

DEVELOPMENT OF FAST AND SIMPLE METHODS FOR PORCINE HAPTOGLOBIN AND CERULOPLASMIN PURIFICATION

Desarrollo de métodos rápidos y sencillos para la purificación de haptoglobina y ceruloplasmina porcina

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ABSTRACT

Two fast and simple methods for porcine haptoglobin (Hp) and ceruloplasmin (Cp) purification are described in this paper. Hp was purified by ammonium sulphate fractionation and a FPLC Superdex-200 gel chromatography. The protein obtained showed two bands with a M_r (molecular mass) of about 44 kDa and 12.9 kDa corresponding to heavy (β) and light (α) chains of Hp respectively, on sodium dodecyl sulphate-polyacrilamide gel electrophoresis, under reducing conditions. Ceruloplasmin (Cp) was isolated by one step of chromatography on amino-ethyl-derivatized Sepharose followed by gel filtration on a Superdex-200 column. The M_r of the protein, as estimated by SDS-PAGE was approximately 150 kDa. In conclusion, two new protocols have been developed for porcine Hp and Cp purification, being less time-consuming and technically demanding than those previously reported. This paper could represent an interesting guideline and be of help to obtain pure protein to use as specie-specific standard material and to produce specific antibodies.

Key words: porcine, haptoglobin, ceruloplasmin, purification.

RESUMEN

En el presente trabajo se describen dos métodos sencillos y rápidos para la purificación de la haptoglobina (Hp) y ceruloplasmina (CP). La Hp fue purificada mediante precipitación con amonio sulfato y posterior cromatografía en gel mediante un sistema de FPLC y columna de Superdex-200. La proteína obtenida mostró en cromatografía en gel de poliacrilamida, bajo condiciones reductoras, dos bandas con un peso molecular de 44 kDa y 12,9 kDa correspondientes a las dos cadenas pesadas (β) y las dos cadenas ligeras (α) de la estructura de la Hp, respectivamente. La ceruloplasmina (CP) fue purificada en un paso de cromatografía de afinidad en gel de sefarosa modificada, seguida de filtración en gel en columna de Superdex-200. El peso molecular de la pro-

teína, estimado por electroforesis en gel de poliacrilamida, fue de aproximadamente 150 kDa. En conclusión, han sido desarrollados dos nuevos protocolos para la purificación de la Hp y Cp porcina, siendo más rápidos y menos exigentes técnicamente que los anteriormente descritos. Este trabajo puede representar una interesante guía y ser de ayuda para obtener proteína pura para ser usada como material estándar especie-específico y para la producción de anticuerpos específicos.

Palabras clave: porcino, haptoglobina, ceruloplasmina, purificación.

INTRODUCTION

The acute phase response is a reaction of the organism to local or systemic disturbances in its homeostasis caused by infection, tissue injury, trauma or surgery, neoplastic growth, immunological disorders or stress (Petersen et al., 2004). During the acute phase response, there is a change in the serum concentration of certain plasmatic proteins named acute phase proteins (APPs). Some of them decrease in concentration (negative APPs) while others increase in concentration (positive APPs), and according to the magnitude of their increase they can be classified in major (10- to 100-fold increases) and moderate (2–10) (Eckersall et al., 2000).

Haptoglobin (Hp) is one of the major APPs in pigs (Eckersall et al., 1999), and it has been used to identify both clinical and subclinical disease. Research suggests that in the future, Hp measurements may routinely be used in pig production to assess animal health, optimize production rates and monitor antibiotic therapy (Eckersall et al., 1992; Eckersall et al., 1999; Petersen et al., 2004). Ceruloplasmin (Cp) is considered as a positive APP in pigs and its biosynthesis and secretion is enhanced by inflammatory cytokines showing a moderate increase (40%) in serum after an inflammatory stimulus such as turpentine oil administration (Martínez-Subiela et al., 2006). So, in pigs, Hp and Cp determination could provide an objective measure of the health status of an animal and could be used as marker of health and welfare of animals which are going to be introduced in the human food chain (Eckersall et al., 1999; Skinner et al., 2001).

