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Myoglobin as an Inhibitor of Exopeptidases from *Lactobacillus sake*

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The effects of myoglobin on exopeptidases of *Lactobacillus sake* were determined. Inhibition of the aminopeptidases increased as the myoglobin concentration increased; aminopeptidase 3 was the most affected (90% inhibition). Aminopeptidases 1, 2, and 4 showed similar inhibition levels (around 60%). Myoglobin did not affect tripeptidase activity. Thus, myoglobin could limit amino acid generation in meat systems.

Proteolytic events that take place during the processing of dry sausages result in an increase in small peptides and free amino acids, similar to that which occurs during cheese ripening. The composition and concentration of these compounds contribute to the overall flavor in cured meat products such as dry sausages (5) and ham (1, 17). In sausages, microbial peptidases have an important role in the hydrolysis of oligopeptides, previously released by proteinases (7). The proteolytic system of lactic-acid bacteria commonly present as part of the adventitious microflora or as a starter culture, such as *Lactobacillus sake*, may have a role in this proteolytic phenomenon (8, 9). Indeed, an aminopeptidase, a tripeptidase, and a dipeptidase from *L. sake* have already been purified and characterized (9, 12, 14). Nevertheless, the activities of these enzymes in meat systems may be affected by a number of factors, and their significance is difficult to predict. For instance, the effects of dry-sausage processing conditions and curing ingredients on the activities of several aminopeptidases from *L. sake* have been studied (13). These factors might be important for taste development by slowing the generation of amino acids. On the other hand, myoglobin, the respiratory pigment responsible for color in meat, has been reported to act as an endogenous inhibitor of muscle proteases, aminopeptidases, and lipases (10, 11, 16). However, our knowledge of the factors that affect microbial peptidase activity in this environment is limited. Our aim was to determine the effects of myoglobin on the activities of four aminopeptidases and a tripeptidase purified from the cell extract of *L. sake*.

L. sake CECT 4808 was previously isolated from the indigenous flora of dry-cured sausages (15). The cell extract obtained by the procedures previously described (12, 13) was used for enzyme purification. A tripeptidase with high specificity for Phe-Gly-Gly was purified according to the method described by Sanz et al. (14). Two aminopeptidases, designated aminopeptidase 1 (AP 1) and AP 3, with high specificity for leucine-7-amido-4-methylcoumarin (AMC) and two aminopeptidases, designated AP 2 and AP 4, showing high specificity for arginine-AMC substrates were purified as described by Sanz and Toldrá (12, 13).

Tripeptidase activity was assayed by using Phe-Gly-Gly as the substrate and monitoring the peptide hydrolysis by capillary electrophoresis (14). Aminopeptidase activity was measured by using Leu-AMC as the substrate for AP 1 and AP 3 and Arg-AMC as the substrate for AP 2 and AP 4. Fluorescence was measured at 360 and 440 nm as excitation and emission wavelengths, respectively, with a multiscan fluorimeter (Fluoroskan II; Labsystems, Helsinki, Finland).

The effect of myoglobin concentration on enzyme activity was determined by addition of muscle myoglobin to the reaction mixture to a final concentration from 0.1 to 1 mg/ml. The enzyme activity in the presence of myoglobin was determined by the procedure outlined above. Controls were run without myoglobin. In all cases, replicate experiments were performed on four different occasions. Results are expressed as means from the replicate experiments and standard errors of the means.

Myoglobin concentration varies depending on the muscle, and it is closely related to the metabolic pattern of the muscle (2, 4, 6). In addition, the amount of myoglobin present in a sausage mixture depends on the percentages of meat and fat.

Myoglobin exerted different effects on the activities of the exopeptidases purified from *L. sake*, as shown in Fig. 1. The purified tripeptidase has broad specificity, releasing a great variety of amino acids from the N-terminal positions of tripeptides (14). As a result, it could contribute to the generation of free amino acids and to the hydrolysis of hydrophobic tripeptides during the ripening of sausages. The presence of myoglobin in the range of concentrations assayed did not inhibit the purified tripeptidase (Fig. 1). In contrast, the remaining exopeptidases were inhibited to different extents in the presence of myoglobin, and in all cases, aminopeptidase activity decreased as the myoglobin concentration increased (Fig. 1). This was also the case for muscle proteases and aminopeptidases, where it was concluded that myoglobin could play a role in meat tenderization by reducing myofibrillar proteolysis and in taste development by slowing the generation of amino acids (10). AP 3 has specificity for amino acids such as leucine, levels of which increase greatly during the ripening of sausages (3). However, this aminopeptidase was strongly inhibited in the presence of myoglobin. Its activity was reduced by approximately 90% at 1 mg of myoglobin per ml (Fig. 1). Therefore, myoglobin should be considered an important limiting factor for AP 3 activity. The presence of myoglobin also inhibited the remaining aminopeptidases (AP 1, AP 2, and AP 4). They retained about 40 to 55% of their initial activities at high myoglobin concentrations (0.7 to 1 mg per ml [Fig. 1]). At low myoglobin concentrations (0.1 to 0.2 mg/ml), the activity of AP 4 was reduced around 10 to 20% and AP 1 and AP 2 showed slight inhibition (about 10%). AP 1 has broad specificity, hy-

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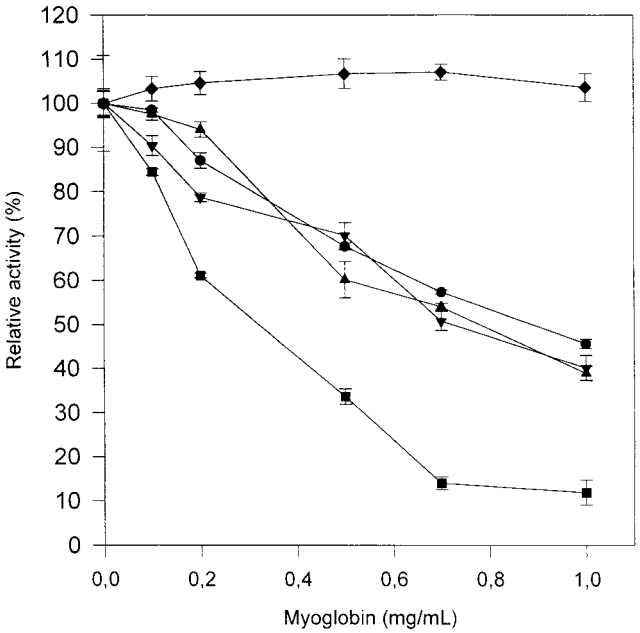

FIG. 1. Effects of myoglobin on the activities of the exopeptidases from *L. sake*: AP 1 (●), AP 2 (▲), AP 3 (▼), AP 4 (■), and tripeptidase (◆). Results shown are means from quadruplicate experiments. Error bars, standard errors of the means.

drolyzing amino acids such as leucine, alanine, and valine, which are known to reach important levels in ripened sausages (3) and also constitute a source of volatile compounds with intense aromatic characteristics (8). On the other hand, the activities of AP 2 and AP 4 mainly contribute basic amino acids. Thus, the pool of amino acids generated during ripening may be the result of the combined activity of both the bacterial and endogenous muscle proteolytic systems (18). The contribution of each enzyme to the proteolytic phenomenon remains to be established, but it has been shown that myoglobin is an additional inhibitor that has to be taken into account in considering microbial aminopeptidase activity in meat systems.

In summary, myoglobin decreases the activities of both microbial and muscle aminopeptidases in meat, although its effect in sausages could be less significant than in lean meat because it is present at lower concentrations in the former.

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