Studies on Proteolytic Activity in Commercial Myoglobin Preparations

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Commercial myoglobin preparations from horse skeletal muscle degraded casein. The maximum activity was at pH 8-8.5. A muscle myofibril preparation was also attacked. The protease could be partly separated from the myoglobin by selective ultrafiltration through a membrane with an exclusion limit of mol.wt. 30000. A greater than 1000-fold purification of the proteolytic activity was achieved by affinity chromatography with soya-bean trypsin inhibitor bound to CM-cellulose. The enzyme preparation hydrolysed *p*-toluenesulphonyl-L-arginine methyl ester and *N*-benzyloxycarbonyl-L-tyrosine *p*-nitrophenyl ester. Its activity was inhibited strongly by soya-bean and ovomucoid trypsin inhibitors, serum and the soluble fraction of muscle homogenates. EDTA, *p*-chloromercuribenzoate and phenylmethylsulphonyl fluoride also caused some inhibition.

During studies on muscle proteinases, which involved observing the breakdown of myoglobin prepared commercially from horse skeletal muscle, it was noticed that the myoglobin preparations possessed proteolytic activity. Some investigations on this are described in the present paper, and may be of interest to others who have used myoglobin as a substrate for proteolytic enzymes and may also be relevant to chromatographic and electrophoretic studies on myoglobin, which have indicated the presence of several fractions (see Satterlee & Snyder, 1969); it is possible that some of these fractions may result from proteolytic breakdown. Moreover, the proteolytic activity may represent undescribed proteolytic enzymes present in skeletal muscle from which the myoglobin was obtained; relatively little is known of skeletal-muscle proteinases.

MATERIALS AND METHODS

Materials. Myoglobin (horse skeletal muscle) was obtained from Miles-Seravac (Pty.) Ltd., Maidenhead, Berks., U.K., and from Koch-Light Ltd., Colnbrook, Bucks., U.K. Individual batches from both these sources displayed wide variation in proteolytic activity. The results presented were obtained with myoglobin from the former source.

Casein (Hammarsten) was from BDH Chemicals Ltd., Poole, Dorset, U.K. A 3% (w/v) solution was prepared with the minimum amount of NaOH necessary to dissolve the casein and was heated at 100°C for 30 min to destroy any proteolytic enzyme that might be present as impurity.

Trypsin (type III, from bovine pancreas, approx. 10000 *N*- α -benzoyl-L-arginine ethyl ester units/mg) was obtained from Sigma (London) Chemical Co. Ltd., London S.W.6, U.K. α -Chymotrypsin (bovine pancreas; 9000-11000 *N*- α -acetyl-L-tyrosine ethyl ester units/mg) was from BDH Chemicals Ltd.

Sephadex 6B was from Pharmacia (G.B.) Ltd., London W.13, U.K. Soya-bean inhibitor bound to CM-cellulose was the product of E. Merck A.-G., Darmstadt, Germany.

Soya-bean trypsin inhibitor and ovomucoid trypsin inhibitor were from Koch-Light Ltd.

Preparation of myofibril fraction. Rats (Wistar strain, 2-5 months old, both sexes) were killed by cervical dislocation; mixed muscles from the hind legs were removed and freed from visible fat and nerve. The muscle was finely chopped and homogenized in 0.05M-KCl (approx. 3 ml/g of muscle) with an Ultra-Turrax homogenizer, sufficiently to release all the contents of the fibres, as seen by phase microscopy. The suspension was diluted with further KCl (approx. 7 ml/g of muscle) and centrifuged at 100000g for 30 min, and the sediment was resuspended twice in water and centrifuged at 600g for 10 min and finally resuspended in water (10 ml/g of muscle).

Measurement of proteolytic activity. The incubations were done in a total volume of 0.3 ml in 0.03M-*N*-tris-(hydroxymethyl)methylglycine (tricine) buffer, pH 8.5, unless otherwise stated. Incubation was at 37°C for 2 h and the reaction was terminated by adding 0.9 ml of 6.7% (w/v) HClO₄; the tubes were kept on ice for 15 min before being centrifuged. Proteolytic activity is expressed as the increase in E_{224} of the supernatant. Increments in E_{260} were relatively small, thus eliminating the possibility that nucleic acid breakdown contributed appreciably to the increments in E_{224} . All assays were done in duplicate.

