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Chicken and Rabbit Antibodies against Porcine Pepsinogen A

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Abstract: Isolated porcine pepsinogen A was used for the preparation of polyclonal rabbit and polyclonal chicken anti-pepsinogen A antibodies. Immunochemical properties of both immunoglobulin fractions were compared. The rabbit anti-serum was further purified using immobilized porcine pepsinogen A on magnetic cellulose beads and the resulting anti-pepsinogen A fraction proved to be applicable for the separation and the determination of porcine pepsinogen A. In contrary, antibodies prepared from chicken eggs by the same way have been found not suitable for the evaluation of the pepsinogen A level. Unexpectedly, the pre-immune fraction of chicken antibodies showed reactivity against porcine pepsinogen A and the affinity separation of specific polyclonal chicken anti-pepsinogen A antibodies on immobilized porcine pepsinogen A did not result in an enrichment of anti-pepsinogen A antibodies.

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Introduction

Immunochemical methods are frequently used to evaluate the content of pepsinogen A (EC 3.4.23.1) and pepsinogen C (EC 3.4.23.3) that are present in human gastric mucosa and in smaller amounts also in blood serum. The decrease in pepsinogen A concentration in serum was suggested as a marker of gastric cancer (Stemmermann et al., 1987; Samloff, 1989; Hallissey et al., 1994; Yoshihara et al., 1998). Similarly a lowered pepsinogen A to pepsinogen C ratio is another sign of gastric malignancy. Thus, the determination of content and mutual relationship of individual forms of human pepsins and their zymogens is important from the diagnostic point of view (Kitahara et al., 1999; Miki et al., 2003; Miki, 2006; Oishi et al., 2006). For the evaluation of the zymogen level mainly rabbit antibodies are used.

Chicken antibodies separated from egg yolks represent a suitable alternative to mammalian antibodies. Laying hens are excellent for a large scale antibody production (~ 40 g IgY/year/chicken). Moreover, the production of antibodies by hens is much more acceptable from the animal welfare point of view than use of blood of mammals to obtain antisera. Another advantage of hens is associated with their better response to mammalian antigens due to the evolutionary distance of these two species. Hence, in our experiments we use hens to develop antibodies against porcine pepsinogen A and evaluate their immunochemical characteristics.

In the present study we have compared properties of immunoglobulins isolated from the egg yolks with those present in rabbit antiserum obtained after the animal immunization with pepsinogen A isolated from porcine gastric mucosa.

Material and Methods

Material

Magnetic bead cellulose was prepared by Institute of Macromolecular Chemistry of Academy of Sciences of the Czech Republic, Prague, Czech Republic (Lenfeld, 1993; Přikryl et al., 2012).

Porcine pepsin A, bovine serum albumin, and ovalbumin were obtained from Sigma-Aldrich (St. Louis, MO, USA). Unless otherwise stated, all chemicals were of analytical grade and were also purchased from Sigma-Aldrich (St. Louis, MO, USA).

Isolation of pepsinogen A from porcine gastric mucosa

Pepsinogen A from porcine gastric mucosa was isolated using chromatography on DEAE-cellulose according to Ryle (1970).

Sample (92 ml) of extracted proteins from porcine gastric mucosa (2.5 mg/ml) was applied to the DEAE cellulose column (32×1.5 cm) equilibrated with 0.01 M phosphate buffer, pH 7.3 and separated at the flow rate of 0.25 ml/min by gradient of NaCl (0–0.6 M). Eluted fractions (3 ml) were collected and pooled based on the protein content (absorbance at 280 nm). After dialysis against the phosphate buffer the pooled fraction was rechromatographed on the same column using gradient of NaCl (0.4–0.6 M) for the protein elution. Finally, pooled fraction from the rechromatography was applied to Sephadex G-100 column (21×1.5 cm) equilibrated with 0.02 M TRIS-HCl buffer, pH 7.3 and separated at the flow rate of 0.4 ml/min. In obtained fractions (3 ml), the content of proteins (absorbance at 280 nm) was determined. Eluted protein peaks were pooled, analysed and lyophilised.

