550) equipped with an electron capture detector was used. A glass column, 0.25 in. × 4 ft, was packed with Chromosorb W-HP (80–100 mesh) coated with 4% SE-30 and 6% QF-1. The column was used at 210–215°. The gas flow was adjusted to give DNPMA a retention time of about 9 min. Five-microliter samples of appropriate DNPMA or extract solutions were analyzed by glc. At attenuation 4, 1.5 ng of DNPMA gave about 0.5 fullscale deflection of the recorder pen. This amount was well within the linear detection range.

RESULTS AND DISCUSSION

The microcolumn apparatus was found invaluable in the glc method developed for methomyl residues in rapeseed oils (Mendoza and Shields, 1974). Consistently good cleanup and recovery of DNPMA were obtained from the microcolumn packed with silica gel G-HR. The apparatus is rapid and efficient in cleaning up the DNPMA extracts before glc analysis. It is routinely used in evaluating methods using DNFB and methylamine moieties of carbamate pesticides.

The following are illustrations of the application and performance of the microcolumn apparatus.

Figure 2 shows typical chromatograms of DNPMA extracts without (no. 1) and with microcolumn cleanup (no. 2) and reaction blank extracts (no. 3 and 3′) with cleanup. Accurate quantitation was not achieved when the peak which appeared after the DNPMA peak (marked with a check) was present. The peak on the front shoulder of the DNPMA peak did not interfere with quantitation unless its height was equal or higher than that of DNPMA. Chromatograms 2, 3, and 3′ show that a column cleanup procedure completely removed the broad interfering peak.

Figure 3 shows typical chromatograms of silica gel G-HR extracts without methomyl (no. 1) and with methomyl added (no. 2). The extracts were allowed to react with DNFB and cleaned up by microcolumn chromatography. Note that the gel did not give any interference.

In another study, methomyl was detected on tlc plates by a tlc-enzyme inhibition technique using indophenyl acetate on 5-bromoxindoxyl acetate and pig liver extracts. The site of inhibition was scraped off the plate, hydrolyzed, treated with DNFB, and cleaned up by microcolumn chromatography. Figure 4 illustrates that good cleanup and complete recovery of DNPMA were obtained after column chromatography. Good recovery of methomyl was achieved even after reaction with enzyme. Neither the enzyme, substrate, nor gel gave interfering glc peaks.

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Nitrite-Induced Inhibition of Purified Fractions of Chicken Muscle Cathepsin D

Isolation and purification of cathepsin D from chicken leg muscle indicated the presence of multiple forms of enzyme in the tissue. Two different fractions (A and B) of cathepsin D obtained during Sephadex G-100 gel filtration showed marked differences in their electrophoretic mobilities and response to treatment with sodium nitrite. At nitrite concentrations simulating those used in meat curing, only fraction A was inhibited, whereas fraction B was unaffected even at a higher concentration (1 mmol/0.8 mg of protein).

The potential risk of using sodium nitrite as a curing agent in meat processing is receiving considerable attention since, under favorable conditions, nitrite reacts with amines to form the nitrosamine group of carcinogenic and hepatotoxic compounds (Ender and Ceh, 1971; Wolff and Wasserman, 1972). Our current knowledge regarding the function of nitrite in meat curing is limited to its role in the enhancement of color (Giddey, 1966) and the inhibition of *Clostridium botulinum* (Emodi and Lechowich, 1969). However, besides bacterial spoilage, deterioration in quality during storage of meat products occurs by the action of hydrolytic enzymes in the tissue, thereby necessitating inactivation of the hydrolases during meat curing processes (Bandack-Yuri and Rose, 1961; Martins and Whitaker, 1968). The mode of action of nitrite on skeletal muscle lysosomal enzymes is, therefore, being carefully scrutinized in our laboratory.

