done with thirteen different antibodies (and antisera) and the results were grouped in four figures, according to the particular IGFBP to be detected. Only the representative results are shown.

WIB analysis of the porcine serum using anti-IGFBP-1 antibodies from different producers is shown in Figure 1. The porcine serum proteins separated by SDS PAGE, under non-reducing or reducing conditions, were blotted to the membranes and probed with the following antibodies: a goat anti-IGFBP-1 antibody (dilution 1:1000) from Santa Cruz Biotechnology, Inc. (Figure 1a and 1b), an affinity purified goat anti-IGFBP-1 antibody (dilution 1:10000) from DSL (Figure 1c and 1d) and a goat anti-IGFBP-1 antiserum (dilution 1:5000) from DSL (Figure 1e). IGFBP-1 bands, according to the literature, were those between the positions of 31 kD and 45 kD mass markers, seen as one faint band in Figure 1b and multiple bands in Figures 1d and 1e (marked with an asterisk). The strong immunoreactive bands above the position of the 45 kD mass marker were also detected (see the blots given in Figure 1a, 1c, 1d and 1e). These bands were the

only immunoreactive bands obtained on blots following the non-reducing electrophoresis. Comparing these blots it appears that an affinity-purified anti-IGFBP-1 antibody, purchased from DSL, was the antibody of choice for the detection of porcine IGFBP-1.

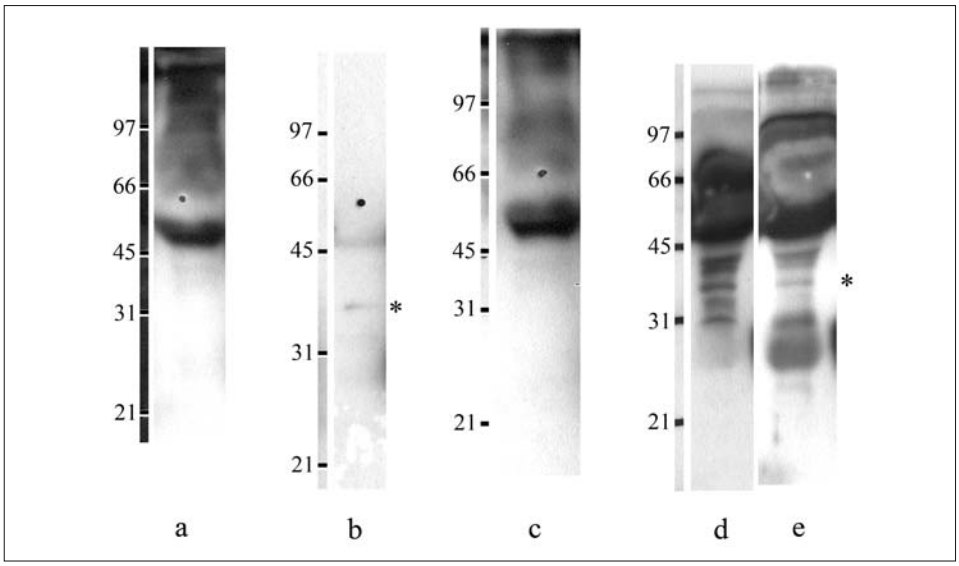

Figure 1. WIB of the porcine serum IGFBP-1 probed with different antibodies: a goat polyclonal antibody from Santa Cruz Biotechnology Inc. (dilution 1:1000), done following a non-reducing (1a) and a reducing SDS PAGE (1b), a goat polyclonal antibody from DSL (dilution 1:10000), done following a non-reducing (1c) and a reducing SDS PAGE (1d) and a goat antiserum (dilution 1:5000) after a reducing SDS PAGE (1e). Secondary antibody was HRP-conjugated SAG IgG (1:10000). The positions of mass markers are indicated on the left and those of IGFBP-1 with an asterisk