Purity and quality of isolated proteins were confirmed by native electrophoresis according to Samloff (1969), SDS electrophoresis, ELISA and Western blotting. Activity of pepsin was determined according to Anson and Mirsky hemoglobin method (1932).

Preparation of rabbit specific antisera and chicken antibodies against porcine pepsinogen A

Pepsinogen A isolated from porcine gastric mucosa was used for immunization of rabbits and laying hens. Chicken antibodies (IgY) were isolated from pooled egg yolks as described elsewhere (Hodek et al., 2013). Leghorn hen was immunized weekly by three subcutaneous injections with pepsinogen A (0.1 mg/dose/animal) emulsified in complete Freund's adjuvant (initial dose) and in incomplete adjuvant for boosters. Antibodies were isolated from egg yolks separated from the whites by two-step procedure consisting of yolk extraction by tap water (8-fold dilution, freezing, and filtration) followed by a specific precipitation of chicken antibodies at pH 4 with 8.8% sodium chloride. Precipitated antibodies were collected by centrifugation (20 min, 3,700× *g*) and the pellet was dissolved in sodium phosphate buffered isotonic saline pH 7.4. The purity of IgY fraction was checked by reduced SDS-PAGE using 8% separation gel.

Anti-pepsinogen A serum was prepared in New Zealand White rabbit by a standard procedure. The animal was immunized monthly with four doses of pepsinogen A (0.16 mg/dose) and the anti-serum was obtained from blood collected by venepuncture.

Activation of magnetic bead cellulose with divinyl sulfone and porcine pepsinogen A coupling

Previously described procedure (Liberda et al., 2002) was used for the activation of magnetic bead cellulose with divinyl sulfone. Magnetic bead cellulose (1 ml) washed with 0.5 M carbonate buffer, pH 10.7 was suspended in the same buffer (1 ml) and divinyl sulfone was added (200 μl). The suspension was shaken at room temperature for 2 hours then washed with 0.5 M carbonate buffer, pH 10.7 and 0.1 M $NaHCO_3$, pH 8.5.

Suspension of divinyl sulfone-activated magnetic bead cellulose (1 ml) was mixed with 6 ml porcine pepsinogen A (5 mg per 1 ml of 0.1 M $NaHCO_3$, pH 8.5) and shaken for 24 hours at room temperature. After washing with 0.1 M $NaHCO_3$, pH 8.5, the 3 ml glycine solution (10 mg/1 ml of 0.1 M $NaHCO_3$, pH 8.5) was added and the suspension was shaken for 24 hours at room temperature, washed with 0.1 M $NaHCO_3$, pH 8.5 and finally with 0.1 M TRIS-HCl buffer, pH 7.2. The

