Studies on the High-Sulphur Proteins of Reduced Mohair

THE ISOLATION AND AMINO ACID SEQUENCE OF PROTEIN SCMKB-M1.2

By DAVID PARRIS and LOUIS S. SWART

National Chemical Research Laboratory, South African Council for Scientific and Industrial Research, Pretoria, Republic of South Africa

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The complete amino acid sequence of a mohair protein, SCMKB-M1.2 (97 residues), was determined. The protein was isolated from reduced and carboxymethylated mohair by chromatography on DEAE-cellulose and cellulose phosphate. Peptides for sequence determination were obtained by digestion with trypsin, pepsin, chymotrypsin, thermolysin and papain, and were fractionated by DEAE-cellulose chromatography, paper chromatography and electrophoresis. The sequences of the peptides were determined by the Edman degradation method (by the use of both the Beckman Sequencer and a non-automatic procedure), and by partial acid hydrolysis. The protein is closely homologous to wool protein SCMKB-IIIB2, and also contains acetylated alanine as *N*-terminal amino acid.

The high-sulphur proteins derived from wool have been more extensively studied than those obtained from any other keratin (Bradbury, 1973) and a number of amino acid sequences have been reported (Haylett *et al.*, 1971*b*; Elleman, 1972; Swart & Haylett, 1973). These proteins can be divided into a number of molecular-weight groups, each group containing microheterogeneous proteins, and with some homology between the groups (Lindley & Elleman, 1972; Swart, 1973).

The most extensive survey on the high-sulphur proteins of mohair was carried out by Joubert (1971*a,b*). Mohair SCMKB was fractionated by a combination of chromatography on DEAE-cellulose and gel filtration. Five major groups of proteins were defined by differences in their molecular weights, electrophoretic mobilities and amino acid compositions. On the basis of these results, a close relationship between the proteins of wool and mohair SCMKB was postulated.

The present paper describes the isolation and amino acid sequence of a homogeneous high-sulphur protein from reduced and *S*-carboxymethylated mohair.

Materials and Methods

Materials

Protein. Mohair SCMKB was prepared from good-quality South African Angora mohair by the method of Joubert (1971*a*).

Enzymes. Trypsin was a twice-crystallized diphenylcarbamyl chloride-treated, salt-free preparation (batch 336A) from Seravac Laboratories (Cape

Town, Republic of South Africa) containing 0.4% relative activity of chymotrypsin; thermolysin was from Daika Kasei K.K., Osaka, Japan; chymotrypsin (batch CD1 6100-1) and pepsin were twice-crystallized salt-free preparations from Worthington Biochemical Corp., Freehold, N.J., U.S.A. Papain was a twice-crystallized preparation (batch PAP5582), suspended in water, containing 13.6mg of papain/ml from Worthington Biochemical Corp., Freehold, N.J., U.S.A.

Acetylalanine. A.R.-grade acetylalanine was obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A.

Methods

DEAE-cellulose chromatography. The initial separation of mohair SCMKB was carried out by chromatography on DEAE-cellulose by the method of Joubert (1971*b*).

Cellulose phosphate chromatography. The fractions obtained by DEAE-cellulose chromatography were rechromatographed on cellulose phosphate under the conditions described by Swart *et al.* (1973).

Enzymic digestions. Tryptic digestion was carried out on 40 μ mol of protein M1.2* in 50ml of 2% (w/v) NH_4HCO_3 (enzyme/substrate ratio 1:100, w/w) for 1 h at 40°C. The digestion was stopped by addition of acetic acid and the material was freeze-dried.

Thermolysin digestion was carried out on tryptic peptides T3 and T4 (9 μ mol of each) in 2% (w/v)

* Abbreviations: M1.2, mohair protein SCMKB-M1.2; BAW, butan-1-ol-acetic acid-water (40:6:15, by vol.); CmCys (in sequences and tables), *S*-carboxymethylcysteine.

NH_4HCO_3 (enzyme/substrate ratio 1:100, w/w) for 1 h at 40°C. The digestion mixtures were acidified to pH 3 with acetic acid and freeze-dried.

