Effect of frozen storage of bovine psoas muscle on cathepsin D activity

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Stored frozen meat and in particular meat products have been found following cooking to have undergone loss of flavour or to have developed characteristic off-flavours. The identity of the compounds or reactions responsible for or contributing to these flavour changes has not been established but lipid and/or protein components or their degradation products have been implicated as possible sources(Lawrie, 1979). In an attempt to assess the role of the endogenous proteolytic enzymes of meat in off-flavour development the effects of frozen storage on the distribution and activity of cathepsin D have been studied. Cathepsin D and other proteolytic enzymes contained in the lysosomes of muscle are believed to function in the continual turnover of cellular proteins in living muscle(Pennington, 1977). They are acid proteases with pH optima in the range pH 3.0-5.0 (de Duve, 1959) contained within lipoprotein membranes which may rupture when the pH falls as a result of post mortem glycolysis or when there has been extensive tissue damage (Hamdy et al, 1969). The extent to which these enzymes are released and thereby available to exert their effect may be deduced from determination of their distribution between subcellular fractions of muscle following homogenisation and differential centrifugation. This paper presents preliminary results of work directed towards a detailed study of proteolytic activity in post mortem muscle. Cathepsin D activity has been determined in muscle as soon as possible after slaughter and thereafter in intact and in ground muscle held for specified periods at room temperature,refrigerated (0-4^{o}C) or frozen (-20^{o} and -80^{o}C)

MATERIALS and METHODS

Whole psoas muscle was removed from 30 month old bullocks immediately following slaughter and evisceration. It was brought to the laboratory (15 min), trimmed of fat, wrapped in several layers of sterile gauze and placed in the incubator at 17^{o}C for 24 hr. A sterile thermometer and sterile spear electrode (Russell pH) were inserted into the centre of the muscle, from which readings were taken at the intervals indicated. Multiple random samples (each approximately 5 cm cube) were cut at each interval. One such sample was ground, homogenised and processed directly. Further samples were taken in pairs, one of which was ground and both held at one of the following temperatures for designated periods: 17^{o}C, 4^{o}C, -20^{o}C and -80^{o}C. Grinding was carried out in a hand mincer (6mm orifices). Fractionation of subcellular components was carried out following homogenisation (Potter-Elvehjem) of a 10%w/v suspension of ground meat in either (a) 0.15M KCl, 0.02M K_3PO_4, 1mM EDTA, pH 7.0 (modified from Iodice et al, 1972) or (b) 0.25M sucrose, 1mM EDTA, pH 7.0 (modified from Bird, 1975). The homogenate, filtered through gauze, was adjusted to pH 7.0 with 0.1M KOH and was subjected to the centrifugation procedure of Iodice et al, (1972). Fractions isolated were nuclear, mitochondrial, lysosomal ('light mitochondrial') and post-mitochondrial supernatant. These were assayed for protein (by Biuret after Gornall et al, 1949, and Kjeldahl, after Bradstreet, 1965)and for the following enzymes: ß-glycerophosphatase (EC 3.1.3.2)as modified by Barrett (1972a, method 1) p-nitrophenyl phosphatase (EC 3.1.3.41) as modified by Barrett (1972a, method 2), carboxypeptidase A (EC 3.4.17.1)by the procedure of Iodice et al(1966) using the ninhydrin assay of Rosen (1957), cathepsin B (EC 3.4.22.1) and dipeptidyl peptidase 1 (EC 3.4.14.1) after Barrett (1972b) and Cathepsin D (EC 3.4.23.5) modified from Barrett 1977, method 2. Having established the localisation of cathepsin D (assayed using urea denatured haemoglobin as described by Venugopal and Bailey, 1978) in the lysosomal fraction and to minimise possible proteolytic changes during prolonged fractionation procedures a modified centrifugation scheme was introduced. The filtered homogenate was centrifuged for 10 min at 500g to remove nuclei and cell debris. The'post-nuclear homogenate' was then centrifuged (Sorvall-RC5) at 18,000g for 30 min. This was found to result in maximal sedimentation of cathepsin D activity and was taken to represent complete sedimentation of lysosomes. Cathepsin D activity was determined in the post-nuclear homogenate, in the resuspended 18,000g sediment and in the 18,000g supernatant. In addition the 18,000gsediment was exposed to a range of treatments expected to increase the detectable enzyme activity by release from membrane-bound organelles or by 'activation' of the enzyme. These treatments included (a) repeated freeze-thaw cycles, (b) exposure to hypotonic media in the absence and presence of detergents Triton X-100 (Rohme and Hass, purchased from Koch-Light, London) or Brij 58 (Sigma, London), (c) acetone (5%v/v, 1 hr, 4^{o}C; Parrish and Bailey, 1967) and (d) incubation for 45 min at 37^{o}C in adrenaline (100µM ; Sigma, London) or in vitamin A palmitate (19mM; Sigma, London) (Parrish and Bailey, 1967).