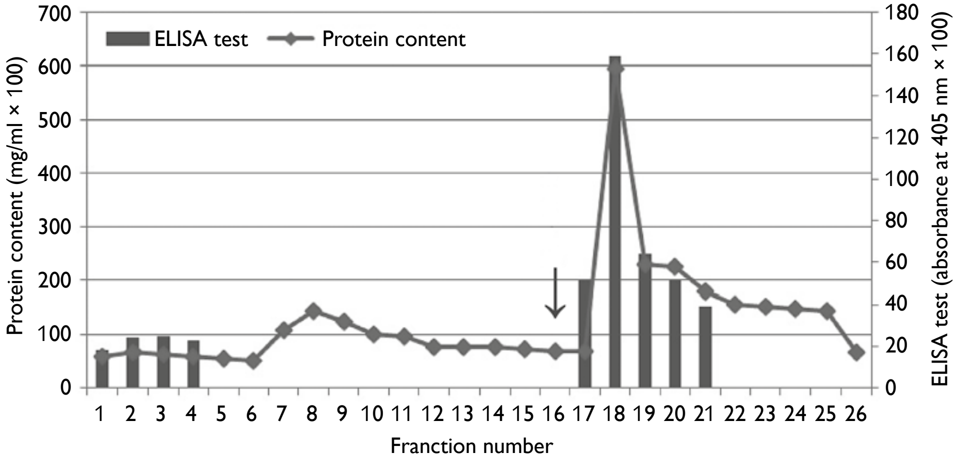

Figure 1 – Chromatogram of the affinity separation of rabbit immunoglobulins on porcine pepsinogen A immobilized to magnetic particles. Solution of rabbit immunoglobulins containing 0.75 mg of proteins per 500 μl of starting buffer; protein content – bicinchoninic acid assay (Smith et al., 1985); arrow – start of the elution with 0.05 M diethylamine (pH 11.5) solution.

immobilized pepsinogen was stored in 0.1 M TRIS-HCl buffer, pH 7.2. The amount of coupled pepsinogen (3.4 mg/ml particles) was determined indirectly from the decrease of protein content (Smith et al., 1985) of the supernatant after the incubation of the pepsinogen with activated magnetic particles.

Separation of antibodies on magnetic particles modified with porcine pepsinogen A
Magnetic particles (500 μl) modified with porcine pepsinogen A (3.6 mg per ml of the carrier) were washed with starting buffer (0.05 M TRIS-HCl pH 7.4 with 0.15 M NaCl); rabbit or chicken polyclonal anti-pepsinogen A fractions in starting buffer (0.75 mg of proteins per 500 μl) were added to washed magnetic particle suspension (500 μl) and the mixture was thoroughly stirred for 24 hours at 4 °C. The supernatant was removed and magnetic particles were washed 10 times with the starting buffer (500 μl) and the adsorbed antibodies were eluted with 0.05 M diethylamine pH 11.5 (0.5 ml fraction, 10 ml in total). The separation was followed by the determination of protein content by the bicinchoninic acid assay (Smith et al., 1985) and immunoglobulin reactivity with pepsinogen A by ELISA.

Affinity separation of rabbit immunoglobulins on immobilized porcine pepsinogen A to magnetic particles is shown in Figure 1.

ELISA testing
The antibody immunoreactivity was tested by ELISA as described elsewhere (Hodek et al., 2013). Instead of ovalbumin gelatine from cold water fish skin was used for blocking the inner surface of microplate wells. To detect rabbit or chicken

immunoglobulin – antigen binding secondary goat antibody against rabbit IgG or chicken IgY labelled with alkaline phosphatase (2,000 times diluted commercial conjugate, Sigma-Aldrich, St. Louis, MO, USA) was applied. As a chromogenic substrate, the solution of *p*-nitrophenyl phosphate (1 mg/ml in carbonate buffer) was used. Wells treated identically but with addition of equal volume of phosphate buffer saline in place of primary antibody solution were used as negative controls. The colour developed in 20 min was measured at 405 nm. The immunoreactivity of antibody samples was expressed as a difference in absorbance at 405 nm of polyclonal rabbit or chicken anti-pepsinogen A antibody samples minus pre-immune polyclonal rabbit or chicken antibody samples treated identically.

Results and Discussion

Pepsinogen A (40 mg of lyophilized protein) was isolated from porcine gastric mucosa using chromatography on DEAE-cellulose. The purified porcine zymogen