Hydrolysis of synthetic substrates. The hydrolysis of toluene-*p*-sulphonyl-L-arginine methyl ester and *N*-benzyloxycarbonyl-L-tyrosine *p*-nitrophenyl ester was measured as described by Hummel (1959) and Martin, Golubow & Axelrod (1959) respectively.

Experiments with labelled muscle proteins. A mouse was given eight subcutaneous injections each of 0.1 ml (total, 44 μ Ci) of 14 C-labelled protein hydrolysate from *Chlorella* (45 mCi/mg-atom of C; The Radiochemical Centre, Amersham, Bucks., U.K.) over a period of 9 days. Then 2 days later it was killed and the myofibril fraction prepared in the usual way from the hind-leg muscles.

Incubations were done in the usual manner. The HClO_4 supernatants were neutralized with KOH, the precipitated KClO_4 was removed by centrifugation and the supernatants were plated on planchets and the radioactivity was measured with a Tracerlab Omniguard end-window counter (background radioactivity less than 1 c.p.m.).

Determination of protein. The method of Lowry, Rosebrough, Farr & Randall (1951) was used, with bovine serum albumin as standard.

RESULTS

When solutions of commercial myoglobin were incubated there was an increase in the E_{224} of the fraction soluble in 5% (w/v) perchloric acid (Table 1). If the myoglobin was previously heated at pH 7.0 for 30 min at 70°C (higher temperatures caused rapid coagulation) its breakdown was almost prevented. On the other hand, preliminary treatment by keeping at pH 1.5 for 30 min at 37°C, then readjustment to pH 7.0, considerably increased its breakdown. Incubation of the myoglobin with casein caused breakdown of the latter; this was decreased to about one-third if the heated myo-

globin was used. Heating the myoglobin at 70°C for further periods of up to 2 h did not decrease its proteolytic activity on casein.

Breakdown of protein was observed also when myoglobin was incubated with the washed myofibril fraction obtained from homogenates of rat skeletal muscle (Table 1); similar results were obtained with mouse muscle. Heating the myoglobin at 100°C for 30 min abolished its activity. The myofibril fraction possessed some proteolytic activity, as shown by incubating it alone, and to confirm that the proteolytic enzyme in the myoglobin was attacking the muscle preparation, muscle proteins were labelled with 14 C as described in the Materials and Methods section, and the release of perchloric acid-soluble radioactivity during the incubation was measured. The presence of unheated and heated (70°C) myoglobin increased the average counts from 89 to 119 and 180/50 min respectively.

The nature of the protein or proteins in the myofibril fraction from rat muscle that were attacked by the proteolytic enzyme in the myoglobin was not ascertained. About one-third of the protein of this fraction was dissolved by extraction with 0.02 M-tricine buffer, pH 8.0 (1 ml/15 mg of protein). Extracts were ultrafiltered through Diaflo membranes (XM-100, exclusion limit 100 000 mol.wt.; Amicon Ltd., High Wycombe, Bucks., U.K.) alone or in the presence of one of the following: urea (8 M), guanidine hydrochloride (5 M), urea (8 M) + 2-mercaptoethanol (10 mM), guanidine hydrochloride (5 M) + 2-mercaptoethanol (20 mM), Triton X-100 (0.2%), or potassium chloride (0.6 M). In all cases the extent of protein breakdown when the high- and low-molecular-weight fractions (after dialysis) were incubated with myoglobin was approximately the same.

Proteolysis of casein by the myoglobin preparations, measured from pH 6.5 to 9.5, was maximum at pH 8–8.5 (Fig. 1). Protein breakdown resulting from the incubation of myoglobin with the washed myofibril fraction increased from pH 7.6 to about pH 10, and decreased sharply between pH 11 and 12 (Fig. 1).

