PROTEOLYSIS OF WOOL AND ITS *S*-CARBOXYMETHYL DERIVATIVES BY PRONASE AND OTHER PROTEASES

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[*Manuscript received February* 18, 1963]

Summary

The proteolysis of native Merino wool by pronase, a protease from *Streptomyces griseus*, was shown to resemble that of other proteases in liberating cortical cells and solubilizing less than 20% of the fibre.

The action of proteases on fully reduced and alkylated wool and on soluble wool proteins was compared. Modified whole wool was digested most rapidly by pronase and the least readily by pepsin (3.4.4.1). High-sulphur proteins extracted from wool were broken down by pepsin initially at least 10 times faster than the low-sulphur proteins. The structural significance is discussed.

An attempt was made to determine whether peptide bonds in the vicinity of the more readily reducible disulphide bonds were the most susceptible to proteolysis. Varying proportions of the carboxymethyl groups of fully reduced and alkylated preparations were labelled with ^{14}C. The release of trichloroacetic acid-soluble ^{14}C and nitrogen was measured after varying periods of incubation with proteases. The rate of liberation of ^{14}C from modified whole wool by pronase and trypsin (3.4.4.4) was independent of the proportion of ^{14}C present in the preparations. With pepsin, the ^{14}C release was variable, but the ratio of percentage ^{14}C release to percentage nitrogen release was constant and thus not related to the proportion of labelled *S*-carboxymethyl groups present. The rate of production of ^{14}C soluble in trichloroacetic acid from the high-sulphur proteins was also independent of the proportion of ^{14}C label present. The more easily reducible portion of the low-sulphur proteins appeared to be somewhat more resistant to proteolysis than the rest of the molecule, but when the nitrogen liberation was taken into account, a lack of dependence on the ^{14}C content could once again be demonstrated.

These and other findings are consistent with a random distribution of the more easily reducible disulphide bonds in the wool fibre.

I. INTRODUCTION

The action of some proteases on wool and reduced wool has been known for some years (Alexander and Hudson 1954). It was of interest to examine the action of pronase, a protease from *Streptomyces griseus* of broad specificity (Nomoto, Narahashi, and Murakami 1960*b*), and to extend the comparative studies to reduced and alkylated wool. Information was also sought regarding the environment of disulphide bonds in wool. Since only about half the –S–S– bonds in some types of wool are readily reduced (Middlebrook and Phillips 1942), and since it is possible to label reduction sites by means of [^{14}C]iodoacetate (Gillespie and Springell 1961; Springell *et al.* 1964), it became feasible to determine whether the bonds nearby the more labile –S–S– bonds were also the most susceptible to proteolysis.

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II. Experimental

(a) *Wool*

Solvent-degreased Merino 64's wool top (sample No. MW129, –S–S– + –SH = 470 μmoles/g) was used throughout.

(b) *Proteolytic Enzymes*

The proteases* used were: crystalline trypsin (3.4.4.4, lot TRSF-717, Worthington Biochemical Corporation, New Jersey), crystalline pepsin (3.4.4.1, lot 13712, Armour Chemical Co., Chicago), and "Pronase-P" (Kaken Chemical Co. Ltd., Tokyo), the purified protease derived from *S. griseus*.

(c) *Preparation of 2-[^{14}C]Iodoacetic Acid*

2-[^{14}C]Bromoacetic acid (3 mc/m-mole, from the Radiochemical Centre, Amersham, England) was converted to iodoacetic acid (Gillespie and Springell 1961) in the presence of unlabelled carrier bromoacetic acid (100–200 μmoles/μc) shortly before use.

