Myoglobin as an Endogenous Inhibitor of Proteolytic Muscle Enzymes

Cristina M. Rosell,* Mónica Flores, and Fidel Toldrá

Instituto de Agroquímica y Tecnología de los Alimentos, CSIC, Apartado de Correos 73, Burjassot 46100, Valencia, Spain

The effect of myoglobin content on the activity of different proteolytic systems, lysosomal enzymes, calcium dependent neutral proteinases, and aminopeptidases was examined concretely. Increasing myoglobin content inhibited cathepsin activities (B, B + L, and H). A similar effect was obtained with μ-calpain, which showed about 10% of its initial activity in the presence of 1 mg/mL of myoglobin. In contrast, *m*-calpain and calpastatin were almost insensitive to different levels of myoglobin in the medium. Exopeptidases, aminopeptidase B and alanyl aminopeptidase, were inhibited by myoglobin to approximately the same extent as the cathepsins. These results suggest that myoglobin might be considered an endogenous inhibitor, which could affect the endogenous proteolytic activity differently in different types of muscles. Consequently, the rate of both meat tenderization and generation of free amino acids may also be regulated by myoglobin.

Keywords: *Myoglobin; inhibition; muscle proteases; cathepsins; calpains; aminopeptidases*

INTRODUCTION

Myoglobin is one of the main proteins in skeletal muscles, being the pigment responsible for muscle color. This protein has an important role during animal life, providing the oxygen necessary for muscle metabolism. The myoglobin content is highly related to the metabolic pattern of the muscle, and its concentration varies to some extent with the kind of muscle examined (Beecher et al., 1965; Leseigneur-Meynier and Gandemer, 1991; Flores et al., 1996b). This variation in myoglobin content was initially used as a simple means of classification of muscle fiber type (Needham, 1926) in red or white, depending on the high or low myoglobin content, respectively. Afterward, Moody and Cassens (1968) used the terms red, white, and intermediate fibers as a criterion of fiber classification according to their predominant activities: oxidative, glycolytic, or both oxidative and glycolytic activities. Typically, red fibers exhibit slower rates of contraction and higher oxidative activity than white fibers; therefore, muscles with a preponderance of red fibers are named slow-twitch red muscles while fast-twitch white muscles, are those with a high proportion of white fibers.

However, these simple terms, white or red, involve a number of biochemical and physiological differences among muscles, for instance, a large variation in enzyme activities like between citrate synthase and lactic dehydrogenase, which are closely related to oxidative or glycolytic metabolism, respectively (Pearson and Young, 1989). Additionally, a wide variability has been reported in the contents of proteinases and their inhibitors in different muscles. This is the case of cathepsin B (Spanier and Bird, 1982) and B + L (Valin and Ouali, 1992) and calcium dependent proteinases (Ouali and Talmant, 1990; Monin and Ouali, 1991; Valin and Ouali, 1992). No clear relationship, however, was found between aminopeptidases and the muscle type (Flores et al., 1996b).

* Author to whom correspondence should be addressed (telephone 34-6 390 0022; fax 34-6 363 6301; e-mail crosell@iata.csic.es).

Dransfield et al. (1980−1981) stated the major role of muscle type in the post-mortem aging rate or meat tenderization rate, and later Ouali (1991) reported that the aging rate was enhanced with increasing contraction speed but was inversely related to the content of heme iron. Therefore, the tenderization rate would be higher in fast-twitch white than in the slow-twitch red muscles. However, this is in contradiction with the highest concentration of calcium dependent proteinases observed in slow-twitch muscles which have the lower aging rate. Similar results are obtained for the major cathepsins B and L. Different hypotheses have been proposed to explain these contradictory results (Valin and Ouali, 1992), on the basis of the distinct (i) sensitivity of muscle proteins to proteolysis, (ii) osmotic pressure, or (iii) levels of endogenous inhibitors, but so far, no agreement has been reached. In fact, Spanier and Bird (1982) indicated the possibility that myoglobin would act as an endogenous inhibitor of cathepsin B in living muscle. If a similar trend could be demonstrated for calcium dependent proteinases, it could explain the controversial findings on the basis of the myoglobin content in the respective muscle.