One critical point in the field of veterinary APPS is the need for species-specific standard material in the assays for their quantification (Eckersall et al., 1999; Skinner et al., 2001). A pilot proficiency testing program, based on patient samples was performed on selected porcine APPs (Hp and serum amyloid A) to create reference materials for APPs tests (EU-Concerted Action Number QLK5-CT-1999-0153).

This program showed the evidence that assays developed in different laboratories could give substantially different results when the same samples were analyzed. In addition, a recent study in which two porcine reactive C protein (CRP) quantification methods were compared, showed discrepancies in the results (Kjelgaard-Hansen et al., 2006), which could be explained in part by the use of calibration material from different species.

Purification from serum of selected APPs would contribute to make species-specific standards available thereby enabling the establishment of a global accessibility to traceable species-specific calibration material as a means to decrease inter-laboratory discrepancy (Kjelgaard-Hansen et al., 2006). Traditional methods for porcine Hp (Connell et al., 1961; Shim et al., 1971; Eurell et al., 1990; Yang & Mao 1999; Hiss et al., 2003) and Cp (Broman et al., 1964; Zgirski et al., 1978; Zgirski et al., 1978; Kovár et al., 1986; Essamadi et al., 2002) purification can be troublesome and time-consuming since use, at least, two chromatography steps which implies several days of processing and demand high serum volumes.

The aim of the present study was the development of methods that allow the purification

of Hp and ceruloplasmin, in an economic and simple way.

MATERIAL AND METHODS

Animals and samples

Pigs used in this study came from the Veterinary Teaching farm of the University of Murcia, located in Southern Spain. This is a farm that integrates all phases of production, with a capacity of 200 sows and 1000 places of finishing pigs.

Samples with high concentration of Hp and Cp obtained from 3 month old pigs with acute inflammatory diseases (three animals with acute rectal prolapse and three animals with acute arthritis), were used for purification of both proteins. Specimens were collected by jugular venipuncture into Vacutainer™ Brand Sterile tubes (no additive), and after 2 hours, samples were centrifuged (4500 r.p.m., 7 min.) and serum was pooled and stored at -20°C .

Purification of haptoglobin

Three mL of the serum rich in Hp were saturated by 50% ammonium sulphate (Yang & Mao, 1999). After a gentle stirring for 30 min at room temperature, the precipitate was discarded by centrifugation and the supernatant was dialyzed against NaCl 0.9% by using NAP™ 10 desalting columns (Amersham Biosciences). After dialysis, the solution was concentrated by Centricon (Amicon, Millipore, USA) and filtered through a 0.22 μm pore-size filter (Millex-GV). The final solution was applied to a Superdex™ 200 10/300 GL column (Amersham Biosciences, Uppsala, Sweden) connected to a FPLC (performance liquid chromatography) system, equilibrated and run with Tris-HCL (TSA buffer), pH 7.5 with a flow rate of 20 mL/h at room temperature. Fractions were collected using a fraction collector (Amersham Biosciences), and the absorbance at 280 nm was recorded.

Purification of ceruloplasmin

A derivatized Sepharose 4B (Sigma-Aldrich Co., Spain) column was prepared by reaction of Sepharose with chloroethylamine. For this purpose, 5 mL of Sepharose 4B, were previously treated at 70°C for 2 h with 1.66 mL of 5 N NaOH and 0.41 mL of epichlorhydrin, and then exhaustively washed with ultrapure water. After that, the resin was suspended in 10 N NaOH and treated with 2.33 mL 100% chloroethylamine for 2 h at 70°C . The resulting derivatized Sepharose was equilibrated at pH 7.4 with 3 mM phosphate buffer, and finally activated-Sepharose was introduced into a small column (empty NAP-10 column; 6.5 cm height and gel bed dimensions 1.3 cm x 1.5 cm) and washed with 25 mL of 3mM phosphate buffer. Next morning, serum (3mL) was diluted with 20 mM ω -aminohexanoic acid (a serum protease inhibitor), until conductivity was lower than 7 ms at 18°C to ensure the ionic strength of the sample was not too high. The total quantity of acid added was 5 ml.