(d) *Introduction of Varying Proportions of ^{14}C into Fully Reduced and Alkylated Wool*

For partial reduction, wool (1 g) was immersed in sodium mercaptoacetate (1M; 25 ml; pH 5–6; 24 hr; 20°C). For almost complete reduction (Maclaren 1962), wool (1 g) was mechanically shaken with toluene-ω-thiol (0·1M; 100 ml in 20% n-propanol) for 48 hr, or was left (24 hr; 20°C) in sodium mercaptoacetate (0·1M; 100 ml in 40% n-propanol). The wool was washed with water, acetone, and anaesthetic diethyl ether, and dried *in vacuo*.

Alkylation with labelled iodoacetate (0·0025–0·005M) was then undertaken for 24 hr at pH 5 (0·2M potassium phthalate buffer in 50% n-propanol with sufficient sodium bicarbonate added to give pH 5 at the glass electrode) or at pH 8 (0·5M sodium borate buffer). After alkylation the wool was dried as above.

Subsequently, the wool was reduced with toluene-ω-thiol (0·1M; 100 ml in 20% n-propanol) as before (Maclaren 1962), followed by a second alkylation. Where only part of the *S*-carboxymethyl (SCM) groups were labelled with ^{14}C, a second alkylation was carried out with unlabelled iodoacetate, followed by a third reduction and alkylation with unlabelled iodoacetate. For full labelling, a second alkylation was carried out with [^{14}C]iodoacetate.

These treatments gave almost fully reduced and alkylated proteins, in which a variable portion of the SCM groups was labelled with ^{14}C. The combination of methods used for any one preparation are given in the legends to Figures 1 and 2.

Soluble proteins were extracted from wool by the long extraction procedure (Gillespie and Lennox 1955; Gillespie 1960; Gillespie and Springell 1961). Separation into high- and low-sulphur proteins was carried out at pH 4·4 and ionic strength 0·5 (Gillespie, O'Donnell, and Thompson 1962).

* The numbering is in accordance with the recommendations of the Enzymes Commission of the International Union of Biochemistry.

(e) *Protein Digestion*

The enzymes trypsin and pronase (1% w/w of substrate) were buffered with 1% ammonium acetate at pH 8 prior to use, but in preliminary experiments with pronase and purified *S. griseus* enzyme, 1% ammonium acetate and 1% ammonium carbonate buffers (pH 7 and 9 respectively) were used. Pepsin was buffered with 1% ammonium acetate–hydrochloric acid at pH 2. Bactericides used were ethylmercuric thiosalicylic acid (finally 1 : 10,000 v/v) for pepsin and trypsin digestions, and ethanol (finally 10% v/v) for the digestions with pronase.

A typical system was: substrate (100 mg), buffer (8 ml), bactericide (10 ml), and enzyme solution (2 ml). Controls contained water in place of the enzyme solution.

Incubation was carried out at 28°C, and occasionally at 37 and 55°C. Mechanical agitation was applied at the lower temperatures. In experiments on native wool the residue was isolated after varying periods, but with modified wool 1-ml aliquots of the supernatants were withdrawn periodically. The protein was removed by adding 1 ml of 20% trichloroacetic acid (TCA).

(f) *Measurement of the Extent of Proteolysis*

With native wool, this was estimated from the loss of weight of the substrate. Otherwise, 0·5 ml of each TCA supernatant was taken for Kjeldahl nitrogen determinations (McKenzie and Wallace 1954) and 1·2 ml for ^{14}C-estimation. The ratio of TCA-soluble nitrogen to total nitrogen or TCA-soluble ^{14}C to total ^{14}C was taken as index of the extent of proteolysis. Allowance was made for the very small amounts of nitrogen and ^{14}C that were found in the controls.

Radioactivity was measured in a "Tricarb" liquid scintillation spectrometer (Packard Instrument Co. Inc., U.S.A.). The counting system consisted of 1·2 ml TCA supernatant, 0·8 ml water, 15 ml scintillation mixture* (Butler 1961), and 25 μl 0·77M tetraethylammonium hydroxide (Leach and Hill 1963). An internal standard was used to correct for quenching when necessary. Counting was carried out for long enough, where practicable, so as not to exceed a standard error of $\pm 1\%$.