The objective of this work is to determine the effect of myoglobin on the activities of the muscle proteolytic system (cathepsins, calpains, and aminopeptidases).

MATERIALS AND METHODS

Extracts were obtained from the skeletal muscle *Biceps femoris* from 6-month-old pigs. The (aminoacyl)-7-amino-4-methylcoumarin (MCA) substrates and casein−fluorescein isothiocyanate (FITC−casein) were purchased from Sigma (St. Louis, MO). All reagents were of analytical grade.

Assay of Cathepsins. Muscle (2.5 g) with no visible fat or connective tissue was homogenized in 25 mL of 50 mM citrate buffer (pH 5.0) containing 1 mM EDTA and 0.2% (v/v) Triton X-100 using a Polytron homogenizer (three strokes of 10 s each at 20 000 rpm with cooling in ice). The homogenate was then centrifuged for 20 min at 10000*g*, and the supernatant obtained by filtering through glass wool was used for further enzyme activity assays.

The cathepsin activities were measured as described elsewhere (Toldrá and Etherington, 1988) by using *N*-CBZ-L-arginyl-MCA at pH 6.0, *N*-CBZ-L-phenylalanyl-L-argininyl-

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MCA at pH 6.0, and L-argininyl-MCA at pH 6.8 as specific substrates of cathepsin B, B + L, and H, respectively. The reaction mixture was incubated at 37 °C for 15 min, and the fluorescence was continuously monitored in a Fluoroskan II instrument (Labsystems) at λ_{ex} = 360 nm and λ_{em} = 440 nm.

Calcium Dependent Neutral Proteinases. The extracts were prepared as reported elsewhere (Rosell and Toldra, 1996). Briefly, 50 g of trimmed muscle was homogenized in 3 volumes of extration buffer [50 mM Tris, 3 mM EDTA, and 10 mM mercaptoethanol (MCE) (pH 8.3)] using a Polytron homogenizer (three strokes of 10 s each at 20 000 rpm with ice cooling). This homogenate was centrifuged at 28000*g* for 30 min at 4 °C. The supernatant filtered through glass wool and adjusted to pH 7.5 was again centrifuged at the same conditions. The later supernatant was filtered through glass wool and further used for calpain separation.

Separation of calpains and calpastatin was performed using the procedure described by Koohmaraie (1990) and Wheeler and Koohmaraie (1991). Eighty milliliters of supernatant was pumped into a DEAE-Sephacel column (2.6 cm × 17 cm) at 1 mL/min. The column was previously equilibrated with 40 mM Tris buffer containing 0.5 mM EDTA, 10 mM MCE, and 1 mM NaN_3 (pH 7.5). The bound proteins were eluted at 1 mL/min, with a NaCl linear gradient (25 to 200 mM) in the equilibrating buffer within 6 h. Calpastatin and μ-calpain were eluted at ca. 75 and 150 mM NaCl, respectively. *m*-Calpain was eluted with 400 mM NaCl in equilibrating buffer for 2 h. Fractions of 6 mL were collected under refrigeration.

Enzyme activity was measured as reported by Wolfe et al. (1989) and Lonergan et al. (1994), with slight modifications. FITC−casein (2 mg/mL) was dissolved in 100 mM Tris-acetate (pH 7.5) containing 10 mM MCE and used as the substrate. μ-Calpain or *m*-calpain activity was determined using either 75 or 50 μL of the collected fraction, respectively. FITC−casein was added to a total volume of 100 μL. Calpastatin activity was assessed by incubating 50 μL of the inhibitor fraction with 20 μL of the *m*-calpain fraction previously to the addition of 50 μL of the substrate. The enzyme reaction was started by the addition of 25 μL of 10 mM $CaCl_2$ or 5 mM EDTA for calcium dependent and independent protease activity determination, respectively. The fluorescence enhancement was continuously monitored at 25 °C in a Fluoroskan II using λ_{ex} = 485 nm and λ_{em} = 538 nm.

