BOVINE MUSCLE 20S PROTEASOME: QUANTIFICATION IN TISSUE CRUDE EXTRACTS USING ELISA, RADIAL IMMUNODIFFUSION TECHNIQUES AND PRACTICAL APPLICATIONS

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Introduction

In the food industry, it was shown that the 20S proteasome contribute significantly to the improvement in meat texture of slow-twitch oxidative muscles and as well to a high pH in meat (Ouali, 1999). Hence, the 20S proteasome might be used as a marker, not only for pathologies but also for meat quality. This work develops two accurate immunochemical quantitative tests, the ELISA method and the less known radial immunodiffusion (RID).

Materials and Methods

The 20S proteasome was purified from bovine *Diaphragma* muscle by Dutaud (1998). SDS-PAGE (Laemmli, 1970) was conducted using acrylamide concentration (12%). Immunoblot was carried out as described by Sentandreu *et al.*, (2003). The monoclonal antibody (mcp 20) directed against alpha subunits of human 20S proteasome was prepared as previously described (Hendil *et al.*, 1995). Preparation of the antiserums were carried out (Dutaud *et al.*, 2002). The polyclonal antibody obtained against proteasome was used at a dilution of 1/1000. *ELISA procedure:* For proteasome quantification, the procedure used was based on a sandwich ELISA technique. The capture antibody was the monoclonal antibody whereas the secondary antibody was Peroxidase-labelled IgG and HRP activity was revelated. *Proteasome quantification by RID:* Radial immunodiffusion (RID) (Mancini *et al.*,1965), is a precipitation reaction carried out by application of antigen solution to a gel into which a monospecific antibody solution has been added. It is an adaptation of gel-precipitation reactions that permits the quantitation of an antigen. From the known standard concentrations, a standard curve can be drawn by plotting the square root of the antigen concentration versus the ring diameter. From this linear calibration curve the concentration of the unknown antigen samples can be determined

Results and Discussion

When analysed by SDS-PAGE, the purified proteasome exhibited a series of bands, characteristic to the 20S proteasome complex (see Figure 1A, lane 2). Pure proteasome preparations were used for the production of polyclonal antibodies and as a standard for the quantification of this complex by ELISA and RID.

The specificity of the rabbit antiserum was tested by western blot against a crude muscle (Figure 1A, lane 1) and against the purified 20S proteasome (Figure 1A, lane 2). As shown in Figure 1B, the antibody recognised several major bands corresponding to the proteasome subunits (Figure 1B, lane 1). When tested against the whole muscle extract, the number of labelled bands were lower, a similar pattern was observed (Figure 1B, lane 2) and no other bands were detected suggesting a high specificity of the antibody.

Assesment of the optimun dilution for mcp 20 was perfomed. mcp 20 therefore used at a dilution of 1/4500 and a typical standard curve was established (Figure 2). As shown in Figure 3, a linear relationship was observed for all muscle extract dilutions tested (r^2= 0.998). For three dilutions (Figure 3) the plots of the measured concentration versus the added amount of purified proteasome were all linear with slope values close to 1 (from 0.99 to 1.05; see Fig 3). The initial proteasome concentration in the muscle extract tested was estimated to be 53.5 ± 3.1 µg/ml (6 replicates).

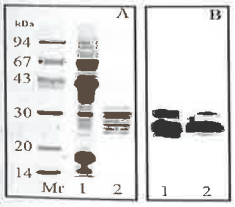

Figure 1: : Immunoblot analysis of the specificity of the polyclonal rabbit anti-20S proteasome (RABPs2) and the monoclonal (mcp 20 and mcp 21) antibodies. (A) Mr markers. Crude muscle extract from *Diaphragma pedialis* muscle stained with coomassie (lane 1); Purified proteasome stained with silver (lane 2). (B) Proteasome revealed by the polyclonal antibody on the purified proteasome (lane 1) and the muscle crude extract (lane 2).

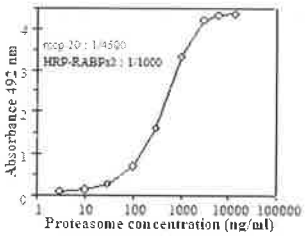

Figure 2: Standard curve obtained with optimum dilution of mcp 20 (capture antibody) and RABPs2.