(g) *Reduction of Wool Powder*

Wool was finely ground up under liquid nitrogen (Inglis and Leaver, unpublished data), reduced with 1M mercaptoacetate at pH 5, and alkylated with non-radioactive iodoacetate at pH 9.

III. Results

(a) *Proteolysis of Intact Wool*

Experiments with pronase and the purified fungal protease can be summarized as follows:

(i) Proteolysis proceeds as far as the release of cortical cells from the disintegrating fibres, and it is accompanied by a 10–20% weight loss. The dialysable portion of the solubilized material was analysed for amino acids after acid hydrolysis. The only significant feature was that the cystine content was about 12% of the value observed for intact wool.

* Composition: 120 g naphthalene, 0·05 g 1,4-bis(5-phenyl-2-oxazolyl)-benzene, and 4 g 2,5-diphenyloxazole made up to 1 litre with *p*-dioxane.

(ii) The proteolysis is fastest at pH 9. Higher pH ranges were not studied because of possible wool damage. Some activity was still found at pH 7.

(iii) Proteolysis is faster at 37°C than at 28°C. However, it does not take place at 55°C, even in the absence of ethanol.

(iv) The release of cortical cells was in evidence after 6–8 days at pH 9 and 28°C. With mechanical shaking under identical conditions or at pH 8 and 37°C, this period was reduced to about half.

(v) Proteolysis is accelerated in the presence of mercaptoethanol, but does not proceed beyond liberating the cortical cells.

(vi) As far as could be determined, the two enzyme preparations behaved similarly towards wool.

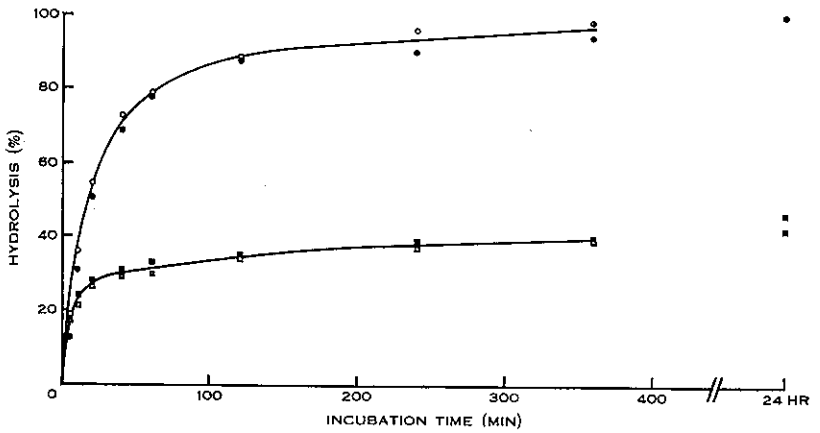

Fig. 1.—Rate of proteolysis of fully reduced and alkylated whole wools by pronase (○, ●) and trypsin (□, ■) at pH 8. ○, □ 24% *S*-carboxymethyl (SCM) groups labelled with ^{14}C. First reduction: mercaptoacetate; first alkylation: [^{14}C]iodoacetate, pH 8; second reduction: toluene-ω-thiol; second alkylation: iodoacetate, pH 8; third reduction and alkylation: repeat of second. ●, ■ 93% SCM groups labelled with ^{14}C. First reduction: toluene-ω-thiol; first alkylation: [^{14}C]iodoacetate, pH 8; second reduction and alkylation: repeat of first; no third cycle. Further details in Section II(*d*).

(*b*) *Proteolysis of Total Insoluble* S-*Carboxymethylated Wool Protein*

Both trypsin and pronase solubilize the fully reduced and alkylated wool within 1 hr, but in the case of trypsin, some opalescence of the digest persists. The release of TCA-soluble ^{14}C is compared in Figure 1. It can be seen that:

(i) The rate of proteolysis by trypsin is slower initially, and even after 24 hr at 28°C only 40% of the ^{14}C is rendered soluble in 10% TCA. By contrast, all the ^{14}C becomes soluble in TCA within 4 hr in the presence of pronase.

