BLOCKING AGENTS FOR ELISA QUANTIFICATION OF CATHEPSIN L IN BOVINE AND PORCINE MUSCLE CRUDE EXTRACTS

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Background

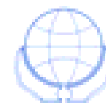
Consumers have considered from long time tenderness as the principal quality attribute of meat. Related to this, Jeremiah (1982) concluded that the most common cause of unacceptability in beef, pork and lamb meat purchases was toughness. This problem is strongly related with the inconsistency and unpredictable variability of meat texture, which avoids the standardization of meat quality and satisfaction of consumer demands, being one of the main problems that Meat Industry actually has to solve. The main origin of this variability is the large biological diversity of skeletal muscle [Pette and Staron, 1990]. Therefore it is not surprising to observe, in standardized processing conditions, a large animal variability in the rate and extent of postmortem meat texture development.
Endogenous muscle proteolytic enzymes are assumed to play a key role in this question, because they are responsible of myofibrilar disruption during ageing, a phenomenon directly related with the development of meat tenderisation [Sentandreu et al., 2002; Ouali, 1992]. So, the possibility to accurately quantify levels of some of these proteolytic enzymes has been proposed as a way to explain and predict meat texture variability. Cathepsin L is one of these target enzymes. Methods actually in use for quantification are based in the determination of its endopeptidase activity. However, quantification of cathepsin L activity in muscle crude extracts is imprecise due to absence of a specific substrate, being the interfering action of other peptidases, such as cathepsin B, always present. Immunochemical methods such as Enzyme Linked Immunosorbent Assay (ELISA) can be an interesting alternative for a more specific, sensitive and faster quantification of cathepsin L directly in crude extracts allowing a correct analysis of large number of samples per day. However, attention must be paid because ELISA can display important sources of error when not working in the appropriate conditions. Normally, the development of an ELISA test includes a first coating step consisting in the adsorption of antigens or antibodies to a plastic surface by non-specific binding (NSB). However, NSB of other undesired protein components during subsequent steps of the assay can give an overestimation of the signal not corresponding with the desired antigen-antibody reaction, which is detrimental for its sensitivity and specificity. Traditionally, undesirable NSB may be minimized by saturating the remaining binding sites of the plastic surface with different protein additives that must not interfere with the immunoassay. Not all blocking proteins that are commonly used for that purpose are adequate for each particular ELISA test, being necessary to determine the suitable one by empirical testing.

Objectives

In a previous work, a specific polyclonal antibody against highly purified bovine cathepsin L has been raised in rabbits [Sentandreu et al., 2004] with the aim to develop an immunoassay able to overcome the actual difficulties existing for cathepsin L quantification. The present work had as main objective the study of various blocking agents in their ability to prevent NSB of the reactants, other that capture IgG in the coating step, that will be utilized for the quantification of cathepsin L from both bovine and porcine muscle crude extracts by sandwich ELISA.

Materials and methods

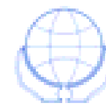
Preparation of muscle crude extracts: 500 grams of either bovine or porcine muscle were used as the starting material to prepare a lysosomal-rich extract as initially described by Lardeaux (1983). The extract was then fractionated with ammonium sulphate, collecting the precipitated protein between 30-70 % saturation. The pellet was redissolved in a minimum volume of 20 mM Bis-Tris buffer, pH 6.0, containing 200 mM NaCl and 1 mM EDTA. The redissolved extract was aliquoted in volumes of 200 µl and immediately frozen and stored at –20 ºC until used.