RESULTS and DISCUSSION

Temperature and pH readings for bovine psoas muscle removed immediately after slaughter, maintained at 17^{o}C and measured at 30min, 90min, 2hr and hourly up to 9hr and at 22hr and 24hr are shown in fig 1. Temperature decreased during 4 hours to the ambient temperature of the incubator and after 24 hr was reduced to 4^{o}C following transfer to the refrigerator. pH decreased from 6.2 to 5.5 after 8hr and remained at that value for a further 16hr at 17^{o}C and when held for a further 24 hr in the refrigerator. The distribution of cathepsin D activity between the 18,000g sediment and supernatant expressed as per cent of the combined values were calculated at 90min. 4hr, 24hr, 7days and 14 days. The results (Table 1A) show that initially 47% of activity remained in the particulate fraction while 53% was recovered in the supernatant. No significant change in distribution was observed at 4hr. The activity detected in the supernatant was believed to represent that of enzyme released from lysosomes due primarily to the grinding and homogenisation of the muscle. At 24hr the proportion of activity recovered in the supernatant increased to 60% after which no further alteration in distribution was observed during 2 weeks storage at 4^{o}C. Corresponding values for meat frozen post rigor and held at -20^{o}C for 2 weeks are shown in Table 1B and for meat frozen pre-rigor at -80^{o}C and analysed at 48hr are shown in Table 1C. These results may be summarised as follows. In muscle from the slaughtered animal grinding and homogenisation procedures cause release of over 50% of lysosomal proteolytic activity due to cathepsin D. A further 10% of the activity may be released by

these procedures in post rigor muscle; no further release occurs during two weeks in the refrigerator. Post rigor muscle, whether ground or whole, allows the release of up to a total of 70% of cathepsin D activity during one or two weeks storage at -20°C. This may simply reflect additional rupture of lysosomes due to freezing and/or thawing. Support for this possibility would appear from the behaviour of pre rigor muscle frozen at -80°C in which an increase of about 10% in activity in the supernatant over that in pre rigor muscle was observed (Table 1C).

TABLE 1 PER CENT DISTRIBUTION OF CATHEPSIN D ACTIVITY BETWEEN THE SEDIMENT AND SUPERNATANT FROM 18000g CENTRIFUGATION OF THE POST NUCLEAR HOMOGENATE IN 0.15M KCl, 0.02M K_3PO_4, 1mM EDTA, pH 7.0, OF BOVINE PSOAS.

1A MUSCLE HELD AT 17°C FOR 24HR AND REFRIGERATED (0-4°C) THEREAFTER.

FRACTION	% TOTAL ACTIVITY OF HOMOGENATE				
TIME	90min	4hr	24hr	7d	14d
SUPERNATANT	53	51	60	62	60
SEDIMENT	47	49	40	38	40

1B MUSCLE HELD AT 17°C FOR 24HR, GROUND AND STORED GROUND AND UNGROUND, FROZEN AT -20°C THEREAFTER

SUPERNATANT	UNGROUND	-	-	60	66	68
	GROUND	-	-	60	67	69
SEDIMENT	UNGROUND	-	-	40	34	32
	GROUND	-	-	40	33	31

1C MUSCLE, PRE RIGOR, FROZEN AT -80°C FOR 48HR.