Alanyl and Arginyl Aminopeptidases. Five grams of muscle was homogenized in 25 mL of 50 mM phosphate buffer (pH 7.5) containing 5 mM EGTA using a Polytron homogenizer (three strokes of 10 s each at 20 000 rpm with cooling in ice). The homogenate was then centrifuged at 10000*g* for 20 min at 4 °C, and the supernatant was filtered through glass wool and used for subsequent purification.

Alanyl aminopeptidase and aminopeptidase B were purified as described elsewhere (Flores et al., 1993, 1996a). The supernatant was fractionated by ammonium sulfate addition. The fraction precipitating between 40 and 60% ammonium sulfate saturation was collected and dialyzed against 100 mM Tris-HCl buffer (pH 7.0) containing 0.02% (w/v) sodium azide, under refrigeration. The dialysate was filtered through a 0.45 μm nylon membrane filter and injected into an anion exchange HPLC column previously equilibrated with 10 mM Tris-HCl buffer (pH 7.0) containing 100 mM sodium chloride, 0.1% (v/v) MCE, and 0.02% (w/v) sodium azide. The column was eluted at 0.5 mL/min with the equilibration buffer and after 9 min with a linear salt gradient (100 to 400 mM NaCl) for 20 min and finally with 400 mM NaCl for 9 min. Aminopeptidase B eluted at 180 mM NaCl, while alanyl aminopeptidase eluted at 310 mM NaCl. Fractions were collected and assayed for enzyme activities.

Alanyl aminopeptidase was incubated in a reaction buffer containing 100 mM phosphate buffer (pH 6.5), 2 mM MCE, and 0.1 mM L-alanyl-MCA as the substrate. The reaction buffer for aminopeptidase B consisted of 50 mM phosphate buffer (pH 6.5) containing 200 mM NaCl and 0.1 mM L-arginyl-MCA as the substrate. The enzymes were incubated at 37 °C during 15 min, and the fluorescence was measured at 360 and 440 nm as excitation and emission wavelengths, respectively, in a Fluoroskan II instrument.

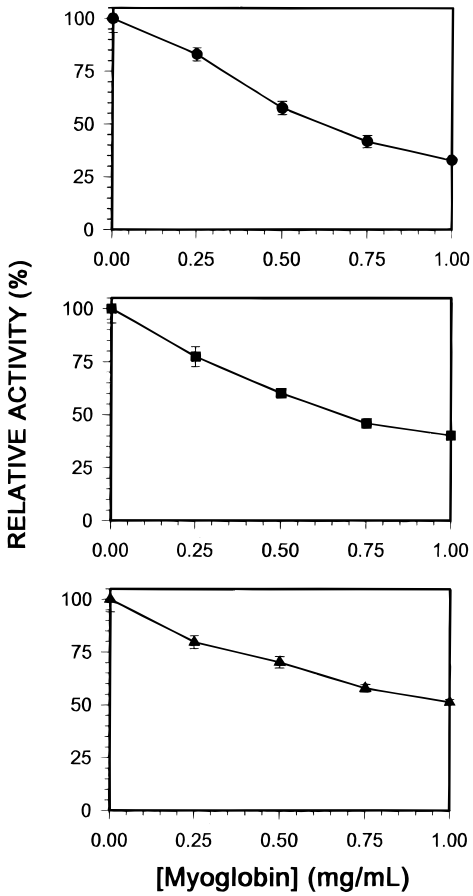

Figure 1. Inhibition of cathepsin B (●), cathepsin B + L (■), and cathepsin H (▲) by myoglobin. Experimental conditions are described in Materials and Methods. Vertical bars represent means of six replicates ± SEM.

Effect of Myoglobin on the Enzyme Activities. Different amounts of myoglobin were dissolved in the reaction media (final concentrations from 0 to 1 mg/mL). The enzyme activities were further assayed in the presence of different myoglobin concentrations. In all cases, six replicates were assayed for each experimental point. In the figures, vertical bars represent means of the replicates ± SEM. No bars are shown when the intervals are smaller than the symbols.

