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Problems Associated with the Assay of Cathepsin D in Meat and Meat Products

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ABSTRACT

Cathepsin D is usually assayed by following the release of the trichloroacetic (TCA)-soluble peptides from denatured haemoglobin at 280 nm, but some artefacts may appear giving false results. Cathepsin D activity has therefore been assayed under different conditions in muscle, liver and dry-cured ham extracts. Substantial errors (around 50–56%) become evident when using the classical standard assay. The assay of cathepsin D activity in muscle extracts should include the use of a blank containing a specific inhibitor such as isovalerylpepstatin.

INTRODUCTION

A method for measuring the activity of cathepsin D (EC 3.4.23.5), a well-known lysosomal proteinase, in the acidic pH range was proposed by Anson (1938). This method involves the use of haemoglobin as substrate and the measurement of TCA-soluble peptides after digestion with cathepsin D. Other proteins, different from haemoglobin, have also been used as substrates but were not sufficiently hydrolyzed by cathepsin D, and the results showed poor reproducibility (Barrett, 1977). Northrop *et al.* (1948) was the first to use absorbance at 280 nm for quantifying the acid-soluble peptides. Since then, it has become a widely used method for measuring the proteolytic activity for both crude muscle extracts (Melo *et al.*, 1974; Deng & Lillard, 1973; Barrett, 1977; Ouali & Valin, 1981; Ouali *et al.*, 1987) and

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purified cathepsin D (Knight & Barret, 1976). However, some artefacts may appear, giving false results for proteolytic activity, such as the formation of ferric ion or acid-soluble oligonucleotides by the action of nucleases (Barrett, 1977).

Dry cured ham represents an important commercial product not only in the countries of the Mediterranean area (mainly in France, Italy and Spain) but also in the United States (Country-style ham). Many biochemical changes are known to take place during the dry-curing process (Bellati *et al.*, 1983; Flores *et al.*, 1984). One of the more important, which is closely related to the quality of the final product, consists of progressive proteolysis of muscle proteins due to the action of muscle proteinases. In fact, cathepsins B, D, H and L were all found to be active even after 8 months of dry-curing (Toldrá & Etherington, 1988). The accurate assay of these proteinases is essential for a better understanding of the proteolytic changes that occur during the dry-curing process.

However, we have observed that the apparent cathepsin D activities measured in muscle extracts are not uniquely due to the action of cathepsin D. This observation is more relevant when assaying low levels of cathepsin D activity which is the case in aged meat or meat products.

The problems associated with the measurement of cathepsin D activity in several extracts (muscle, liver and dry-cured ham) as well as a modification for its correct determination are reported in the present paper.

MATERIALS AND METHODS

Preparation of enzyme extracts

Enzyme extracts were prepared according to Okitani *et al.* (1981) but with slight modifications. Twenty-five grams of porcine muscle (*Biceps femoris*) were homogenized in 75 ml of 50 mM sodium citrate, pH = 5·0, containing 1 mM EDTA and 0·2% (v/v) Triton X-100. The extract was centrifuged 20 min at 10 000 *g*. The supernatant was collected, filtered through glass wool and used for enzyme assays. Extracts of liver and dry-cured ham aged for 8 months were prepared in the same way.

Assays of cathepsin D activity

The assay was based on the Anson's procedure (1938). Some 0·75 ml of enzyme extract was added to 4 ml of 0·2M sodium citrate buffer, pH 3·7, containing 0·75% acid-denatured bovine haemoglobin as substrate and incubated at 45°C for 1 h.

TABLE 1
Different Conditions for the Assay of Cathepsin D Activity in Crude Extracts

	Condition					
	A	*B*	*C*	*D*	*E*	*F*
Substrate	−	−	+	+	+	+
Inhibitor	−	+	−	+	−	+
Enzyme extract	+	+	−	−	+	+
Activity[a,b]						
Muscle	0·085	0·090	0·010	0·005	0·241	0·135
Liver	0·080	0·085	—	—	0·400	0·120
Dry-cured ham	0·095	0·090	—	—	0·180	0·095

[a] Activity expressed as increase of absorbance (A_{280}) per hour.
[b] Standard error of means ($P < 0{\cdot}01$) = ±0·010.