Our results (Warrier *et al.*, 1973; Harikumar *et al.*, 1974) have demonstrated that enzymes associated with tissue autolysis, *viz.*, cathepsin (EC 3.4.4.23), arylsulfatase (EC 3.1.6.1), and β-glucuronidase (EC 3.2.1.31), are readily inhibited by nitrite treatment at concentrations varying from 5 to 50 μmol. A combination of nitrite and mild heat treatment was found to be more effective than either of the single treatments in suppressing the activities of these enzymes. Among these the inhibition of cathepsin D merits special attention since it is known to be the major proteinase in muscle tissues (Weinstock and Iodice, 1969; Caldwell and Grosjean, 1971; Harikumar *et al.*, 1974). The compounds which are known to inhibit cathepsin D, *viz.*, tetranitromethane (Keilova, 1971), methyl esters of diazoacetylphenylalanine (Barrett, 1971), diazoacetylnorleucine (Smith *et al.*, 1969), and heavy metal ions (Misaka and Tappel, 1971), have limited scope as food

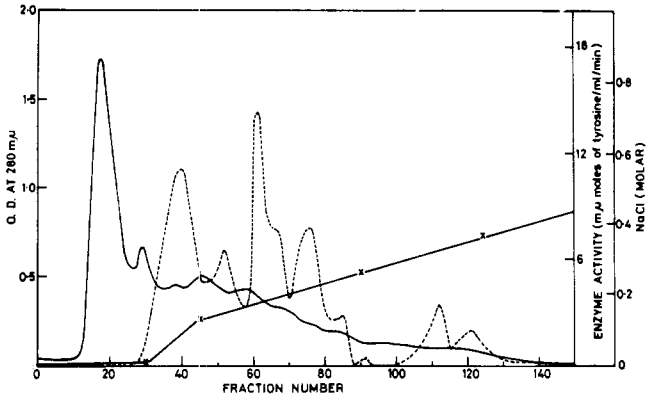

Figure 1. Elution pattern of cathepsin D on DEAE-cellulose. A dialyzed ammonium sulfate fraction of chicken muscle cathepsin D, prepared as described in the text, was applied on a DEAE-cellulose column (2 × 30 cm) and eluted into 150-ml fractions (3 ml) using a continuous gradient of 0–0.5 *M* NaCl in 0.01 *M* phosphate buffer (pH 6). The protein was monitored by 280-mμ absorption and cathepsin D activity was determined according to the method of Gianetto and de Duve (1955): (—) protein; (- - -) enzyme activity; (× – ×) NaCl concentration.

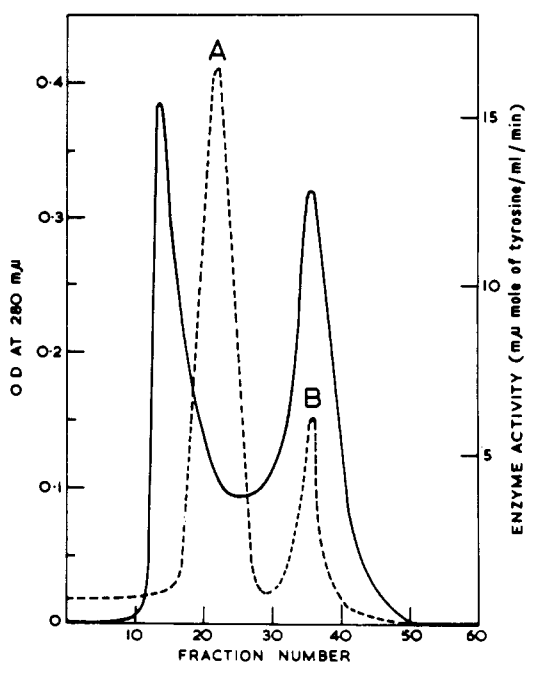

Figure 2. Elution pattern of cathepsin D on Sephadex G-100. Fractions 32–80 obtained from DEAE-cellulose were pooled together, dialyzed overnight against distilled water, and centrifuged. The supernatant was lyophilized and dissolved in 0.01 *M* phosphate buffer (pH 6). An aliquot of the fraction (1.2 mg of protein) was applied on a Sephadex G-100 column (3 × 30 cm) and eluted with the same buffer into 3-ml fractions: (—) protein; (- - -) enzyme activity.

additives. Therefore, studies on the mechanisms of nitrite inhibition with pure enzymes may help in catalyzing research on suitable substitutes for nitrite in meat curing. The results presented in this communication indicate profound differences in the response of two fractions of cathepsin D, containing isoenzymic forms of the enzyme purified from chicken leg muscle, to nitrite treatment.