WIB analysis of the porcine serum using different anti-IGFBP-2 antibodies is shown in Figure 2. The porcine serum proteins separated under non-reducing or reducing conditions, were blotted and probed with the following antibodies: a goat anti-IGFBP-2 antibody (dilution 1:1000) from Santa Cruz Biotechnology, Inc. (Figure 2a and 2b), an affinity-purified goat anti-IGFBP-2 antibody (dilution 1:10000) from DSL (Figure 2c and 2d), a rabbit anti-IGFBP-2 antiserum (dilution 1:50) from GroPep (Figure 2e) and a rabbit anti-IGFBP-2 antibody (dilution 1:1000) from Biogenesis (Figure 2f). IGFBP-2 bands were those seen between the positions of 31 kD and 45 kD mass markers, marked with an asterisk. The clearly visible bands were obtained both with anti-IGFBP-2 antibody from Santa Cruz Biotechnology, Inc. and from DSL. These two blots looked very similar to each other. The blot done using GroPep antiserum contained a faint doublet below the 31 kD marker. The literature data suggested the IGFBP-2 band to be positioned

above the 31 kD marker. This antiserum could not be used for the identification of porcine IGFBP-2 because of the bad resolution and strong background staining. It appears that a low concentration of specific anti-IGFBP-2 antibody was present in the antiserum. Stronger bands of IGFBP-2 were obtained when the blots followed reducing SDS PAGE (Figures 2b and 2d) than those obtained on the blots after non-reducing electrophoresis (Figures 2a and 2c). Similarly to anti-IGFBP-1 blotting, the immunoreactive bands above the position of the 45 kD mass marker were also detected (see the blots given in Figures 2a and 2c), but only after non-reducing electrophoresis. The IGFBP-2 band obtained using anti-IGFBP-2 antibody purchased from Biogenesis, most likely, was a doublet just above the 31 kD marker.

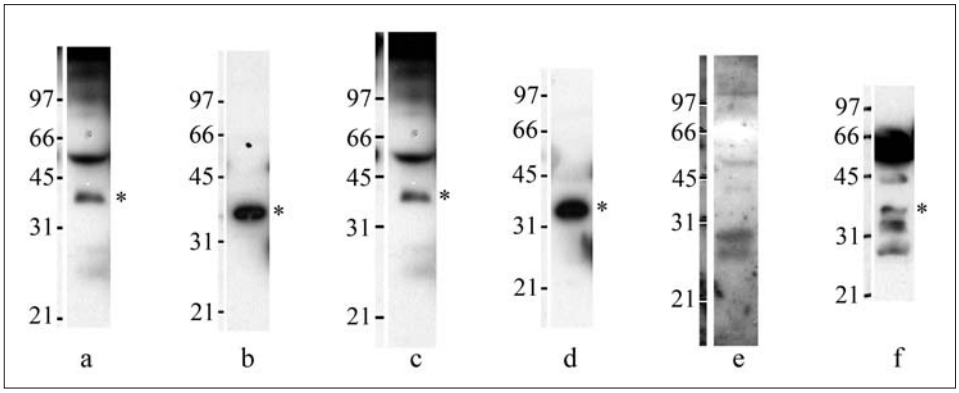

Figure 2. WIB of the porcine serum IGFBP-2 probed with different antibodies: a goat polyclonal antibody from Santa Cruz Biotechnology Inc. (dilution 1:1000), done following a non-reducing (2a) and a reducing SDS PAGE (2b), a goat polyclonal antibody from DSL (dilution 1:10000), done following a non-reducing (2c) and a reducing SDS PAGE (2d), a rabbit antiserum from GroPep (dilution 1:50) after a non-reducing SDS PAGE (2e) and a rabbit antiserum from Biogenesis (dilution 1:1000) after a reducing SDS PAGE (2f). Secondary antibody was either HRP-conjugated SAG IgG (1:10000, 2a to 2d) or HRP-conjugated DAR IgG (1:7000, 2e and 2f). The positions of mass markers are indicated on the left and those of IGFBP-2 with an asterisk

WIB analysis of the porcine serum using different anti-IGFBP-3 antibodies is shown in Figure 3. The porcine serum proteins separated under non-reducing or reducing conditions, were blotted and probed with the following antibodies: a goat anti-IGFBP-3 antibody (dilution 1:1000) from Santa Cruz Biotechnology, Inc. (Figure 3a and 3b), an affinity purified goat anti-IGFBP-3 antibody (dilution 1:10000) from DSL (Figure 3c and 3d) and a rabbit anti-IGFBP-3 antiserum (dilution 1:100) from GroPep (Figure 3e). IGFBP-3 bands were marked with an asterisk. In the WIB obtained using DSL anti-IGFBP-3 antibody after the reducing SDS PAGE, the porcine serum exhibited the characteristic doublet in the region of approximately 45 kD (Figure 3d). A faint doublet band was present on the blot

obtained using the same anti-IGFBP-3 antibody, but only after the non-reducing electrophoresis (Figure 3c). Anti-IGFBP-3 antibody from Santa Cruz Biotechnology, Inc. could also be used for the identification of IGFBP-3 in the porcine serum, but only one band was present (Figures 3a and 3b). A band of IGFBP-3 was also obtained using anti-IGFBP-3 antiserum from GroPep, although of relatively poor resolution (Figure 3e).