After adjusting the pH to 7.4 by addition of small aliquots of H_3PO_4 , the serum was applied to the gel bed previously packed in the small column. Cp retention was evidenced by the appearance of a blue band at the top of the column. The resin was washed by 15 mL of 50 mM, 20 mL of 80 mM, 10 mL of 100 mM, 10 mL of 150 mM and 10 mL of 200 mM phosphate buffer (pH 7.4). Fractions were collected manually in eppendorf tubes, with a volume of 500 μL , approximately. In each fraction, the absorbance at 280 nm was measured. Time of chromatographic process depends on the time needed to reach zero optical density after each wash with phosphate buffer of different molarities. To eliminate impurities, fractions obtained after first chromatography step, which contained Cp, were pooled and applied to a Superdex™ 200 10/300 GL column (Amersham Biosciences, Uppsala, Sweden), with 50 mM phosphate buffer pH 7, as eluent buffer, and

a flow rate of 20 mL/h at room temperature. Fractions of 500 μ l were obtained and the absorbance at 280 nm represented graphically in a chromatographic profile.

Electrophoretic analyses

One dimensional sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) with 12% polyacrylamide gel for Hp, and 7.5 % for Cp, were performed according to Laemmli's method (Laemmli, 1970) under reducing conditions (100°C for 5min in presence of the β -mercaptoethanol) (SDS-PAGE denaturant). The SDS was carried out with a Mini-PROTEAN 3, Bio-Rad Laboratories, (Inc. California, USA), and for M_r calibration, Mark12™ Unstained Standard, Invitrogen TM Corporation (USA) for Hp, and BenchMark Protein Ladder, Invitrogen TM Corporation (USA) for ceruloplasmin, were used. After the electrophoresis separation, the protein bands were revealed with a solution of 40% methanol, 10% acetic acid and 0.1% of brilliant blue Comassie R-250.

Image analysis

Electrophoretic gels were analyzed using the Gel-Pro Analyzer 3.1 software (Media Cybernetics 1993-1997). The lane profile was corrected subtracting the background baselines. The background-corrected intensity of every pixel in the box was combined to represent the protein amount in the band. The exposure intensity was automatically calculated along the centreline for each lane by the computer software. The peak area under the protein band was integrated and correlated to the M_r .

Determination of protein concentration and chromatography efficiency

Initial serum Hp and Cp concentration was measured by means of:

- Hp: commercial Tridelta Phase range serum Hp kit was used to determine the concentration of this protein (Tridelta Development Limited, Ireland). All determinations were performed on duplicate by using a biochemical analyzer Cobas Mira Plus, ABX Diagnostics (Montpellier, France).
- Cp: Cp was measured with the method developed previously (Cerón et al., 2004; Martínez-Subiela et al., 2006). All determinations were performed on duplicate by using a biochemical analyzer Cobas Mira Plus, ABX Diagnostics (Montpellier, France).

Protein concentration of fractions containing purified proteins was determined with RC DC Protein Assay, BIO-RAD Laboratories (Inc. California, USA), by using bovine serum albumin as calibrator. Performance of chromatography column was calculated as: [Purify protein obtained/ Protein introduced in the column] x 100.

RESULTS

Haptoglobin

Initial Hp concentration was 4.81 mg/mL. After gel filtration, the chromatogram obtained by FPLC showed 3 peaks (Figure 1), with the following Hp values: peak 1: 0.076 mg/mL; peak 2: 1.162 mg/mL; peak 3: 2.601 mg/mL. The second peak corresponded mainly to Hp as it could be seen by SDS-PAGE with 12% polyacrylamide gel under reducing conditions. The content of this peak yielded two bands, the first one with a M_r of 44 kDa, and the second one with 12.9 kDa (Figure 2). OD (Optical density) lane profile versus M_r (kDa) was represented (Figure 3) for Hp. First and third peak from the gel filtration corresponded to impurities. The recovery of Hp from the gel filtration chromatography was 16.11% and the time spent in the process was about 3 hours.