(ii) The percentage of ^{14}C released by pronase and trypsin is independent of the proportion of SCM groups labelled with ^{14}C.

Pepsin acts much more slowly on the modified wool; even after 14 days digestion, fragments are still visible. A comparison of peptic action with that of trypsin and pronase is presented in Table 1. It is evident that:

TABLE 1

COMPARISON OF THE ENZYMIC RELEASE OF TRICHLOROACETIC ACID-SOLUBLE ^{14}C AND NITROGEN FROM REDUCED AND ALKYLATED WOOLS CONTAINING DIFFERENT PROPORTIONS OF ^{14}C-LABELLED *S*-CARBOXYMETHYL (SCM) GROUPS

Enzyme	Incubation Period (hr)	Percentage of SCM Groups Labelled with ^{14}C	Hydrolysis (%) as Measured by:		% ^{14}C Released / % Nitrogen Released
			^{14}C Release	Nitrogen Release	
Pepsin	6	24*	28‡	22‡	1·3
	4	24	21	15	1·4
	6	59†	8	7	1·1
	6	93*	17‡	14‡	1·2
	4	93	8	7	1·1
Trypsin	6	24	39	34	1·1
	4	24	37	33	1·1
	6	93	40	36	1·1
	4	93	39	36	1·1
Pronase	6	24	98	75	1·3
	4	24	96	74	1·3
	6	93	94	74	1·3
	4	93	90	70	1·3

* For details of preparation see legend to Figure 1, and Section II(*d*). The same preparations were used for all three enzymes.

† For details of preparation see legend to Figure 2 and Section II(*d*).

‡ Mean of duplicate experiments.

(1) The percentage of ^{14}C and nitrogen released by pepsin is less than for either trypsin or pronase.

(2) The rate of peptic digestion is much more variable from one preparation to another.

(3) The percentage of ^{14}C released by pepsin shows no relationship to the proportion of SCM groups labelled with ^{14}C.

(4) The percentage of nitrogen released is closely parallel to the percentage of ^{14}C liberation in all cases, so that the ratio percentage ^{14}C release to percentage nitrogen release is close to unity. The ratio is constant regardless of the content of ^{14}C-labelled SCM groups in the preparations.

(c) *Proteolysis of Soluble* S-*Carboxymethylated Wool Proteins*

The rates of peptic digestion of high- and low-sulphur proteins are compared in Figure 2. It appears that:

(i) The ^{14}C is released rapidly from the high-sulphur proteins, reaching completion in 1 hr.

(ii) The ^{14}C release from the low-sulphur proteins is at least 10 times slower initially, and even after 24 hr is less than 70% complete.

(iii) Only in the case of high-sulphur proteins is the percentage of ^{14}C release independent of the proportion of SCM groups labelled.