Development of specific IgG against bovine cathepsin L: A polyclonal antibody against purified bovine cathepsin L was raised in rabbits as described in a previous work [Sentandreu et al., 2004]. The IgG fraction was obtained by chromatography on Q-Sepharose Fast Flow, being a part of this IgG fraction biotinylated.
ELISA protocol for the present study: Microlon microtiter plates, each one containing 96 flat-bottom wells of high binding capacity (600 ng/cm^2) were routinely used for the assays. Effectiveness of each blocking agent in preventing NSB was assayed over a range of eight different concentrations of crude extracts ranging from 0 to 4,600 and 0 to 8,600 µg/ml of bovine and porcine muscle crude extract concentrations, respectively. 1) In a first step columns of eight wells, corresponding to the different muscle crude extract concentrations, were incubated with either solutions of goat serum, skimmed milk, bovine serum albumin (BSA), fish gelatine (surcoating step, 5 % in PBS) or with PBS only (no surcoating step), for 1 h at 37 ºC. At the end of each incubation step wells were routinely washed five times with PBS containing 0.1 % Tween 20 (washing buffer); 2) After washing, wells were incubated with the eight different concentrations of bovine or porcine muscle crude extracts during 1 h at room temperature, using the washing buffer containing the corresponding blocking agent (1%) as diluent (assay buffers). Additionally, two columns per plate were not exposed neither to a surcoating step nor addition of any blocking agent to the assay buffer, thus serving as reference for determining total NSB on the plastic surface at each muscle crude extract concentration; 3) Wells were incubated with the biotinylated IgG fraction of the developed polyclonal antibody against cathepsin L in a 1/2000 dilution with each one of the assay buffers for 1 h at room temperature. After washing, wells were incubated with Extravidin®-horseradish peroxidase conjugate (Sigma, St. Louis, MO; 1/1000 dilution in assay buffers) for 1 hour at room temperature. Peroxidase activity was determined by incubation of wells with 3,3' 5,5'- tetramethyl-benzidine liquid substrate (TMB), 20 min for bovine samples and 10 min for the porcine ones. Reaction was stopped with a commercial stop reagent (Sigma), reading the O. D. values at 650 nm in an automated plate reader.

Results and discussion

The ELISA protocol developed in the present work aimed at the evaluation of different types of blocking agents in their capacity for avoiding unwanted NSB. So, the experimental protocol design presented here is basically the sandwich ELISA that will be used for cathepsin L quantification but avoiding the initial coating step of binding the capture antibody. In these conditions, the effectiveness of blocking agents will be proved by their ability to avoid any kind of binding to the plastic surface, which will be reflected in the absence of O. D. at 650 nm due to peroxidase activity.
The developed polyclonal antibody was raised against bovine cathepsin L, but previous studies have shown that this antibody cross-react with porcine cathepsin L so that quantification of this latter would be also feasible. In figure 1.A we can see results obtained for the ELISA carried out with different concentrations of bovine muscle extract, whereas figure 1.B shows the same ELISA but performed with a porcine muscle crude extract as antigen. In both assays the positive control, serving as reference for total NSB, was given by the curve developed in absence on any blocking protein.
In the case of bovine (figure 1.A), we can observe that skimmed milk was the most effective blocking agent, avoiding NSB for all of the assayed crude extract concentrations, obtaining fully comparable results with or without the surcoating step. Only for the highest concentration (4,600 µg/ml extract) we can observe a little increase in the O. D. value but negligible compared to the reference curve. This would indicate that using skimmed milk as blocking agent the surcoating step could be suppressed, always that it was contained in the reaction buffer. Similar results were obtained for porcine species except for the highest crude extract concentration (8,600 µg/ml extract), in which the blocking capacity of skimmed milk is lost (see figure 1.B). As reported in the literature, this can be due to electrostatic interactions between antigen, antibody and/or maybe other sample compounds because of the high concentration of the reactants. Like bovine, the surcoating step wouldn't be necessary, as previously pointed out [Vogt et al., 1987]. Other authors working with completely different samples also observed the effectiveness of skimmed milk in preventing NSB [Kaur et al., 2002; Zimmermann and Regenmortel, 1989].
Even if they yielded slightly higher O. D. values, both BSA and goat serum proved to be also effective blocking agents for bovine samples. However, and contrary to skimmed milk, results clearly indicated that the surcoating step is necessary to achieve an effective blocking effect (see figure 1.A, differences of BSA and goat serum with and without surcoating). This is in opposition to results obtained by Pruslin et al. (1991), where BSA exerted an optimal blocking effect with and without the previous blocking step. The similar behaviour between serum and BSA can be explained by the fact that albumin is one of the major



components of the serum [Kaur et al., 2002]. Opposite to bovine, neither BSA nor goat serum exerted an effective blocking of NSB for pork crude extracts. With the previous surcoating step, only a partial blocking effect was achieved, whereas in its absence similar O. D. values to the reference curve were obtained (figure 1.B), in accord to results obtained by Zimmermann and Regenmortel (1989).