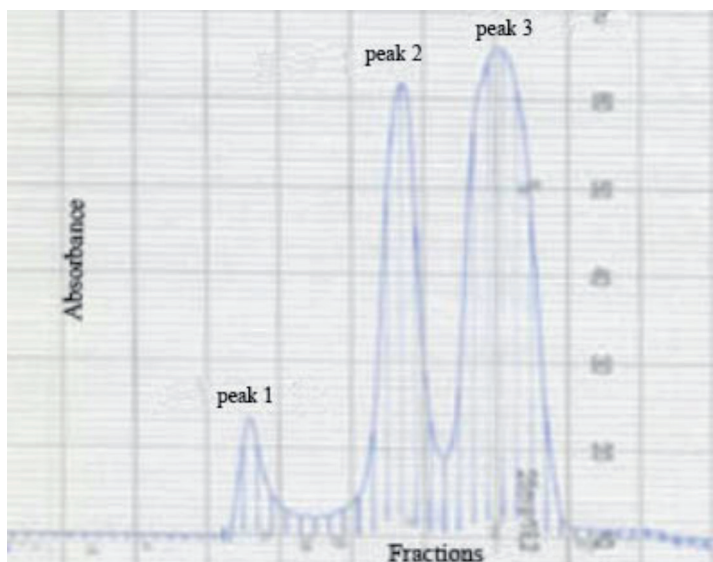


Figure 1. Chromatogram obtained after elution through Superdex 200 column, in Hp purification.

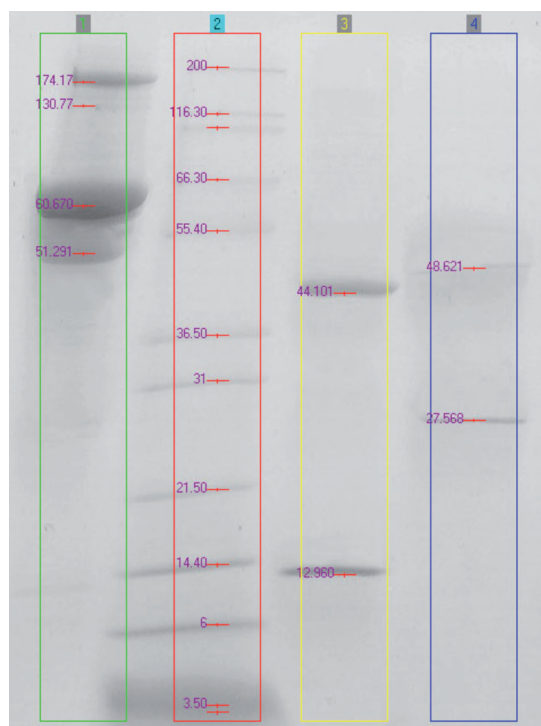


Figure 2. SDS-PAGE profile of purified Hp. Lane 1: peak 3 / Lane 2: M_r markers / Lane 3: peak 2 (5 μ g. of pure Hp) / Lane 4: peak 1.

