## Proteolysis of sarcoplasmic proteins during in vitro incubation of bovine muscle extracts

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### Introduction

Proteolytic degradation by endogenous proteinases of muscle proteins contributes to the constant metabolic turnover occurring in the live animal, has been implicated in several aspects of the conditioning of muscle as meat in post mortem muscle, probably plays an important role in further tenderisation of meat during processing and cooking and may, by providing peptides and amino acids, be important in flavour development in cooked meat.

Despite early reports (e.g. Smith 1948) that aqueous extracts of muscle showed little or no proteolytic activity against endogenous proteins over a pH range of 4.0 - 8.0, a large number of proteolytic activities have been demonstrated in minced muscle tissue. The enzymes responsible have been characterised. They exert their activities at acid, neutral or alkaline pH and all can be classified (on the basis of their active sites) into the four groups (Barrett 1980) of aspartic, thiol, metallo - and serine proteinases. (Reviewed by Goll et al, 1982). Many of the proteinases described are clearly lysosomal in origin and may thus be expected to exert their activity at acid pH. Proteinases active at alkaline pH appear to arise from non muscle tissue, particularly from mast cells (Drabikowski et al., 1977) fibroblasts and leucoytes (Etherington 1981). Of the enzymes shown to act at neutral pH, the $Ca^{2+}$ activated neutral proteinase (CANP) (Busch et al 1972) and the neutral serine proteinase (Beynon & Kay 1978) are believed to be muscle enzymes which however would exert their activity only in the early post-mortem period when muscle pH is still near neutral.

In many studies on proteolytic activities in muscle the main focus of attention has been on the degradation of myofibrillar proteins and on its role in meat tenderisation (e.g. Penny, 1980). In this Laboratory, studies on proteolysis in muscle originated from an interest in flavour and off-flavour development in frozen stored meat products. (Henahan et al., 1981). Because of the highly labile nature of sarcoplasmic proteins, which constitute up to 30% of muscle protein and which have so far received little attention (Etherington, 1981), we have undertaken a general study of proteolytic degradation of these proteins by enzymes extractable with them in solutions of low ionic strength. Anzanza et al., (1979) have shown that CANP is water soluble and we have found (unpublished observations) that all detectable cathiptic activity of muscle (assayed as Cathepsins B and D) is fully recoverable by extraction with low ionic strength buffer. In this paper we report on the SDS-PAGE Electrophoresis profiles of proteins in muscle extracts incubated for up to 72 hr over the range of pH of 5.0 - 8.0 in the absence and presence of inhibitors selected to identify the active site type of the enzymes involved.

### Materials and Methods

**Extraction of muscle:** Beef psoas muscle, five-days post mortem was obtained from a commercial source. Appropriate segments were taken from the centre of the muscle, chopped into 1 mm³ cubes and extracted by either of two procedures using 4 volumes of relevant buffer.

1) A single muscle sample was extracted for 3 hr at 0-4°C with mechanical stirring in Universal buffer, (boric acid, citric acid, diethyl barbituric acid, $KH_2PO_4$) 0.038 M pH 6.5. Four aliquots of the extract were individually titrated to pH 5.0, 6.0, 7.0 and 8.0 with 1N-HCl or 1N-NaOH as appropriate. Precipitation was found to occur in the extract titrated to pH 5.0. Extracts were then treated as set out below.

2) Individual samples of muscle were similarly extracted in buffers 0.04 M at pH 5.0 (citrate-phosphate) pH 6.0(citrate-phosphate) pH 7.0(phosphate) pH 8.0(Tris-HCl) and treated as set out below.

The protein concentration in each extract or titrated aliquot in the case of Universal Buffer extraction was determined by the Biuret method (Gornall et al., 1949) using bovine serum albumin as standard.

**Incubation:** Aliquots of each extract were immediately denatured, or stored at 0°C or 37°C in the absence or presence of inhibitors for up to 72 hr prior to denaturation.

**Denaturation:** An aliquot of each extract was added to 0.05 M Tris-HCl buffer pH 6.8, (Laemli, 1970), 5% SDS, 5% β-mercaptoethanol, 30% glycerol and 0.5% bromophenol blue to yield a protein concentration of 2 mg/ml.