The worst blocking results were obtained with fish skin gelatine. Even if it is commonly used and it has been recommended as a model blocking agent between gelatine preparations [Vogt et al., 1987], we realized that fish skin gelatine was only able to partially prevent NSB of bovine crude extract when the surcoating step is done (figure 1.A), whereas in the case of pork we did not observe any blocking effect, being the O. D. values obtained for all the concentrations in both cases (with and without the surcoating step) similar to that of the reference curve (figure 1.B).

Comparing the O. D. values obtained for all curves in the absence of muscle crude extract (zero values of the X-axis) we can observe that even if there are some differences, these are minimal. This indicated that in the absence of crude extract, biotinlylated anti-cathepsin L IgG and/or Extravidin®-peroxidase conjugate did not inespecifically bind to the plastic surface, being also true for the reference curve.

Conclusions

In the development of a sandwich ELISA test for bovine or porcine cathepsin L coming from muscle crude extracts, the choice of and adequate protein agent is essential to prevent non-specific binding (NSB) of components itegrating the immunoassay with the solid support. As previously suggested [Engvall and Perlmann, 1972; Mohammad and Esen, 1989], NSB of the anti-cathepsin L polyclonal antibody and/or Extravidin®-peroxidase conjugate can be minimized just with the inclusion of Tween 20 in the reaction buffer. However, and contrary to them, for reduction of background due to NSB of muscle crude extracts (antigen) to the matrix, it is necessary the use of a suitable blocking agent in addition to Tween 20. Binding of some components of the crude extract to the plastic surface is detrimental for sensitivity and reproducibility of the assay because we do not know if the obtained O. D. values were exclusively due to the detection of bound cathepsin L or to the union of the antibody to other absorbed components from the extract.

Skimmed milk revealed to be the most efficient of the assayed blockers, as also observed by other authors. In the case of pork, this would be the only suitable blocking agent but avoiding the highest assayed concentration. All the same attention must be paid, since some authors pointed out the possibility that skimmed milk may interfere the antigen-antibody reaction under certain conditions [Mohammad and Esen, 1989; Vogt et al., 1987]. For the ELISA quantification of cathepsin L from bovine species, goat serum and BSA, together with skimmed milk, could be used as effective blockers always that a surcoating steps be included in the assay in addition to be present in the assay buffer.

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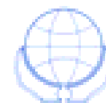

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Acknowledgements

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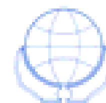