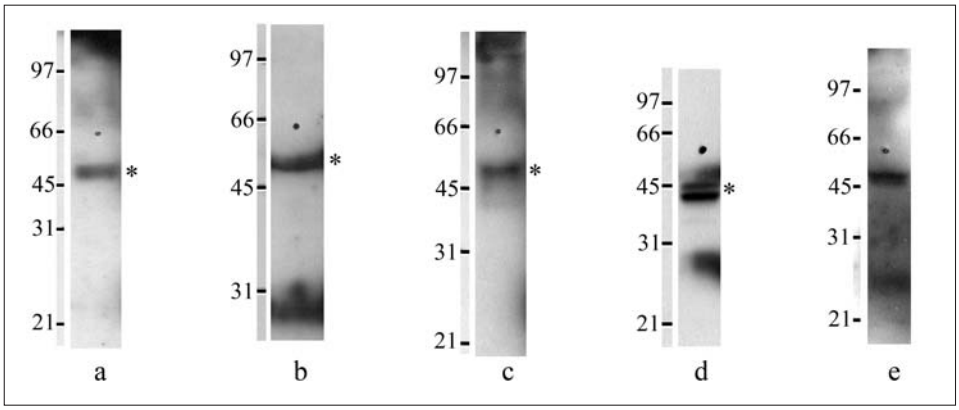

Figure 3. WIB of the porcine serum IGFBP-3 probed with different antibodies: a goat polyclonal antibody from Santa Cruz Biotechnology Inc. (dilution 1:1000), done following a non-reducing (3a) and a reducing SDS PAGE (3b), a goat polyclonal antibody from DSL (dilution 1:10000), done following a non-reducing (3c) and a reducing SDS PAGE (3d) and a goat antiserum from GroPep (dilution 1:100) after a non-reducing SDS PAGE (3e). Secondary antibody was either HRP-conjugated SAG IgG (1:10000, 3a to 3d) or HRP-conjugated DAR IgG (1:7000, 3e). The positions of mass markers are indicated on the left and those of IGFBP-3 with an asterisk

WIB analysis of the porcine serum using different anti-IGFBP-4 antibodies is shown in Figure 4. The porcine serum proteins separated under non-reducing or reducing conditions were blotted and probed with either a goat anti-IGFBP-4 antibody (dilution 1:1000) from Santa Cruz Biotechnology, Inc. (Figure 4a and 4b) or a rabbit anti-IGFBP-4 antiserum (dilution 1:100) from GroPep (Figure 4c). No clearly visible immunoreactive IGFBP-4 bands could be detected under the experimental conditions used in this work. IGFBP-4, according to the literature, should be below the 31 kD marker. The antibody and antiserum, directed against human IGFBP-4, thus, cannot be used for the detection of porcine serum IGFBP-4.

Besides molecular mass markers, the positions of IGFBPs on autoradiograms were confirmed using samples that are known to contain particular IGFBPs (data not shown): non-glycosylated rhIGFBP-3 (29 kD) from DSL (Webster, USA), IGFBP-1, isolated from the citosol of human placenta (Masnikosa *et al*., 2003) and the seminal plasma from healthy men, having predominantly IGFBP-2 and IGFBP-4 (Nikolić *et al*., 2003). As a general rule, the immunoblotting done with the porcine serum prepared under the reducing

conditions preceding the SDS PAGE gave better resolution of IGFBP bands on the blots. Strong immunoreactive bands, which were seen in the range of higher molecular masses after the non-reducing electrophoresis, almost disappeared upon protein reduction.