Myoglobin Hydrolysis during Enzymes Assays. Myoglobin (1 mg/mL) was dissolved in the appropriate reaction media. The hydrolysis of myoglobin was directly assayed, after 15 min and 1 h of incubation with the enzymes in the absence of the specific substrates. The hydrolysis was monitored using an Applied Biosystems Model 270A capillary electrophoresis apparatus. Samples were vacuum injected for 1.5 s and run in 100 mM sodium phosphate buffer (pH 2.5) at 20 kV and 35 °C in a standard 72 cm capillary (50 μm internal diameter and 50 cm to detector). UV absorbance was monitored at 200 nm.

RESULTS AND DISCUSSION

Myoglobin can act as an endogenous inhibitor of muscle proteolytic enzymes. However, as it could act as a competitive substrate, the possible enzymatic hydrolysis of myoglobin was previously determined by incubating with the respective enzymes in their specific reaction conditions. The potential degradation of myoglobin was monitored by capillary zone electrophoresis (results not shown). No hydrolysis of myoglobin was detected during the reaction time even after 1 h of incubation at 37 °C. That suggests that myoglobin might act as a true inhibitor of these enzymes, discarding its possible role as a competitive substrate.

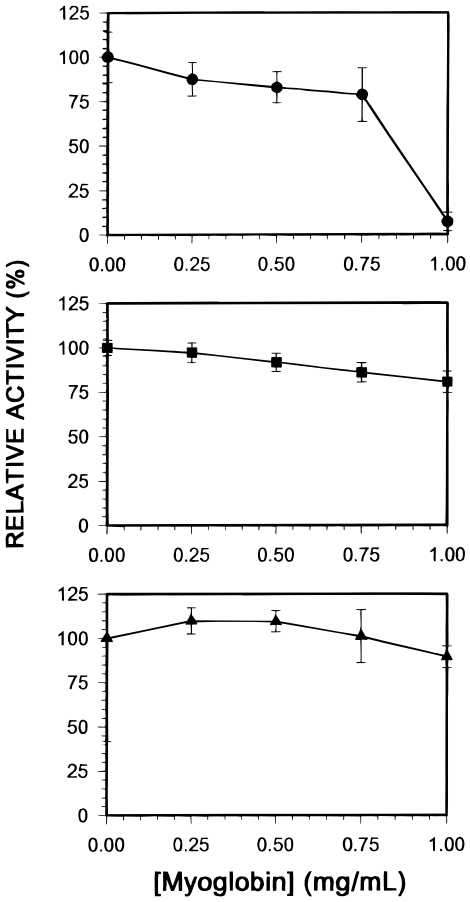

Figure 2. Effect of myoglobin concentration on the calcium dependent neutral proteinase system: μ-calpain (●), *m*-calpain (■), and calpastatin (▲). Experimental conditions are detailed in Materials and Methods. Vertical bars represent means of six replicates ± SEM.

Effect of Myoglobin Concentration on the Activities of Cathepsins. The effect of various myoglobin concentrations on the activity of cathepsin B, B + L, and H is shown in Figure 1. All the assayed lysosomal enzymes follow similar inhibitory trends. Catheptic activity decreases as myoglobin concentration increases. This inhibition is more pronounced for cathepsin B (only 32% of the initial activity remains at 1 mg/mL myoglobin) but closely followed by that of cathepsin B + L. Cathepsin H is less affected, and its activity falls to 51% at the maximum myoglobin content assayed. Therefore, the activity of cathepsins during meat aging will strongly depend on the muscle myoglobin content, with the cathepsin activity more inhibited in slow-twitch red muscle which has been reported to have a lower tenderization rate despite its higher level of lysosomal enzymes (Valin and Ouali, 1992).

Effect of Myoglobin on the Calcium Dependent Proteinase System. The behavior of μ-calpain, *m*-calpain, and calpastatin in the presence of different myoglobin concentrations is shown in Figure 2. Regarding μ-calpain, myoglobin promotes a slight inhibition till concentrations of 0.75 mg/mL, but a great drop in activity is observed at 1.0 mg/mL, with only about 6% of the initial μ-calpain activity remaining. *m*-Calpain and calpastatin activities are barely affected by myoglobin content. The former is slightly inhibited by myoglobin, keeping 80% of its initial activity at myoglobin concentrations up to 1 mg/mL, whereas the inhibitor is practically unaffected by the presence of myoglobin.