**Electrophoresis:** Discontinuous sodium dodecyl sulphate - polyacrylamide gel electrophoresis (Disc-SDS-PAGE, Laemli, 1970) was carried out on slab gels (7.5%, 12.5% or 15% PAGE, 0.4%SDS, 0.75 mm thick) in the 2001 LKB vertical electrophoresis unit. Sample volumes corresponding to 25 µg protein in original extract were applied and separation was effected by a constant current of 40 mA for 6 hr. Molecular weights of separated proteins were estimated by comparison with concurrently run marker proteins (Bio-Rad, Richmond, California, 94804, USA). BBR (Sigma, U.K.) and destained for 24-48 hr in 5% methanol - 7.5% acetic acid (Weber and Osborn, 1969). Gels were stained overnight in Coomassie a computer-linked densitometer (LKB, Stockholm, Sweden) before and after drying. Gels were examined visually and analysed in

### Results

Differences between the protein profiles of extracts made in different buffers and at different pH values were detected. In most cases these were seen as comparatively small alterations in the relative proportions of individual proteins extracted. In the case of extraction at pH 5.0 a major difference was observed. A protein, M.W. 93,000 extracted by Universal buffer was not found in citrate-phosphate extract. Separation of the precipitate formed on titration of the Universal buffer extract to pH 5.0 confirmed that the precipitate consisted predominantly of this protein (Fig.1). Components which show variable extractability by different buffers are indicated by asterisks

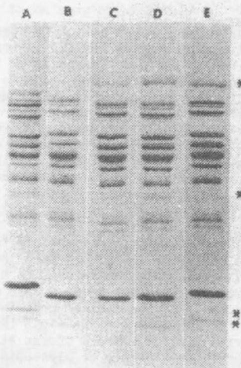


Fig.1. SDS-PAGE profiles of protein units extracted with different buffer systems.
A: Universal buffer pH 6.5 and adjusted to pH 5.0
B: Citrate phosphate buffer, pH 5.0
C: Citrate-phosphate buffer pH 6.0
D: Phosphate buffer, pH 7.0
E: Tris-HCl buffer, pH 8.0

*Proteins showing variable extractability with different buffers.

Extracts were incubated in closed vessels for period up to 72 hr during which samples were removed at suitable intervals, their pH determined and then denatured as described in methods. In the Universal buffer extract, adjusted to pH 5.0, proteolytic degradation was evident after 12 hr and increased progressively to 72 hr; an extract held at 0-4°C for 72 hr showed no detectable proteolysis. (Fig.2). In both the adjusted estracts prepared in Universal buffer and in extracts prepared in specific buffers it was found that degradation of proteins was extensive at pH 5.0, considerable at pH 6.0, negligible at pH 7.0 and slight but selective at pH 8.0 (Fig.3).

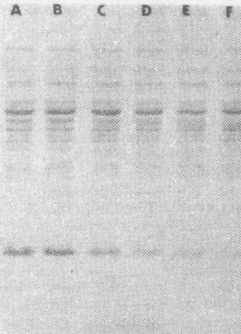


Fig.2. SDS-PAGE profiles of samples removed at intervals from incubation of extracts in Universal buffer pH 6.5, adjusted to pH 5.0.
A: immediately denatured
B: stored at 0°C, 72 hr
C: incubated at 37°C, 12 hr
D: " " " 24 hr
E: " " " 36 hr
F: " " " 72 hr

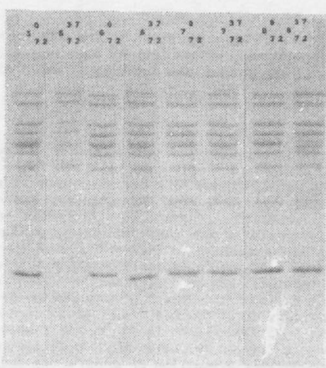

Fig.3. Comparison of SDS-PAGE profiles of proteins in extracts in specific buffers and held at 0°C or incubated at 37°C for 72 hours as indicated by the labels. e.g. channel $5^{0}_{72}$ and $5^{37}_{72}$ indicate incubations of pH 5.0 extracts at 0°C and at 37°C for 72 hours respectively.

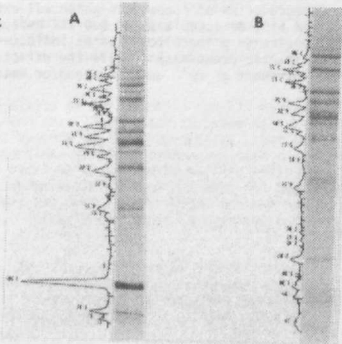


Fig.4. Correlation of densitometer trace and SDS-PAGE profile of extract in pH 5.0 buffer.
A: stored at 0°C, 72 hr
B: incubated at 37°C, 72 hr.