SUPERNATANT 60% SEDIMENT 40%

In the preceding experiments enzyme activity of the resuspended sediment was determined without further treatment. Treatments such as repeated freeze-thaw or with the detergent Triton X-100 caused negligible increase in enzyme activity. It appeared that homogenisation in KCl medium made the sedimentable enzyme activity accessible to the protein substrate, possibly by altering the permeability characteristics of the lysosomal membrane. This conclusion was supported by the observation that when lysosomes were prepared in sucrose medium and resuspended in KCl medium such accessibility was not evident. This will be further investigated.

Despite the observation of Andersen et al (1979) that sucrose interfered with assay of cathepsin D the possible advantage of sucrose medium in preserving lysosomal integrity was examined. It was found that homogenisation in 0.25M sucrose, 1mM EDTA, pH 7.0 resulted in isolation of a sediment fraction with negligible detectable cathepsin D activity while the supernatant fraction contained all the activity of the postnuclear homogenate. Inhibition of activity by sucrose was not evident. Attention was then focussed on the estimation of cathepsin D in the lysosomal sediment. Of the methods tested (see METHODS) maximum cathepsin D was detectes when the sediment was resuspended in 0.2% aqueous Brij 58. Considerably less activity was found with Triton X-100 and with vitamin A palmitate. Examination of the effects of Brij 58 on the activity of all of the fractions was complicated by the presence of isotonic homogenisation medium and so the use of 0.2% Brij 58 in that medium was examined. The results of a typical experiment (Table 2) show that inclusion of the detergent causes an increase in activity in all three fractions. The recovery of greater summed activity in the separated fractions than in the homogenate from which they were isolated remains unexplained. It may point to the existence of an inhibitory component which is more effective in the homogenate. The increase in activity in the lysosomal fraction, while large, does not represent the full activity potential of the fraction as expressed following treatment with aqueous Brij 58. The significance of this latter extremely high activity remains to be determined. Resuspension of the lysosomal sediment in water resulted in the release of some 60% of the activity released in aqueous Brij 58. This would suggest that a specific effect of Brij 58 on the lipid environment of the enzyme was necessary for the full expression of enzyme activity.

TABLE 2. EFFECT OF BRIJ 58 (0.2%w/v) ON CATHEPSIN D ACTIVITY OF FRACTIONS ISOLATED FROM BOVINE PSOAS MUSCLE HOMOGENISED IN 0.25M SUCROSE, 1mM EDTA, pH 7.0.

TREATMENT	CATHEPSIN D ACTIVITY (nmoles tyrosine equivalents solubilised/min/mg wet wt. of muscle)		
	POSTNUCLEAR HOMOGENATE	18000g SUPERNATANT	18000g SEDIMENT
AS ISOLATED	15.5	11.4	—
RESUSPENDED IN HOMOGENISATION MEDIUM	—	—	0.0
HOMOGENISATION MEDIUM PLUS BRIJ 58	21.7	25.4	22.4
RESUSPENDED IN AQUEOUS BRIJ 58	—	—	52.3

The effect of removal of homogenisation medium by dialysis is being investigated. In the meantime further observations on enzyme distribution during ageing and refrigerated and frozen storage involved the use of Brij 58 in homogenisation medium. The results (Table 3) show an interesting alteration in the distribution of activity compared with that observed using KCl medium in that at least 67% of cathepsin D activity of muscle was recovered in the sediment. However the pattern of release appears similar; up to 10% of lysosomal activity is released during 24hr immediately post mortem with only a slight further release occurring during refrigeration for one week.

Preliminary results using the sucrose procedure suggest that, as for the KCl procedure, freezing of meat, unground or ground increases by about 10% the release of lysosomal proteolytic activity.

Work currently in progress aims at determining the nature and degree of proteolysis which occurs during

post mortem conditioning of muscle and subsequent long term storage under the conditions described here.