The proteolytic activity associated with the myoglobin was strongly inhibited by the supernatant fraction obtained when an homogenate of skeletal muscle in water (1:10, w/v) was dialysed overnight against 0.05 M-potassium chloride and then centrifuged at 100 000 g for 30 min. Inhibition was obtained also with diluted blood serum and with trypsin inhibitors from soya-bean and ovomucoid (Table 2). When labelled myofibril fraction (0.05 ml) was incubated alone and in the presence of the muscle supernatant (0.05 ml, approx. 150 μ g of protein) the mean perchloric acid-soluble radioactivity released was 89 and 61 counts/50 min respectively, thus demonstrating that the muscle

Table 1. *Proteolytic activity of commercial myoglobin preparations*

Enzyme activity was determined at pH 8.5, as described in the text. It is expressed as the E_{224} of the perchloric acid-soluble products. Except when indicated, the myoglobin was heated for 30 min at 70°C at pH 7.0.

Materials added	Proteolytic activity
Myoglobin* (3 mg)	0.143
Myoglobin† (3 mg)	0.955
Myoglobin (3 mg)	0.017
Myoglobin* (3 mg) + casein (3 mg)	1.570
Myoglobin (3 mg) + casein (3 mg)	0.515
Myoglobin (3 mg) + myofibrils (0.05 ml)	0.457
Myoglobin (3 mg) + heated myofibrils (0.05 ml)	0.378
Myofibrils (0.05 ml)	0.073

* The myoglobin was untreated.

† The myoglobin was acid-treated as described in the text.

Fig. 1. Proteolytic activity in myoglobin preparations as a function of pH. The buffers were tricine-NaOH (pH 6.5–9.8) and glycine-NaOH (pH 10.1–11.5). Δ , Myoglobin (1 mg) + casein; $\circ$, myoglobin (3 mg, heated at 70°C for 30 min) + casein; $\bullet$, myoglobin (3 mg, heated at 70°C for 30 min) + myofibrils.

supernatant also inhibits the proteolytic activity associated with the myofibril fraction.

When myoglobin solutions were ultrafiltered through Diaflo membranes (PM-30, exclusion limit 30000 mol.wt.) the proteolytic activity was retained whereas a large proportion of the myoglobin passed through the membrane. By repeated redilution and refiltration of the retained solution a severalfold increase in the ratio of proteolytic activity to myoglobin was attained. Some increase in the absolute amount of proteolytic activity was also observed during this procedure, indicating that the myoglobin or other compound which passed through the filter was capable of inhibiting the proteolytic activity.

A considerably greater purification of the proteolytic enzyme (or enzymes) was achieved by a simple procedure involving affinity chromatography with soya-bean trypsin inhibitor bound to an insoluble matrix. Both inhibitor attached to Sepharose 6B (Sepharose-SBI), prepared by the procedure of Cuatrecasas, Wilchek & Anfinsen (1968), and inhibitor bound to CM-cellulose (CM-cellulose-SBI) were used.

When solutions of myoglobin in 0.05M-tricine buffer, pH 8.5, were passed through small columns of Sepharose-SBI, about 30% of the proteinase activity was bound to the matrix; the remainder passed through with the myoglobin. The bound enzyme could be eluted with 0.2M-potassium chloride, adjusted to pH 2.0 with hydrochloric acid. Purifications of up to 300-fold were obtained by this method.

Better purification was obtained with a batchwise technique involving soya-bean inhibitor bound to CM-cellulose. Solutions of 5% myoglobin in 0.05M-

Table 2. Natural inhibitors of proteolytic activity of commercial myoglobin

Conditions were as for Table 1. Myoglobin (heated for 30 min at 70°C) (3 mg) was present in all cases together with myofibrils (0.05 ml; Expts. 1–3) or casein (3 mg; Expt. 4).

Expt. no.	Addition	Inhibition (%)
1	Muscle supernatant (50 μ g of protein)	86
	(10 μ g of protein)	49
2	Blood serum (7 μ g of protein)	95
	(0.9 μ g of protein)	36
3	Soya-bean trypsin inhibitor (1 μ g)	80
	Ovomucoid trypsin inhibitor (1 μ g)	71
4	Muscle supernatant (50 μ g of protein)	61

Table 3. Effect of enzyme inhibitors upon activity of purified proteinases

In each case the enzyme was incubated for 1 h at 4°C with the inhibitor and buffer in a volume of 0.2 ml. The substrate (casein, 3 mg/0.1 ml) was then added and proteolytic activity was measured as usual; the inhibitor concentrations given were those present after the addition of the casein solution.