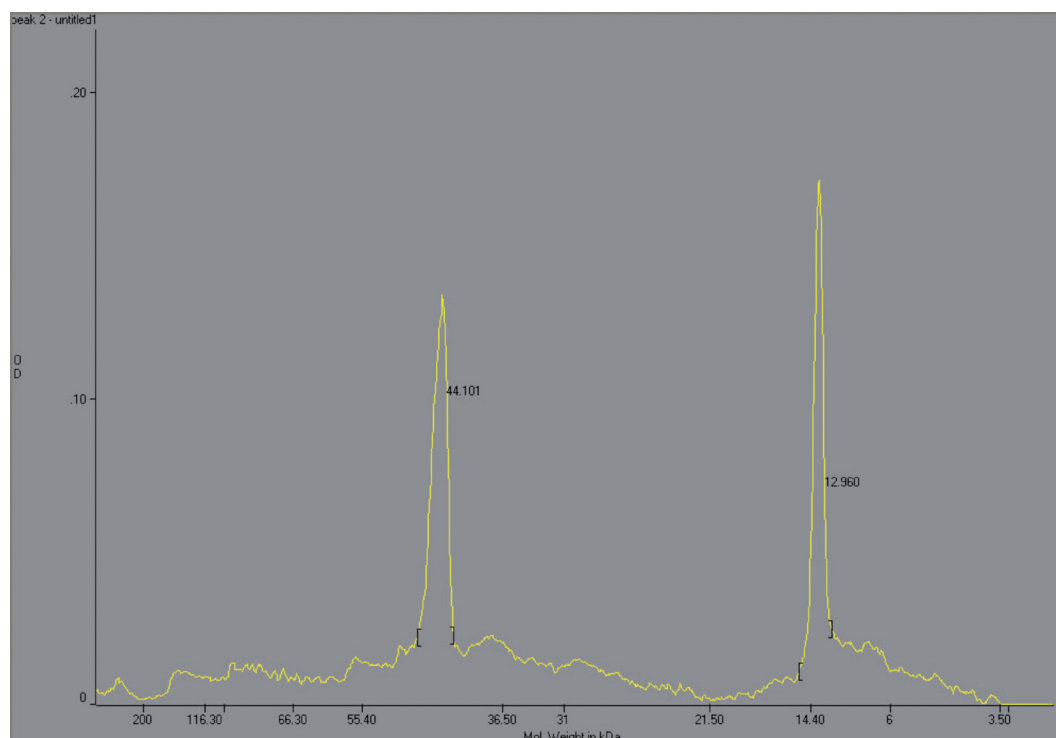


Figure 3. OD lane profile versus M_r (kDa) for Hp.

Ceruloplasmin

Initial serum Cp concentration was calculated, obtaining a value of 0.0457 Δ abs/min (absorbance increase / minute). The pig Cp bound to the derivatized Sepharose with chlorethylamine as evidenced by an intense blue colour (characteristic of copper) on the top of the column. After adsorption, the resin was washed by different phosphate buffers and the protein was eluted as a single peak with 80 mM buffer. Small peaks were also observed with 100 and 150 mM buffer (Figure 4). The electrophoresis analysis (SDS-PAGE denaturant 7.5%) of the absorbance peak fractions showed the presence of a band with a M_r of approximately 150 kDa; although less M_r impurities were also observed (Figure 5). The chromatogram obtained after gel filtration showed 2 peaks (Figure 6). The

electrophoresis analysis (SDS-PAGE denaturant 7.5%) of the second one, showed the presence of a clear band with a M_r of approximately 150 kDa (Figure 7). OD (Optical density) lane profile versus M_r (kDa) was represented (Figure 8) for ceruloplasmin. In this case, the recovery of Cp from the affinity and gel filtration chromatography could not be calculated because serum Cp concentration could not be initially determined as mg/mL, since the method employed uses Δ abs/min units, as reference standard solutions were not available. The time spent in the process was about 4 hours.

DISCUSSION

Porcine Hp and Cp, assessed in this study, might be used to determinate an APP profile integrated by a major (Hp) and a moderate (Cp)