Densitometric profiles confirmed the visual observations as can be seen in Fig.4 which shows correlation of the densitometer traces for the Universal buffer extract adjusted to pH 5.0 and stored at 0-4°C or incubated at 37°C for 72 hr. Stain binding protein was reduced to approx. 50% and this was shown to reflect a similar decrease in all but two of the protein bands. A protein, M.W. c77,000 was virtually unaffected while a protein, M.W. c15,000 was almost completely degraded. Up to eight peptides of varying molecular weights appeared as degradation products, which however corresponded to only a small fraction of the loss in total protein. It is therefore concluded that degradation of most of the protein proceeded to a level outside the detection limits of the present methodology. Experiments to confirm this and to identify the products are currently proceeding.

In an attempt to characterise the proteolytic activities responsible for the degradation occurring at each pH level, a series of inhibitors were incorporated into the incubation media. It is clear from the results to hand that enzymes of different active site types are involved in each case of detectable proteolysis. This may be deduced from the altered protein profiles associated with inhibition by specific inhibitors. In fig.5 the profiles are shown for the pH 5.0 (Universal buffer) extract stored at 0°C or incubated at 37°C for 72 hr in the absence or presence of pepstatin or iodoacetate (IAA).

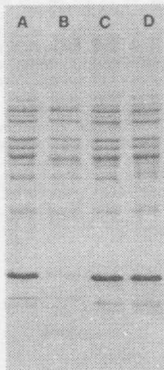


Fig.5. SDS-PAGE profiles of pH 5.0 extracts (A)stored at 0°C or incubated in the absence (B) or in the presence (C) of pepstatin or (D)of iodoacetate for 72 hr at 37°C.

It will be seen that both inhibitors reduced the extent of total proteolysis. However, in the case of pepstatin the proteins whose degradation was not inhibited were of molecular weights 107,000 and 43,700 whereas those whose degradation was not inhibited by IAA had in addition molecular weights of 45,000, 29,000 and 14,500.

This variation in susceptibility to proteolysis is of course primarily a reflection of the inherent characteristics of the substrate. In the present context it seems reasonable to assume that it also indirectly relates to the nature and identity of the enzyme, the activity of which will be modified in the presence of its specific inhibitor.

## Discussion

The results of a preliminary survey of proteolytic activity in extracts of muscle sarcoplasmic proteins during incubation at 37°C are presented. The aim of the study was to demonstrate the occurrence of and to identify the nature of the activity over a range of pH values. The use of 5-day aged meat and of an incubation temperature of 37°C sheds no light on the roles of these activities in either conditioning of muscle as meat or in flavour development during cooking. The relevance of the results presented here to these two processes, so important for the meat industry, remains to be elucidated, and this will now be undertaken. Meanwhile it is tempting to speculate, on the basis of the extent of degradation of sarcoplasmic proteins even in 12 hours at 37°C, that a similar process occurring during cooking may prove to be a significant factor in the complex sequence of reactions which enhance flavour in cooked meat. In particular, the identification of the considerable production of amino acids and peptides which resulted from proteolysis observed here and which were not detected by the methods used, would provide components for Maillard reactions, thereby enhancing flavour development and browning. Much attention has already been paid to the proteolytic degradation of myofibrillar proteins by enzymes such as those studied here and to its role in tenderisation of meat. It is suggested that this process is likely to be modified by competition for degradative enzymes from the highly susceptible alternative substrates such as the sarcoplasmic proteins discussed here.

## Summary

The SDS-PAGE profiles of extracts of sarcoplasmic proteins in low ionic strength buffers vary with the pH and composition of the buffer. Incubation of the extracts for periods up to 72 hr at 37°C resulted in proteolytic degradation of components which was extensive at pH 5.0, considerable at pH 6.0, detectable at pH 8.0 and undetectable at pH 7.0. At pH 5.0 and 6.0, preliminary inhibitor studies indicated that the enzymes responsible were aspartic proteinases, while the effect of EDTA at pH 8.0 suggested the involvement of $Ca^{2+}$ activated and/or metallo-proteinases.

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