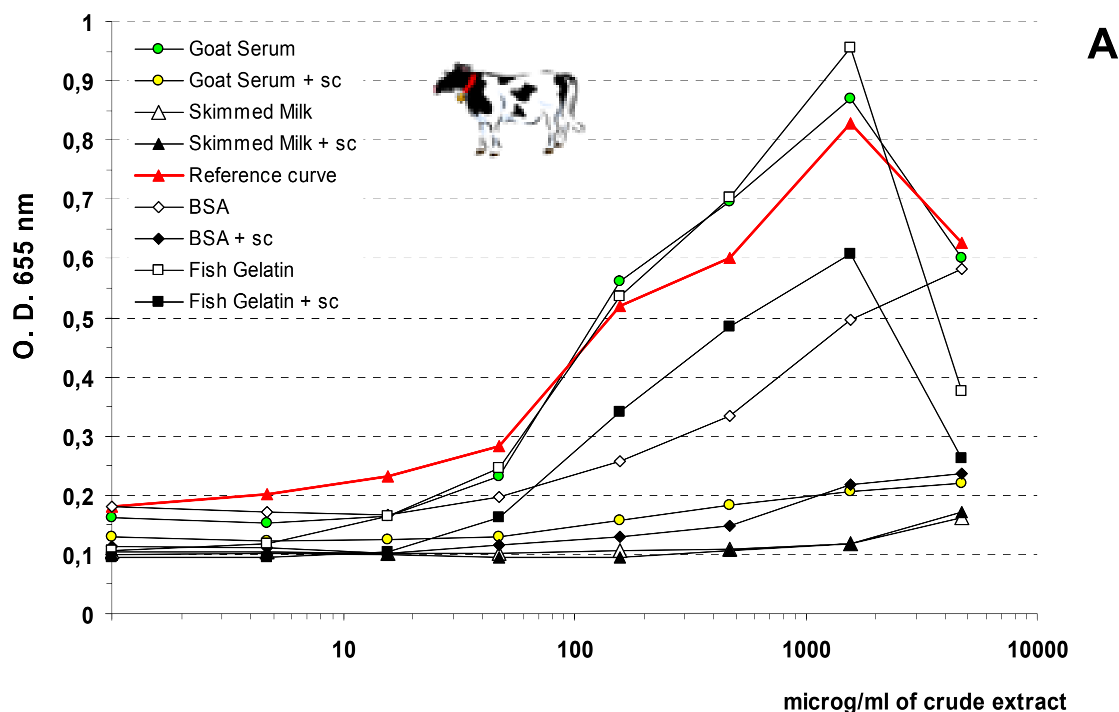

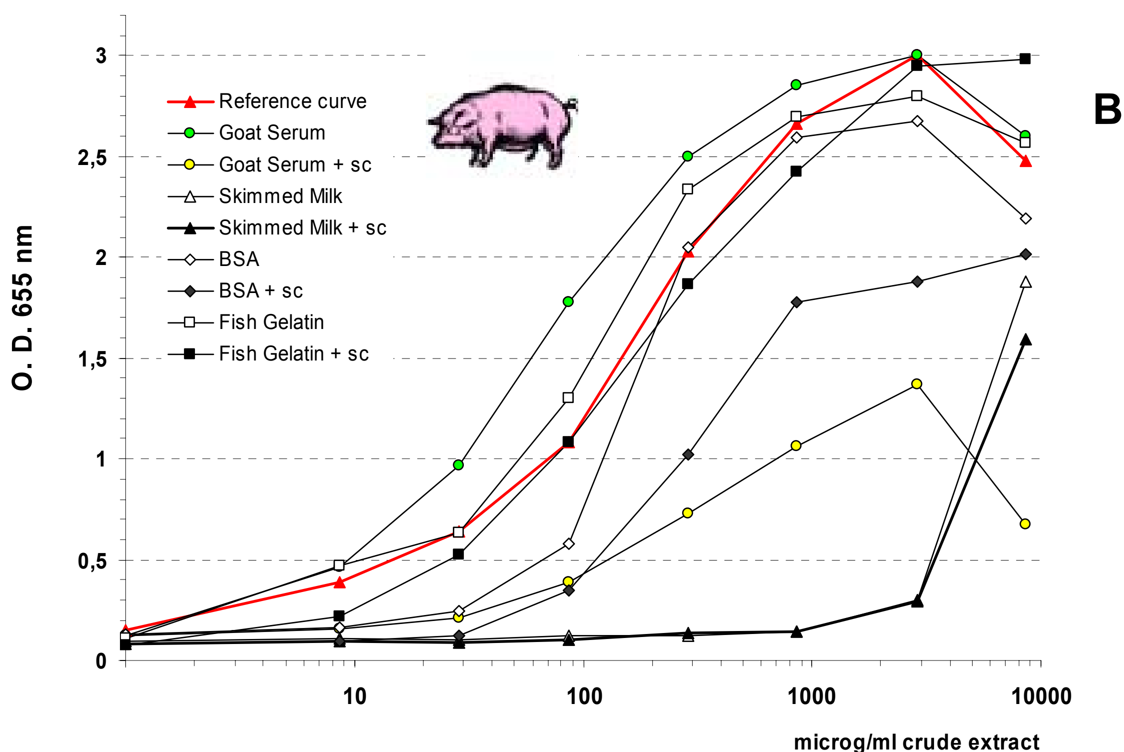

Figure 1: Effectiveness of blocking agents for preventing non-specific binding in indirect ELISA for cathepsin L using increasing concentrations of A) bovine or B) porcine muscle crude extracts. Legends showing "+ sc" indicate the curves in which a surcoating step has been carried out with the corresponding blocker. Those not showing "+ sc" belong to curves for which the surcoating step was not carried out, the corresponding blocking agent being only contained in the reaction buffer. Experimental points placed on the Y-axis correspond to 0 microg/ml of crude extract.