EXPERIMENTAL SECTION

White leghorn chickens belonging to the age group of 2–4 months were used in the present investigation. Birds were slaughtered by exanguination and bled thoroughly. Leg muscle tissue was excised, washed with chilled water, and held at 0–4°.

Sephadex G-100 and DEAE-cellulose (Whatman New Fibrous DE-22, catalog No. 2422) were the products of Pharmacia Fine Chemicals, Uppsala, Sweden, and W & R Balston Ltd., London, respectively. Denatured hemoglobin type II was obtained from M/s Sigma Chemicals.

Purification of Cathepsin D. The method of Iodice *et al.* (1966) was suitably modified for the purification of chicken muscle cathepsin D. Chicken leg muscle was finely minced with scissors and homogenized at top speed for 2 min in 0.1 *M* acetate buffer (pH 3.8) containing 0.2 *M* KCl employing a Sorval omnimixer. The homogenate was frozen and thawed ten times and centrifuged at 10,000*g* for 10 min. The extract thus obtained was heated at 50° for 10 min and centrifuged. The supernatant was saturated to 60% with solid $(NH_4)_2SO_4$; the precipitate obtained was dissolved in 0.01 *M* phosphate buffer (pH 6) and dialyzed free of $(NH_4)_2SO_4$ against 0.005 *M* phosphate buffer (pH 6).

Chromatography on DEAE-Cellulose. The dialyzed fraction was applied on a DEAE-cellulose column (2 × 30 cm) equilibrated with 0.01 *M* sodium phosphate buffer and eluted into 150 fractions (3 ml) using a continuous gradient of 0–0.5 *M* NaCl in 0.01 *M* phosphate buffer (pH 6). The protein was monitored by 280-mμ absorption. Cathepsin D activity was determined from the individual fractions according to the method of Gianetto and de Duve (1955).

Chromatography on Sephadex G-100. Fractions 32–80 obtained from DEAE-cellulose were pooled together and dialyzed overnight against distilled water. The dialyzed fraction was lyophilized and then dissolved in a minimum volume of 0.01 *M* phosphate buffer (pH 6). Suitable aliquots (1.2 mg of protein) were chromatographed on a Sephadex G-100 column and eluted employing 0.01 *M* phosphate buffer. Protein values as well as cathepsin D activities were determined from the fractions obtained.

Acrylamide Gel Electrophoresis. Fraction A (eluate 20–25) and fraction B (eluate 32–38) obtained from Sephadex G-100 were lyophilized and dissolved in a minimum volume of distilled water. Aliquots of the samples containing 100–200 μg of protein were electrophoresced by the method of Davis (1964) using 7.5% polyacrylamide gel in 0.01 *M* Tris-glycine buffer (pH 8.3).

Nitrite Treatment of Cathepsin D. The conditions of nitrite treatment for purified fractions of cathepsin D were suitably modified from our previous observations (Warrier *et al.*, 1973; Harikumar *et al.*, 1974) on the effects of nitrite at concentrations ranging from 5 to 200 μmol and heat at 37, 50, and 60° on the degree of inhibition of lysosomal enzymes in skeletal muscle extracts. Fractions A and B of cathepsin D obtained from Sephadex G-100 were separately subjected to treatment with 10 μmol of $NaNO_2$ at 50° in a reaction system (1.5 ml) containing 0.8 mg of protein. Nitrite treatment was carried out at 50° since it facilitates maximum inhibition of cathepsin D without itself inactivating the enzyme.

Protein Values. The protein content of the tissue preparations was determined according to the method of Lowry *et al.* (1951).

RESULTS AND DISCUSSION

The chromatographic profile of purified cathepsin D on DEAE-cellulose indicated a distribution of activity as shown in Figure 1. When fractions 32–80 were chromatographed on Sephadex G-100, enzyme activity was distributed into two distinct peaks corresponding to tubes 20–25 (fraction A) and tubes 32–38 (fraction B) as shown in Figure 2. Fraction A was purified to the extent of 113-fold while fraction B showed a purification of 21-fold. The data on the stepwise purification of cathepsin D are presented in Table I. On polyacrylamide gel electrophoresis, fraction A resolved into six bands while fraction B remained as a

Table I. Purification of Cathepsin D from Chicken Leg Muscle[a]

Fraction	Sp act.[b]	Purification fold	Act. recovd, %
KCl extract	1.0	1.0	100
Supernatant after heat treatment	2.1	2	96
0–60% $(NH_4)_2SO_4$ fraction dialyzed	3.5	3.5	54
DEAE-cellulose eluate 32–80	25.0	25	41
Sephadex G-100 eluate 20–25 (fraction A)	113.0	113	12
Eluate 32–38 (fraction B)	21.0	21	5

[a] Enzyme activity was determined according to the method of Gianetto and de Duve (1955). [b] The specific activity of the enzyme is expressed as nanomoles of tyrosine equivalents per milligram of protein per minute.