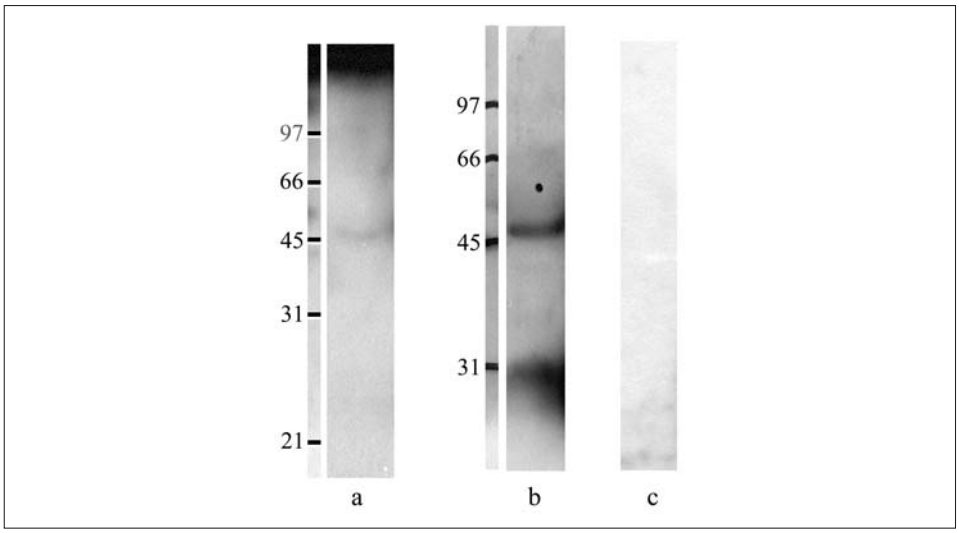

Figure 4. WIB of the porcine serum IGFBP-4 probed with different antibodies: a goat polyclonal antibody from Santa Cruz Biotechnology Inc. (dilution 1:1000), done following a non-reducing (4a) and a reducing SDS PAGE (4b) and a goat antiserum from GroPep (dilution 1:100) after a non-reducing SDS PAGE (4c). Secondary antibody was either HRP-conjugated SAG IgG (1:10000, 4a and 4b) or HRP-conjugated DAR IgG (1:7000, 4c). The positions of mass markers are indicated on the left

DISCUSSION

In this study we report the use of WIB analysis for the detection of IGFBP-1, -2, -3 and -4 in the porcine serum. Most of the available literature on detection and identification of IGFBPs in porcine sera is based on ligand blotting (McCusker *et al*., 1991; Hathaway *et al*., 2003). Specific antibodies and antisera, raised against IGFBPs from domestic animal sera, are seldom found on the laboratory market. Taking this fact into account, we aimed to investigate the extent of usefulness of the antibodies directed against human IGFBP-1 to -4 in the detection of IGFBPs from porcine serum.

Human (h) IGFBP-1 has 234 amino acid residues and a deduced Mr of 25.3 kD (Brinkmann *et al*., 1988). It migrates with an apparent Mr of 25-31 kD on non-reducing SDS PAGE and as a 32-34 kD band on reducing SDS PAGE (Rechler, 1993; Shimasaki and Ling, 1991). hIGFBP-2 is a 289-residue protein (Binkert *et al*., 1989). On non-reducing PAGE, hIGFBP-2 migrates as a non-

glycosylated protein of approximately 34 kD (Yang and Rechler, 1993). hIGFBP-3 is composed of 264 residues and its Mr would be 29 kD based on its sequence (Wood *et al*., 1988). However, its actual Mr ranges from 40 to 45 kD, because it is heavily glycosylated (Firth and Baxter, 1999). On non-reducing SDS PAGE gels, hIGFBP-3 is usually found as a characteristic doublet (Firth and Baxter, 1999). hIGFBP-4 contains 237 amino acids and one N-linked glycosylation site (LaTour *et al*., 1990). The deduced Mr of hIGFBP-4 is 26 kD (Shimasaki *et al*., 1990) and it occurs both in N-glycosylated and non-glycosylated forms. On non-reducing SDS PAGE, glycosylated IGFBP-4 migrates with an apparent Mr of 28 kD, whereas the non-glycosylated form migrates as a 24 kD band (Zhou *et al*., 2003).

We detected IGFBP-1, IGFBP-2 and IGFBP-3 in the porcine serum using WIB and polyclonal antibodies directed against human IGFBP-1 to -3. The affinity-purified goat polyclonal anti-IGFBP-1 antibody from DSL recognised porcine IGFBP-1 as multiple bands placed above the position of the 31 kD mass marker. DSL antiserum directed against human IGFBP-1 gave somewhat poorer resolution of IGFBP-1 bands. Considering porcine IGFBP-2, the immunoreactive protein having an apparent molecular mass of 34 kD was detected in our study using polyclonal antibodies directed against human IGFBP-2. Almost identical blots were obtained using anti-IGFBP-2 antibodies from Santa Cruz Biotechnology Inc. and DSL. A doublet band characteristic of IGFBP-3 was detected in our work to be at a position of 45 kD mass marker using WIB and a polyclonal antibody from DSL raised against human IGFBP-3. We were unable to detect porcine IGFBP-4 using a polyclonal antibody directed against human IGFBP-4.