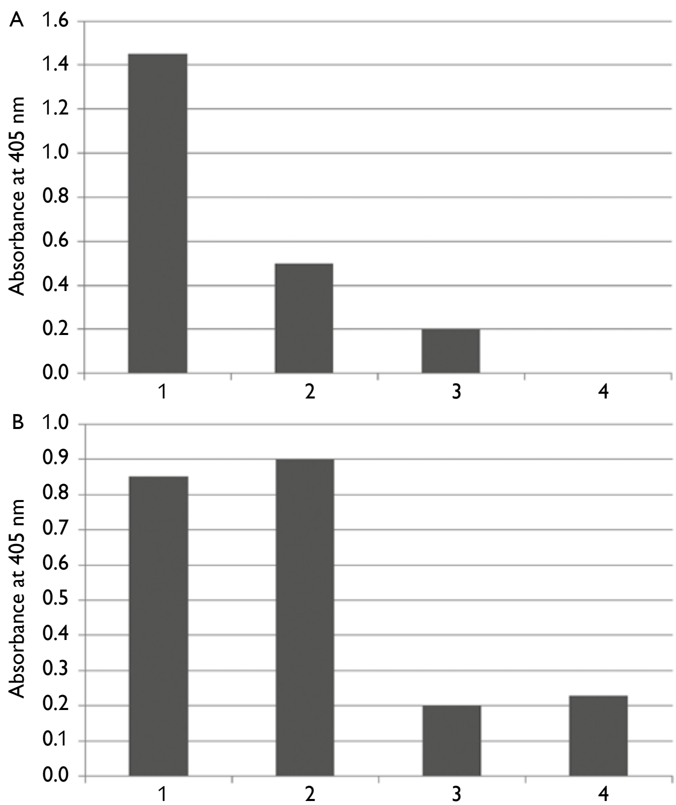

Figure 2 – ELISA test using anti-porcine pepsinogen A rabbit (A, dilution 100 times) and chicken (B, 80 μg/ml) immunoglobulins. Tested antigens: 1 – porcine pepsinogen A; 2 – bovine serum albumin; 3 – ovalbumin; 4 – porcine pepsin A. The immunoreactivity of antibody samples is expressed in the plot as a difference in absorbance at 405 nm of polyclonal rabbit or chicken anti-pepsinogen A antibody samples minus pre-immune polyclonal rabbit or chicken antibody samples treated identically.

was characterized by native electrophoresis (Samloff, 1969), SDS electrophoresis, peptic activity estimation (Anson and Mirsky, 1932), ELISA and Western blotting and further used as an immunogen to develop rabbit and chicken specific antibodies. According to SDS electrophoresis results isolated porcine pepsinogen A was contaminated by porcine serum albumin slightly. Regarding to this fact affinity purification of developed rabbit and chicken antibodies was carried out on immobilized bovine serum albumin.

The specificity of the obtained immunoglobulin samples (chicken and rabbit anti-porcine pepsinogen A antibodies) was tested by ELISA (Figure 2). Both kinds of specific antibodies reacted differently. In rabbit serum antibodies interacted specifically with the antigen, porcine pepsinogen A, much less with bovine serum albumin (2.8×) and ovalbumin and almost no positive interaction with porcine pepsin A was observed. In pre-immune rabbit serum almost no positive interaction with porcine pepsinogen A, bovine serum albumin, ovalbumin and porcine pepsin A was observed. In the case of chicken antibodies, however, the anti-porcine pepsinogen A polyclonal antibodies interacted with porcine pepsinogen A and also with bovine serum albumin (Figure 2). Similarly, in pre-immune chicken antibody sample obtained from the same hen before its immunization, an intense interaction with porcine pepsinogen A and bovine serum albumin was also observed (Figure 3). In the case of serum albumin this phenomenon might be related to the described immune responses of chickens to dietary protein antigens (Klippper et al., 2000). It is likely that bovine serum albumin is present as a protein supplement in commercial poultry feed. However, the occurrence of chicken antibodies