Table II. Effect of Nitrite Treatment on Purified Cathepsin D[a]

Treatment	% retention of enzyme act. Fraction A	Fraction B
Control (heat at 50° for 10 or 60 min)	100	100
Nitrite		
(i) 10 μmol of $NaNO_2$ + heat at 50° for 60 min	66	100
(ii) 1 mmol of $NaNO_2$ + heat at 50° for 10 min	50	100

[a] Fractions A and B were separately subjected to a combination of nitrite and heat treatment. For controls, only heat treatment in the absence of nitrite was employed. The reaction system (1.5 ml) contained 0.8 mg of protein. After the treatments, cathepsin D activity was determined in all the samples at pH 3.8 according to the method of Gianetto and de Duve (1955).

single band (Figure 3). This is probably the first instance indicating the occurrence of different isoenzymic forms of cathepsin D in chicken muscle, although a similar phenomenon has been reported in a number of tissues including human and chicken liver (Barrett, 1970), bovine spleen (Press *et al.*, 1960), and bovine uterus (Woessner and Shamberger, 1971).

In addition to differences in the elution pattern on DEAE-cellulose and Sephadex G-100 and electrophoretic mobilities on polyacrylamide gel indicative of differences in the net charge and molecular weights, the two fractions A and B showed marked differences in their response to nitrite-induced inhibition of enzyme activity. Thus, as shown in Table II, fraction A was inhibited by 34% while fraction B was unaffected by a combination of nitrite and heat treatment (10 μmol of $NaNO_2$/1.5-ml reaction system containing 0.8 mg of protein which was heated at 50° for 60 min). The differential response of fractions A and B persisted even at a concentration as high as 1 mmol. The requirement for heat treatment for inhibition was not obligatory for cathepsin D; however, other lysosomal enzymes, particularly β-glucuronidase, showed marked inhibition by nitrite when subjected to mild heat treatment (Warrier *et al.*, 1973; Harikumar *et al.*, 1974). The details of inhibition characteristics employing purified enzymes will be published elsewhere.

The inhibitory effect of nitrite could be ascribed to the formation of oxidative species such as nitrous acid, nitric oxide, and nitrogen dioxide (Shank *et al.*, 1962). In nitrite cured meat products, it is thus likely that the inhibition of autolytic activity is due to the cumulative effect of modifications caused by these oxidative species on the enzyme as well as substrate molecules.

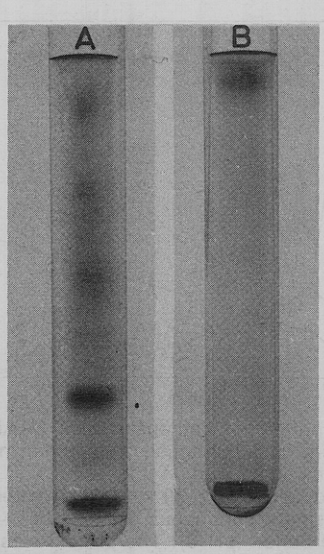

Figure 3. Electrophoretic pattern of cathepsin D on polyacrylamide gel. Fractions A and B obtained from Sephadex G-100 were lyophilized and dissolved in a minimum amount of distilled water. These fractions were separately run on polyacrylamide gel in 0.01 *M* Tris-glycine buffer (pH 8.3).

The differential responses of fractions A and B of cathepsin D to nitrite inhibition, which have not been reported so far, merit further investigations. Such studies may provide not only useful probes in the elucidation of the structural differences among the isoenzymic forms of cathepsin D but may also help in evolving suitable substitutes for nitrite for the inhibition of tissue autolysis.

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