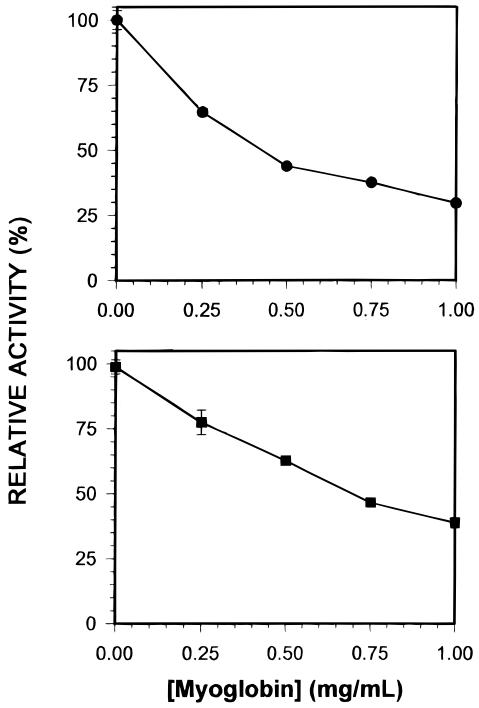

Figure 3. Inhibitory effect of myoglobin on alanyl (●) and arginyl (■) aminopeptidase activities. Experimental conditions are described in Materials and Methods. Vertical bars represent means of six replicates ± SEM.

The neutral proteinase system is composed of two isoenzymes, μ-calpain and *m*-calpain, requiring micromolar and millimolar concentrations of calcium ions, respectively, for its activity, and a specific inhibitor, calpastatin. This proteolytic system promotes the degradation of the Z-disk and as a consequence disrupts the myofibrillar structure (Goll et al., 1992). However, only μ-calpain allows the proteolysis due to the low calcium concentrations generally found in the muscles. It has been reported that *m*-calpain could constitute a reservoir of calpain activity and would be active after some proteolysis that decreases its calcium sensitivity (Yoshizawa et al., 1995). Regarding the myoglobin effect, the μ-calpain in red muscles would therefore be reduced by both the myoglobin inhibition and the higher levels of calpastatin (Ouali and Talmant, 1990) which would result in a retardation in the proteolysis rate of the Z-disk and, thus, a lower post-mortem aging rate. However, other factors such as the intracellular pH, the presence of other endogenous inhibitors, and proper conditions in the cell should also be taken into account. These results would contribute to explain the contradictory findings described by Valin and Ouali (1992).

Myoglobin Effect on Aminopeptidases. Aminopeptidase B and alanyl aminopeptidase are also inhibited by myoglobin as can be seen in Figure 3. The inhibition increases with increasing myoglobin concentration, with the alanyl aminopeptidase and aminopeptidase B enzymes having 26 and 38% of their initial activity, respectively, at a myoglobin concentration of 1 mg/mL (Figure 3).

The myoglobin effect seems to be more pronounced on alanyl aminopeptidase which is described as the major aminopeptidase in muscle cells (Mantle et al., 1983) and having a wider specificity (Toldrá et al., 1995). The last step in the proteolysis chain of muscle proteins consists of the action of exopeptidases. Aminopeptidase B and alanyl aminopeptidase are those muscle exopeptidases that play a significant role in post-mortem

generation of free amino acids (Toldrá et al., 1992, 1995) with a great contribution to taste development (Nishimura et al., 1988; Kato et al., 1989). Thus, the myoglobin inhibition would result in a slower generation of free amino acids in red muscles than in the white ones. Studies are currently carried out to determine the influence of myoglobin on the amino acid generation in different types of muscles during aging.

In summary, it can be concluded that acid lysosomal proteases and calcium dependent proteases (namely μ-calpain) and aminopeptidases are inhibited by myoglobin. Thus, myoglobin would play an important role in meat tenderization by controlling myofibrillar proteolysis rate and in the development of meat taste by slowing the generation of free amino acids.

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