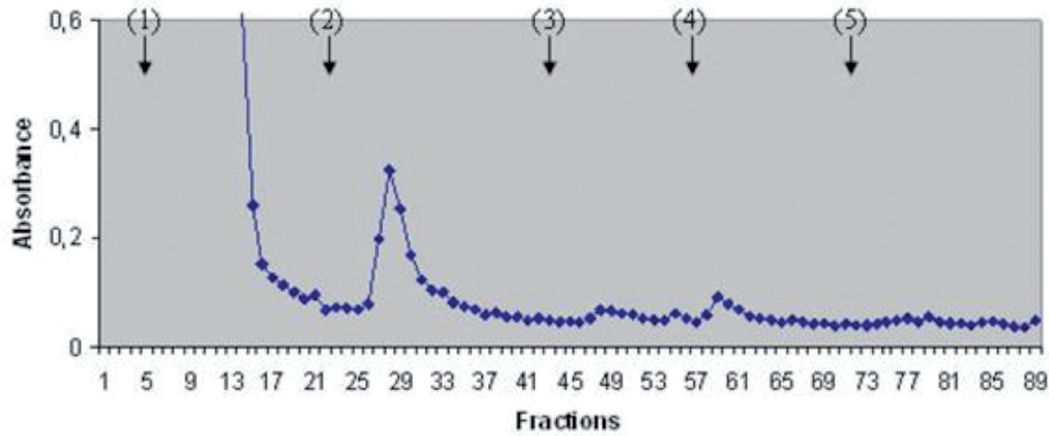


Figure 4. Elution profile of pig Cp. The resin was sequentially washed with 50mM (1), 80mM (2), 100mM (3), 150mM (4) and 200mM (5) phosphate buffer, pH 7.4.

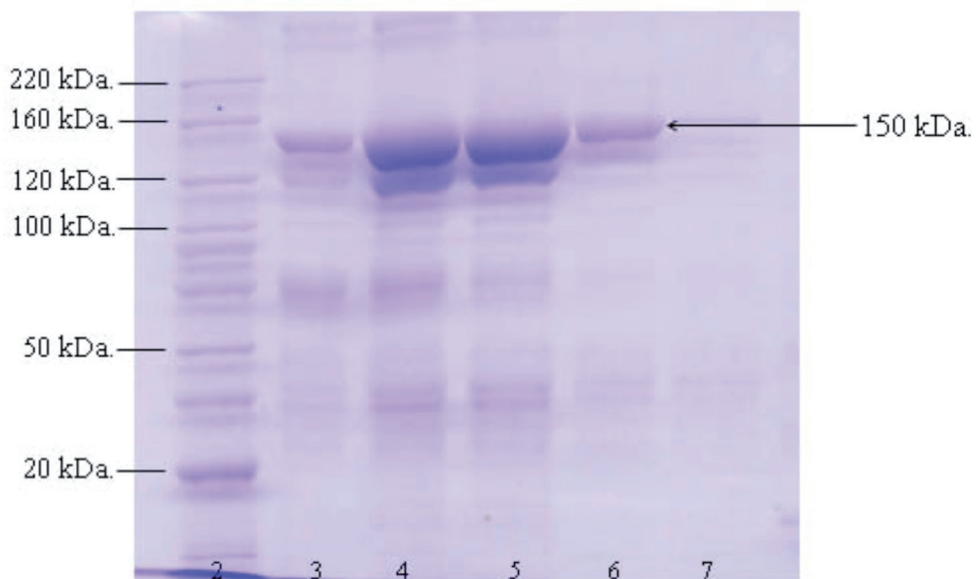


Figure 5. SDS-PAGE profile of purified Cp. Lane 2: M_r markers / Lane 3-6: absorbance peak (fractions 27-30) (15 μ g pure Cp).

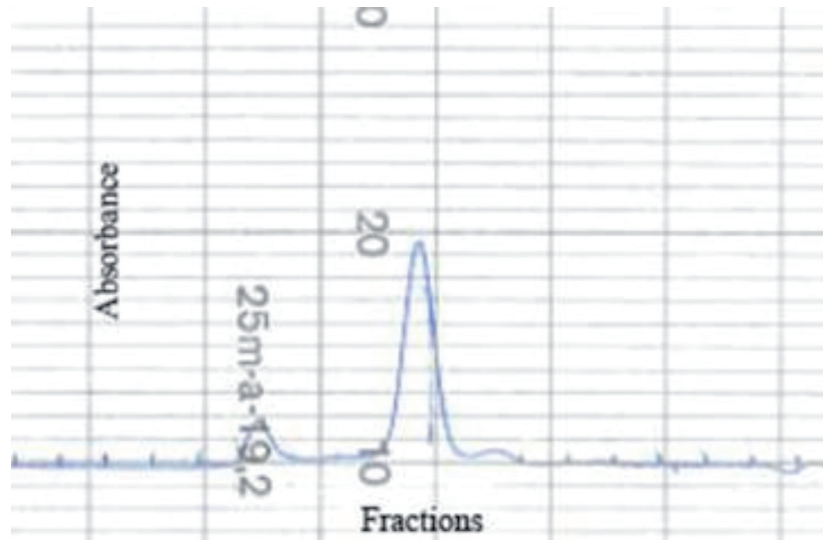


Figure 6. Chromatogram obtained after elution through Superdex 200 column, in Cp purification.