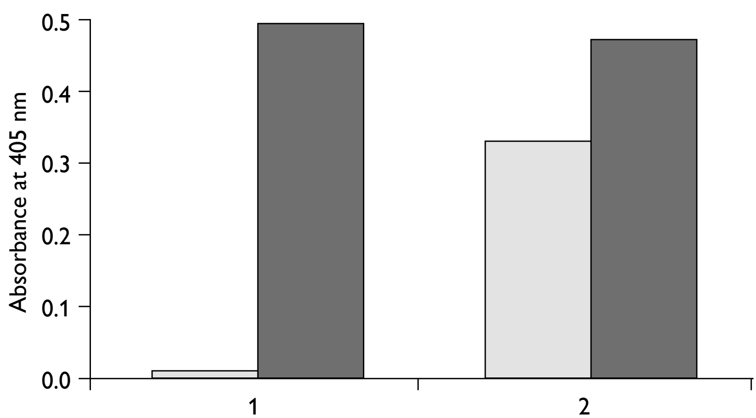

Figure 3 – ELISA test: activity of pre-immune and specific rabbit (1, dilution 100 times) and chicken (2, 80 µg/ml) immunoglobulins against porcine pepsinogen A. Pre-immune samples and samples after immunization are depicted in black and grey bars, respectively. The immunoreactivity of antibody samples is expressed in the plot as a difference in absorbance at 405 nm of polyclonal rabbit or chicken anti-pepsinogen A antibody samples minus pre-immune polyclonal rabbit or chicken antibody samples treated identically.

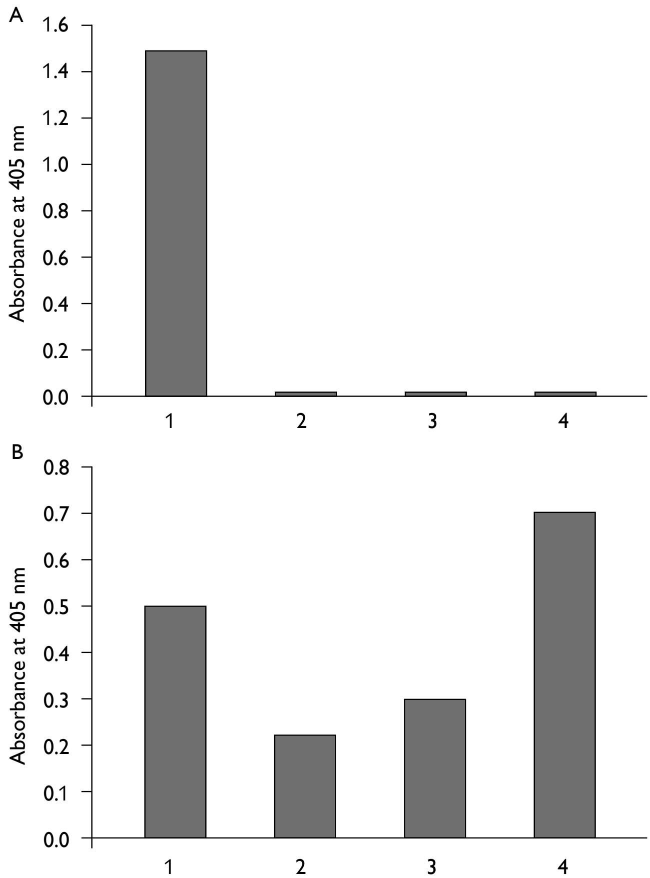

Figure 4 – ELISA test of fractions separated by affinity purification. Affinity purification of polyclonal rabbit (A) or chicken (B) antibodies on magnetic bead cellulose with immobilized porcine pepsinogen A. Tested antigens: 1 – porcine pepsinogen A; 2 – bovine serum albumin; 3 – ovalbumin; 4 – porcine pepsin A. The immunoreactivity of immunoglobulins is expressed as absorbance at 405 nm.

recognizing porcine pepsinogen A in pre-immune fraction may be caused by some cross-reactivity of the antibodies.

To obtain the anti-porcine pepsinogen A specific immunoglobulins, rabbit or chicken immunoglobulin fractions were subjected to the separation on porcine pepsinogen A immobilized to magnetic bead cellulose. This procedure resulted in purification of antibodies against pepsinogen A from rabbit anti-sera (Figure 4A) while in the case of chicken antibodies the level of these antibodies specific to pepsinogen A was not enriched; in eluted fractions from the affinity sorbent the specificity of chicken antibodies was almost the same as in non-separated ones (compare Figures 2B and 4B). The reason for the failure in the preparation of IgYs specific for pepsinogens A is unclear.

Conclusion

In conclusion our results show that in the particular case of porcine pepsinogens, the hen is not an appropriate antibody production organism.

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