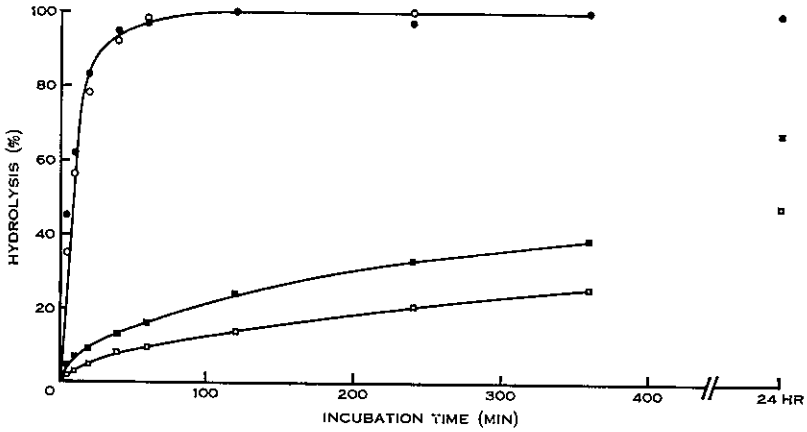

Fig. 2.—Rate of peptic digestion of high- (○, ●) and low-sulphur proteins (□, ■) extracted from fully reduced and alkylated wool. ○, □ 59% *S*-carboxymethyl (SCM) groups labelled with ^{14}C. First reduction: mercaptoacetate; first alkylation: [^{14}C]iodoacetate, pH 5; second reduction: toluene-ω-thiol; second alkylation: iodoacetate, pH 5; third reduction and alkylation: repeat of second. ●, ■ 90% SCM groups labelled with ^{14}C. First reduction: n-propanol–mercaptoacetate; first alkylation: [^{14}C]iodoacetate, pH 5: no further cycles. Further details in Section II(*d*).

The somewhat anomalous situation with the low-sulphur proteins can be resolved by taking the nitrogen release into account (Table 2). The table shows:

(1) The percentage of nitrogen released is again similar in value to that of ^{14}C, giving a value close to unity for the ratio percentage ^{14}C release/ percentage nitrogen release at both concentration levels of ^{14}C-labelled SCM groups.

(2) The rate and extent of ^{14}C and nitrogen release is greater in the soluble proteins than in the modified whole wool (Table 1).

(d) *Extent of Reduction of Wool Powder at pH 5*

Measurement of –S–S– bonds which had survived the grinding treatment (416 μmoles/g) revealed that 165 μmoles/g or about 40% had remained unreduced at pH 5, i.e. the same proportion as in intact wool (Gillespie and Springell 1961).

IV. Discussion

The results of studies on native wool indicate that pronase is unable to break the fibre down any more extensively than other proteases (Lennox 1952; Lennox and Forss 1953; Alexander and Hudson 1954; Crewther 1956). This is in spite of the fact that pronase has exo- as well as endopeptidase activity (Nomoto, Narahashi, and Murakami 1960*a*) and that the enzyme has a very broad spectrum of specificity (Nomoto, Narahashi, and Murakami 1960*b*).

Table 2

Comparison of the peptic release of trichloroacetic acid-soluble ^{14}C and nitrogen from soluble high- and low-sulphur proteins containing different proportions of ^{14}C-labelled *S*-carboxymethyl (SCM) groups

Protein Sulphur Content	Incubation Period (hr)	Percentage of SCM Groups Labelled with ^{14}C	Hydrolysis (%) as Measured by:		% ^{14}C Released / % Nitrogen Released
			^{14}C Release	Nitrogen Release	
High	6	59*	99	98	1·0
	2	59	99	95	1·0
	6	90*	100	84	1·2
	2	90	100	86	1·2
Low	6	59	25	26	1·0
	2	59	14	15	0·9
	6	90	39	35	1·1
	2	90	24	19	1·3

* For details of preparation see legend to Figure 2 and Section II(*d*).

Modification of wool frequently results in a marked increase in susceptibility to proteolysis (Alexander and Hudson 1954); reduction followed by alkylation is no exception. Pronase, as might have been expected, was the most effective of the proteases examined. Not only were the initial rates faster, but the enzyme was also the only one which could convert all the protein nitrogen from the modified whole wool fibre into a TCA-soluble form in under 24 hr.

The peptic digestion of high-sulphur proteins is faster than that of the low-sulphur proteins (Table 2; Fig. 2). This behaviour is in keeping with what one would expect from their known conformations. It has been shown that in the case of collagen (Von Hippel and Harrington 1959) and myosin (Mihalyi and Harrington 1959), differences in susceptibility to enzyme attack are a reflection of their structural features. The finding that high-sulphur proteins undergo rapid proteolysis (Fig. 2) is consistent with a random-coil configuration (Gillespie 1962; Gillespie and Harrap 1963). Conversely, the low-sulphur proteins, which are less readily degraded, are partly helical (Harrap 1963). Other factors, however, could contribute towards

the observed difference between these two proteins. The high-sulphur proteins, being of lower molecular weight, may require breakage of fewer peptide bonds before its fragments become soluble in TCA. On the other hand, taking into account the specificity of pepsin (Green and Neurath 1954) and the composition of the substrates (Gillespie 1960, 1962), the low-sulphur proteins contain considerably more peptide bonds susceptible to pepsin.