Inhibitor	Inhibition (%)
<i>p</i> -Chloromercuribenzoate (0.1 mM)	31
<i>p</i> -Chloromercuribenzoate (1 mM)	44
Phenylmethylsulphonyl fluoride (1 mM)	12
EDTA (1 mM)	33

tricine buffer, pH 8.5, were gently stirred with CM-cellulose-SBI (1 mg/ml of myoglobin solution) for 24 h, and in some cases for 48 h. After centrifugation and removal of the unbound proteins, the sedimented material was washed four to six times with 0.05M-tricine buffer, pH 8.5, and then with 0.2M-potassium chloride, pH 2.0. The proteinase was eluted by the latter solution; about 30% of the original activity was recovered. Purifications of greater than 1000-fold were attained by this method.

The purified (approx. 1000-fold) enzyme hydrolysed toluene-*p*-sulphonyl-L-arginine methyl ester and *N*-benzyloxycarbonyl-L-tyrosine *p*-nitrophenyl ester. Its activity/ μ g of protein on the former substrate was 1/38 that of trypsin; its activity on the latter substrate was 1/36 that of chymotrypsin. (The activity of the chymotrypsin used was, in our hands, only about one-third of that specified.) The enzyme activity on casein was 1/13 that of trypsin or chymotrypsin.

In an attempt to establish the class of proteinase to which the enzyme belongs, the effect of treatment with a number of enzyme inhibitors upon the activity of the purified enzyme was studied

(Table 3). *p*-Chloromercuribenzoate (a thiol-binding agent), phenylmethylsulphonyl fluoride (a serine proteinase inhibitor) and the metal chelator EDTA all caused only partial inhibition. Dialysis overnight against 1 mM-EDTA produced a 23% loss of activity, which was not restored by magnesium chloride (1 mM), zinc sulphate (1 mM) or calcium chloride (1 or 5 mM).

DISCUSSION

The proteolytic enzyme present as an impurity in the commercial myoglobin is presumed to originate from the horse skeletal muscle from which the myoglobin was prepared; possibly, however, it may come from blood present in the muscle. The muscle used by the manufacturers is collected immediately after slaughter and deep-frozen, hence a bacterial origin for the enzyme is unlikely. There has been little study of muscle proteinases with maximum activity in the alkaline range. Huston & Krebs (1968) purified a proteolytic enzyme with maximum activity at pH 7.5 from rabbit skeletal muscle. It was Ca^{2+} -dependent and hence appears to be different from the enzyme present in the myoglobin. An insulin-degrading enzyme from rat muscle reported by Brush & Cheng (1968) had a sharp optimum at pH 7.5 and was completely inhibited by 0.2 mM-*p*-chloromercuribenzoate. The enzyme found in the myoglobin may not be readily detectable in muscle homogenates or extracts, as it is strongly inhibited by a factor or factors in the particle-free muscle fraction; this inhibitor is presumably lost during purification of the myoglobin. It is possibly of biological importance that the enzyme will attack the protein of the myofibril fraction. It remains to be seen whether or not the slow autolytic breakdown of the myofibril fraction is caused by identical enzyme, although it was less susceptible to inhibition by the muscle supernatant. Noguchi & Kandatsu (1969, 1970) have reported that the autolytic breakdown of a rat muscle myofibril preparation is strongly inhibited by the muscle supernatant fraction.

It seems likely that more than one proteolytic enzyme is present in the myoglobin. The loss of about two-thirds of the activity after heating at 70°C for 30 min with little change on further heating may be caused by selective destruction of one enzyme. It may be significant that about the same proportion of the activity failed to bind to the Sepharose-SBI columns. The failure to obtain more complete inhibition by any of the reagents shown in Table 3 could also be caused by the presence of more than one enzyme.

The nature and origin of the proteinase inhibitor in the muscle supernatant fraction remains to be investigated. Inhibitors of trypsin have been reported in other animal tissues. The extent to which proteinase inhibition by muscle and other tissues is caused by plasma inhibitors present in blood vessels or extracellular space has not been determined.

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