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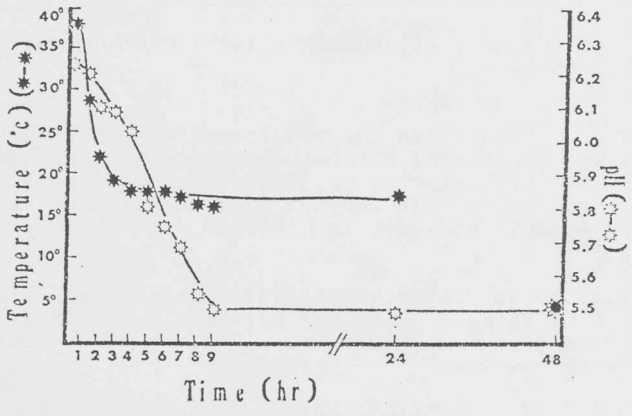
TABLE 3 DISTRIBUTION OF CATHEPSIN D BETWEEN 18000g SUPERNATANT AND SEDIMENT FROM POSTNUCLEAR HOMOGENATE IN 0.25M SUCROSE, 1mM EDTA, pH 7.0 OF BOVINE PSOAS MUSCLE HELD AT 17°C FOR 24HR AND REFRIGERATED (0-4°C) THEREAFTER. ACTIVITY DETERMINED IN PRESENCE OF 0.2%w/v BRIJ 58.

FRACTION	% TOTAL ACTIVITY			
TIME:	90min	4hr	24hr	7d
SUPERNATANT	33	38	42	46
SEDIMENT	67	62	58	54

FIG. 1. TEMPERATURE AND pH CHANGES IN BEEF PSOAS MUSCLE KEPT IN AN INCUBATOR AT 17°C FOR 24HR AND REFRIGERATED AT 0-4°C FOR A FURTHER 24 HR.



SUMMARY

The distribution of cathepsin D activity between subcellular fractions of bovine psoas muscle was different when sucrose based medium or KCl based medium was used for homogenisation. Sucrose prepared lysosomes showed no cathepsin D activity until exposed to detergent Brij 58, the effect of which was greatest in aqueous solution.

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REFERENCES

Andersen,KJ, Dobrota,M and Haga,HJ (1979) J.Biochem. Biophys. Methods, 1, 309-311.
Barrett,AJ, (1972a) in Lysosomes: A Laboratory Handbook (Dingle,JT, ed.) p. 112-113.
Barrett,AJ, (1972b) Anal. Biochem.,47, 280-293.
Barrett,AJ, (1977) in Proteinases in mammalian cells and tissues (Barrett,AJ, ed.) p.209-249, Elsevier.
Bird,JWC, (1975) in Lysosomes in Biology and Pathology (Dingle,JT & Dean,RT, eds.) 4, 75-109.
Broadstreet,RB, (1965) in The Kjeldahl Method for Organic Nitrogen, Academic Press, N.Y.
de Duve,C, (1959) in Subcellular Particles, p.128, Ronald Press, N.Y.
Gornall, AG, Bardawill, CJ, & David,MM, (1949) J. Biol. Chem. 177, 751-
Handy,MK, May,KN, & Powers,JT, (1961) Proc. soc.Exp.Biol.Med. 108, 185-
Iofice,AA, Leong,V,& Weinstock,JM (1966) Arch. Biochem. Biophys. 117, 477-486.
Iofice,AA, Chin,J, Perker,S & Weinstock,JM, (1972) Arch. Biochem. Biophys. 152, 166-174.
Lawrie,RA, (1979) in Meat Science, 3rd.ed. p.353-366, Pergamon Press, Oxford.
Parrish,FC, & Bailey,ME, (1967) J. Agr. Food Chem. 15, 88-94.
Pennington,RJT, (1977) in Proteinases in Mammalian Cells and Tissues,(Barrett,AJ, ed.) p.353-366, Elsevier.
Rosen,H, (1957) Arch. Biochem. Biophys. 67, 10-15.
Venugopal,B, & Bailey,ME, (1978) Meat Science, 2, 227-239.