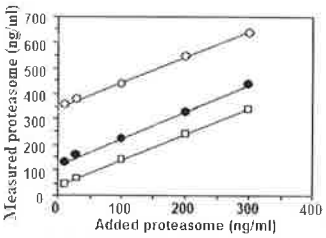

Figure 3: Dose-response curves of 20S proteasome measured by the sandwich-ELISA test. Proteasome concentration was measured on serial dilutions of a crude muscle extract (1/1500, open square; 1/500, closed circles; 1/150, open circles) supplemented with various amounts of purified proteasome. The initial proteasome concentration was determined by linear regression analysis of the plots.

The complex concentration in crude extracts was determined using radial immunodiffusion (RID). The results were compared to those obtained using the ELISA. The agarose gel with the wells containing either purified proteasome of known concentrations or crude extracts from *Longissimus* (L) and *Supraspinatus* (SS) muscles of unknown concentration is presented in Figure 4a. From the diameter of the precipitin rings corresponding to the purified proteasome, a standard curve was plotted (Figure 4b). From this curve the concentration of proteasome in the crude muscle extracts were estimated to be 265 and 200 µg/g for *Longissimus* and *Supraspinatus* muscles, respectively. These values are not significantly different from those obtained using the ELISA technique which yielded values of 275 and 209 µg/g, respectively. Both methods provided therefore very close values.

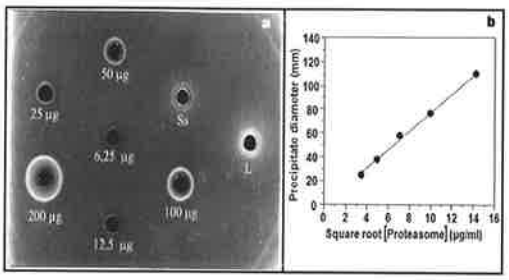

Figure 4: Quantitative determination of proteasome concentrations by radial immunodiffusion (RID). (a) The RABPs2 is incorporated into liquefied agar and allowed to gel. The purified proteasome is introduced at different concentration as indicated in the figure in the circular wells. Diluted crude extracts from *Longissimus* (L) and *Supraspinatus* (SS) muscles were introduced in two different wells as indicated. Note the radial diffusion of the antigen which is precipitated by the polyclonal antibody. (b) Plot of the ring diameter versus the square root of the proteasome concentration. The equation and r^2 value of the plot were: $y = 7.8x + 0.01$, $r^2 = 0.99$.

Conclusion

In this study, we further provide strong evidence for a possible accurate measurement of proteasome content in bovine tissues using another immunochemical technique referred as radial immunodiffusion (RID). RID is a more easy-to-use method than ELISA and requires only low cost material. We showed that RID provided very similar proteasome concentration values compared to ELISA for all bovine tissues tested. The plot of concentration values obtained with ELISA versus values obtained with RID gave linear relationship with a correlation coefficient of 0.99 and a slope close to 1, suggesting that each of these methods can be used independently for proteasome quantification.

References

Dutaud, D. (1998). Quantification et caractérisation de protéasome 20S de muscle de bovin .en relation avec l'attendrissage de la viande bovine. PhD Thesis, Blaise Pascal University, Clermont-Ferrand, France.

Dutaud, D., Aubry, L., Henry, L., Levieux, D., Hendil, KB., Kuehn, L., Bureau, J.P. and Ouali A. (2002). Development and evaluation of a sandwich ELISA for quantification of the 20S proteasome in human plasma. *Journal of Immunological Methods*, *260*, 183-193.

Hendil, K.B., Kristensen, P. and Uerkvitz, W. (1995). Human proteasomes analysed with monoclonal antibodies. *Biochemical Journal*, *305*, 245-252.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, *227*, 680-685.

Mancini, G., Carbonara, A.O. and Heremans J. F. (1965). Immunochemical quantitation of antigens by single radial immunodiffusion. *Immunochemistry*, *2*, 235-254.

Ouali A. (1999). Strructure and biochemistry of muscle as related to meat texture. Proc. XIV European Symposium on the Quality of Poultry Meat (Bologna, Italy). *1*, 91-121.

Sentandreu, M.A., Aubry, L. and Ouali, A. (2003). Purification of bovine cathepsine B: proteomic characterization of the different forms and production of specific antibodies. *Biochem. Cell. Biol.*, *81*, 317-326.