Ligand blot analysis of porcine granulosa cell conditioned medium revealed five bands of IGF binding activity with apparent molecular sizes of 44, 40, 34, 29, and 22 kD. Mondschein and coauthors identified 40-44 kD bands by immunoprecipitation with an antibody to porcine IGFBP-3, the 34 kDa band was immunoprecipitated by an antibody to the rat IGFBP-2, whereas the authors were unable to immunoprecipitate the 29 and 22 kDa bands with any of the antibodies tested including a panel of monoclonal antibodies to human IGFBP-1 (Mondschein *et al*., 1990). Bands at 43, 39, 32 and 24 kD were detected by ^{125}I-IGF-I ligand blotting of the porcine serum (Hathaway *et al*., 2003).

Coleman and colleagues reported three distinct species of IGFBP in porcine serum by NH_2-terminal amino acid sequence analysis: IGFBP-2 (34 kD), three isoforms of IGFBP-3 (43, 40 and 30 kD) and two isoforms of IGFBP-4 (30 and 26 kD). The three isoforms of IGFBP-3 were found to have a common NH_2-terminal amino acid sequence, as were the two isoforms of IGFBP-4 (Coleman *et al*., 1991). Mondschein and coauthors could not detect porcine IGFBP-1 using any of the antibodies tested, including antibodies to human IGFBP-1 (Mondschein *et al*., 1991). In contrast, McCusker and collaborators successively used anti-human IGFBP-1 antiserum for porcine IGFBP-1 blotting (Mc Cusker *et al*., 1991).

We concluded that heterologous antibodies can be used to identify IGFBP-1, IGFBP-2 and IGFBP-3, but not IGFBP-4 in the porcine serum. We recommend an affinity-purified goat polyclonal antibody (Cat No DSL-R00337) from Diagnostic System Laboratories Inc. (DSL, Webster, USA) for the detection of porcine IGFBP-

1, then an affinity-purified goat polyclonal antibody from DSL (Cat No DSL-R00437) or a goat polyclonal antibody from Santa Cruz Biotechnology, Inc., Santa Cruz, USA (Cat No sc-6002) for the detection of porcine IGFBP-2 and, finally, an affinity-purified goat polyclonal antibody from DSL (Cat No DSL-R00536) for the detection of porcine IGFBP-3, all following a reducing SDS PAGE.

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DETEKCIJA VEZUJUĆIH PROTEINA ZA INSULINU-SLIČNE FAKORE RASTA U SERUMU SVINJA

MASNIKOSA ROMANA, BARIČEVIĆ IVONA, LAGUNDŽIN DRAGANA i NEDIĆ OLGICA

SADRŽAJ

U serumu velikog broja vrsta sisara nalazi se veći broj različitih vezujućih proteina za insulinu-slične faktore rasta (IGF-vezujući proteini, IGFBP). Cilj ovog rada je bio da se ispita da li se IGF-vezujući proteini iz seruma svinja mogu detektovati korišćenjem antitela i antiseruma protiv humanog IGFBP-1, -2, -3 i -4. Za identifikaciju IGF-vezujućih proteina korišćena je tehnika Western ligand blota. Rezultati ovog rada su ukazali da se antitela protiv humanih IGF-vezujućih pro-

teina mogu upotrebiti za identifikaciju IGFBP-1, IGFBP-2 i IGFBP-3 iz seruma svinja, ali ne i za detekciju IGFBP-4.

Mi preporučujemo da se za detekciju IGFBP-1 koristi afinitetno prečišćeno poliklonsko antitelo, dobijeno na kozi (kataloški broj DSL-R00337), firme Diagnostic System Laboratories Inc. (DSL, Webster, SAD) Za detekciju IGFBP-2 je najbolje uzeti afinitetno prečišćeno kozje poliklonsko antitelo (kataloški broj DSL-R00437) istog proizvođača (DSL) ili takođe kozje poliklonsko antitelo od proizvođača Santa Cruz Biotechnology, Inc., Santa Cruz, SAD (kataloški broj sc-6002). Za detekciju IGFBP-3 iz seruma svinja preporučuje se afinitetno prečišćeno kozje poliklonsko antitelo firme DSL (kataloški broj DSL-R00536). Sve uzorke za SDS PAGE treba pripremiti pod redukujućim uslovima.