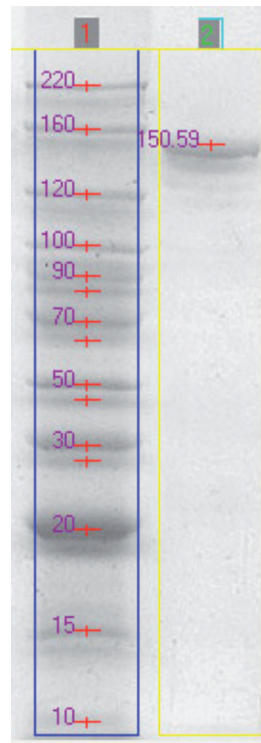


Figure 7. SDS-PAGE profile of purified Cp. Lane 1: M_r markers / Lane 2: absorbance peak (20 μ g pure Cp).

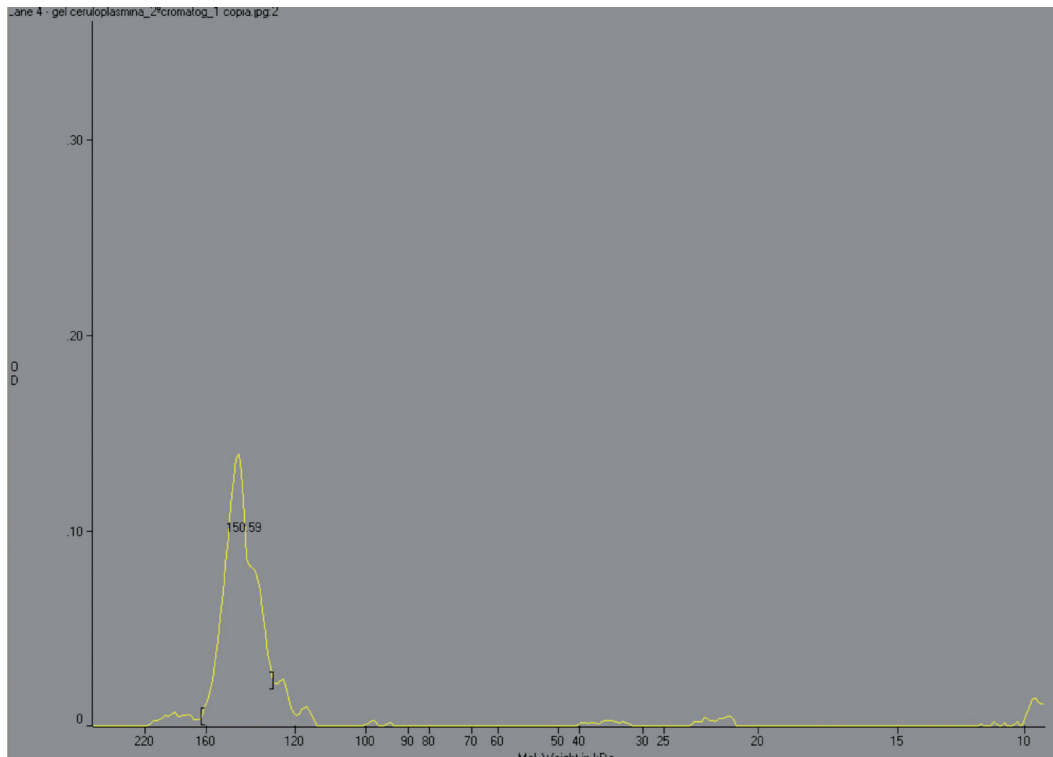


Figure 8. OD lane profile versus molecular weight (kDa) for Cp.

protein, in an easy and cheap way since both can be measured by automated spectrophotometric methods (Martínez-Subiela et al., 2006; Parra et al., 2006). However the inexistence of a validated and harmonized calibration material can represent a limit in their routine use, especially in the use of Cp. The development of simple and fast methods for Hp and Cp purification could contribute to solve the current situation.