The proteolysis of fully reduced and alkylated wool fibres containing varying amounts of ^{14}C-labelled SCM groups revealed some differences among the three enzymes. Pronase and trypsin (Fig. 1; Table 1) are unable to distinguish those portions of the intact wool proteins which contain the more easily reducible –S–S– bonds (Gillespie and Springell 1961) from the rest of the fibre. With pepsin there is the complication that different preparations vary in their digestibility. To make direct comparisons possible, the ^{14}C release had to be examined in terms of a fixed amount of nitrogen (Table 1). It will be seen that the ratio of ^{14}C released to nitrogen released is almost as constant for pepsin as for trypsin and pronase. It can be calculated that if the pepsin were capable of distinguishing the labelled portions of the fibre from the rest, then ratios* of 3·9 and 1·6 would be expected for the preparations containing 24 and 59% ^{14}C-labelled SCM groups respectively. It is therefore concluded that pepsin also digests both labelled and unlabelled portions of the wool to the same extent.

A study of the digestion of extracted wool proteins (Fig. 2) shows that pepsin is also unable to distinguish the more easily reducible portion of the high-sulphur protein molecule from the rest of the protein. In the low-sulphur proteins the anomalous difference between the two lower curves is again attributable to varying susceptibility of the two preparations towards pepsin. Since the ^{14}C release/nitrogen release ratio (Table 2) shows some degree of constancy, preferential digestion of the ^{14}C-labelled portions of the low-sulphur proteins can be ruled out; otherwise a ratio of 1·5 would be found in the preparation containing 59% ^{14}C.

The variability in the peptic digestion of the modified whole wool (Table 1) could be due to differences in the physical properties of the various preparations. These differences may affect penetration of the fibre by the enzyme. If the more readily reducible –S–S– bonds were concentrated around the periphery of the fibre, then one might expect pepsin to preferentially digest the ^{14}C-labelled portions of the part-labelled wools. The absence of evidence for any such preferential digestion, suggests that the more readily reducible –S–S– bonds are randomly distributed throughout the fibre.

By contrast, the Merino fibre appears to be much more accessible to trypsin and pronase. The fact that even in the earliest stages of the digestion (Fig. 1) there is no evidence of preferential ^{14}C release also supports the idea of random distribution of the label.

* The ratios are calculated on the following assumptions:

(1) that the ^{14}C release is inversely proportional to the ^{14}C content; and

(2) that the nitrogen release is independent of the ^{14}C content of the preparation.

The finding that powdered wool was reduced to the same extent as the intact fibre provides evidence against preferential peripheral reduction. Other evidence from preliminary radioautographic studies of cross-sections of Merino fibres partly labelled with ^{14}C (Rogers and Springell, unpublished data) shows a random distribution of radioactivity throughout the fibre. However, Gillespie and Springell (1961) concluded that the more readily reducible –S–S– bonds were evenly distributed between the *ortho*- and *para*-cortex of the Merino fibre.

V. Acknowledgments

I am grateful to Mr. A. S. Inglis for amino acid and nitrogen analyses, to Dr. S. J. Leach for –S–S– determinations, to Mr. J. M. Gillespie for extraction and fractionation of alkylated wools, and to Dr. M. Nomoto for the gift of purified *S. griseus* enzyme. I also wish to express my appreciation of discussion with my colleagues, notably with Mr. W. G. Crewther and Dr. S. J. Leach.

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