Different reaction mixtures were prepared as indicated in Table 1. Forty microlitres of 0·003M isovalerylpestatin was used as a specific inhibitor of cathepsin D (Knight & Barrett, 1976). Previous tests with different levels of inhibitor (20, 40, 80 and 160 µl of 0·003M isovalerylpepstatin) showed complete inhibition in all cases. Three millilitres of 5% (w/v) TCA were added at the end of incubation, except for blanks where TCA was added at the beginning. The TCA-soluble peptides were measured at 280 nm versus their respective blanks.

The activity was defined as the increase in absorbance at 280 nm, (A_{280}), per hour at 45°C and pH 3·7. Four experiments were carried out for each condition. Blanks and samples were assayed in quadruplicate.

Cross-flow filtration of enzyme extracts through a 10000 dalton polysulfone membrane was performed in a Minitan Ultrafiltration system (Millipore Bedford, MA).

RESULTS AND DISCUSSION

The activities of cathepsin D in different extracts (muscle, liver and dry-cured ham) and assay conditions are presented in Table 1. Conditions A and B represent the changes of each extract with no added substrate in both the presence and absence of isovalerylpepstatin, which is a specific inhibitor of cathepsin D (Knight & Barrett, 1976). The intrinsic proteolysis of the substrate (haemoglobin) was also examined in the presence and absence of the inhibitor (conditions C and D). Finally, cathepsin D activities were obtained using the standard assay without (condition E) and with the addition of isovalerylpepstatin (condition F).

Incubation of the enzyme extracts (muscle, liver and dry-cured ham) in both presence and absence of isovalerylpepstatin (conditions A and B in Table 1, respectively) gave similar activities, demonstrating that pepstatin did not affect the observed increase in absorbance at 280 nm. The apparent activity, then, could be due to non-aspartic proteinases, although under the assay conditions it is rather unlikely (Asghar & Bhatti, 1987).

On the other hand, other chemical changes in the extract may be involved. In order to elucidate the enzymatic origin of such changes, enzyme extracts were filtered through a 10000 dalton membrane, boiled for 10 min and incubated under the same conditions. Similar increases in absorbance at 280 nm were obtained. Thus, chemical changes due to the activity of enzymes seems rather unlikely. When substrate was added, greater increases in absorbance at 280 nm were obtained (condition F vs B in Table 1). Intrinsic proteolysis of the substrate during the incubation was not observed (conditions C and D in Table 1).

Substantial errors (up to 56%) in the measurement of cathepsin D activity in crude muscle extracts were found on using the standard assay (condition E in Table 1). The difference between conditions E and F (see Table 1) would reflect more correctly the real cathepsin D activity in the extract because it only differs from reaction mixture in its content of pepstatin, which completely inhibits aspartate proteinases (Knight & Barret, 1976). The proteolytic activity in the majority of tissues, including muscles, was mainly due to cathepsin D (Asghar & Bhatti, 1987).

This phenomenon has also been observed with other tissues such as liver extracts. In this case, cathepsin D activity was found to be much higher (Condition E in Table 1: $A_{280} = 0{\cdot}400$) while the other conditions (B, C, D and F) remained similar to those obtained with muscle extracts, and, as a consequence, resulted in relatively small errors.

This was not the case of dry-cured ham extracts, where low cathepsin D activities would be expected. In fact, the apparent activity (condition E in Table 1: $A_{280} = 0{\cdot}180$) was anomalously high as compared to the corrected cathepsin D activity (condition E minus F in Table 1, $A_{280} = 0{\cdot}085$). The error (53%) was substantial.

Thus, the use of a blank containing isovalerylpepstatin is recommended when assaying cathepsin D in crude muscle extracts.

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