Porcine Hp is an α_2 -glycoprotein present in the plasma, a tetramer of two identical light chains with a M_r of 12 kDa (corresponding to the light chain, α chains) and two identical heavy glycosidase chains (corresponding to the heavy chain, β chains), with a M_r of 40 kDa (Yang & Mao, 1999). The present work can provide a more simplified technique of purifying Hp than those previously reported in pigs

(Shim et al., 1971; Hiss et al., 2003), since we only use one chromatographic step whereas two or three chromatographic steps were employed in protocols reported before. On the other hand, our method might be an alternative to the one described in pigs based on a unique chromatographic step on a Superose 12 HR column by using a HPLC controller system (Yang & Mao, 1999), being a suitable choice for laboratories in which FPLC, but no HPLC is available. M_r of the Hp purified in this study agrees with previous reports and therefore the procedure described here could be widely used for porcine Hp purification. Cp is an α_2 -glycoprotein with a M_r of 132 kDa in human (Takahashi et al., 1984) and has been recognized since the 1950s as the primary copper-containing component of the blood plasma (Cerveza et al., 2000). M_r of

Cp is slightly different in other species, such as sheep and bovine with a M_r of 134 kDa (Calabrese et al., 1981; Calabrese et al., 1983), birds with 140 kDa (Calabrese et al., 1988), reptiles with 150 kDa (Musci et al., 1990) and pigs with 150 kDa (Rydén et al., 1972; Cerveza et al., 2000). The present study describes a simple porcine Cp purification protocol, based on the method described by Essamadi and Calabrese (2002) in camel but with some modifications.

The chromatographic profile of pig Cp was similar to the ones observed when Cp of other species was purified, but with differences in the ionic strength. The ionic strength of pig Cp seems to be lower than those described for human, chicken and sheep Cp which were eluted with 300 mM of phosphate buffer (Calabrese et al., 1983; Calabrese et al., 1988) and in contrast, the turtle Cp was eluted with 80 mM (Musci et al., 1990). The reported differences in the amino acid sequences in the different species studied (Katnik et al., 1998) might explain this finding. Other differences in Cp properties among different species have been found, for example the PPD (-phenylenediamine) oxidase activity/ μ g indirect-reacting copper of the plasmas, which are in the order pig > man > cattle, dog, sheep and cat, bear an approximate ratio to each other of 8 : 4 : 2 (McCosker et al., 1961), or differences in the M_r (Rydén et al., 1972). The subsequent use of a gel filtration chromatography by using a Superdex-200 column let us obtaining a high purity in the process. Our results agree with those reported by Rydén (1972) and Cerveza et al. (2000) in their Cp purifications in porcine, obtaining a single band in the polyacrilamide gel electrophoresis, of the same M_r (about 150 kDa). Therefore, we carried out a simple and fast method for porcine Cp purification, in which a significant concentration of purified protein with high purity was obtained, by using a very small serum volume (3 mL.), compared to other protocols, such as the methods described by Essamadi et al. (2002) who used 500 mL. of serum for camel Cp purification, or

methods described in human Cp purifications in which up to 100 litres were required (Noyer et al., 1980; Oosthuizen et al., 1985).

In conclusion, two methods for purification of two APPs in porcine serum: Hp and Cp have been developed and described; these methods will allow obtaining in a simple way purified proteins that might be used in several practical applications. Such as a standard specie-specific material, necessary for calibration of any quantification method, and for the international harmonization of calibration by use of common reference material. Or as a control material for spectrophotometric methods. In addition, they could even be used to produce species-specific antibodies, an essential step in the development of immunological methods.

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