Chymotryptic digestion was performed on 7 μmol of tryptic peptide T5 in 3 ml of 2% (w/v) NH_4HCO_3 for 2 h at 40°C. The digestion was stopped by the addition of acetic acid and the material was freeze-dried.

Peptic digestion was carried out on 15 μmol of protein M1.2 in 18 ml of 5% (v/v) formic acid (enzyme/substrate ratio 1:100, w/w) for 2.5 h at 40°C. The digestion was stopped by the addition of pyridine and the material was freeze-dried.

Papain digestion was carried out under N_2 on 6 μmol of peptide T1 dissolved in 6 ml of 0.2M-pyridine-acetate buffer, pH 5.5, 10 μl of the enzyme suspension being used. The digestion was initiated by addition of 2 μl of 2-mercaptoethanol and terminated after 18 h at 40°C by freeze-drying.

Partial acid hydrolysis. Partial cleavage of the peptide bonds of peptide T1Q1 was achieved by the method of Haylett & Swart (1969). The peptide (1 μmol) was incubated with 1 ml of 10M-HCl at 40°C for 18 h and the solution was freeze-dried.

Fractionation of peptides. The peptides were separated by chromatography on DEAE-cellulose by using a linear gradient of NH_4HCO_3 . The tryptic peptides were loaded on a column (1.9 cm $\times$ 50 cm; Whatman DE-52, with fines removed), and eluted with an 8 litre linear gradient from 0.015M- to 0.30M- NH_4HCO_3 at a flow rate of 100 ml/h and 10 ml fractions were collected. A Beckman DB spectrophotometer was used to monitor the effluent at 230 nm, and the peptides were recovered by freeze-drying. Other peptide mixtures were separated under similar conditions except that a 0.9 cm $\times$ 50 cm column and a 2 litre gradient at a flow rate of 25 ml/h were used, and 2.5 ml fractions were collected.

Paper chromatography with BAW as eluent and paper electrophoresis at pH 1.9 and 4.5 were carried out as described by Haylett & Swart (1969). Peptides were located on paper by the chlorine-tolidine method of Reindel & Hoppe (1954). Aq. 10% (v/v) propan-1-ol solutions, containing 5% (v/v) acetic acid or 5% (v/v) pyridine, were consecutively used to elute the peptides from the paper.

Amino acid analysis. Amino acid compositions of the protein and peptides were determined as described by Haylett *et al.* (1971a). Threonine and serine values were corrected for destruction during 24 h hydrolysis by factors of 1.04 and 1.08 respectively. All the values reported in the tables were results from single determinations.

Sequence analysis. Edman degradations were carried out on small peptides by the non-automatic method of Peterson *et al.* (1972), but with dimethylallylamine being replaced by dimethylaminopropyne in the coupling buffer (Braunitzer & Shrank, 1970).

Larger peptides were sequentially analysed (Edman & Begg, 1967) on the Beckman Sequencer (model 890) with the use of dimethylaminopropyne buffer as described by Swart & Haylett (1973). Sequencer experiments were repeated using triple cleavages at glycine and proline residues to increase the yield of these and subsequent steps.

The conversion of the thiazolinone derivatives of the amino acids into phenylthiohydantoin and their identification by gas chromatography, without internal standards, and by t.l.c. were carried out as described by Swart & Haylett (1973).

Graphical plots of the absolute heights of peaks and the relative intensities of spots for all the steps were used for identification of residues. In t.l.c. experiments the intensities of spots were estimated

Fig. 1. Isolation of protein M1.2 from mohair SCMKB

(a) Elution pattern of mohair SCMKB (4g) obtained with a Beckman Spectrochrom analyser. Chromatography was performed on a DEAE-cellulose column (150 cm $\times$ 1.9 cm) at 4°C with a 6 litre linear gradient of 0.05–0.70M-sodium acetate buffer, pH 4.5 (flow rate, 100 ml/h; fraction volume 15 ml). (b) Elution pattern of fraction M1 obtained with a Beckman Spectrochrom analyser. Chromatography was performed on a cellulose phosphate column (50 cm $\times$ 3.8 cm) at 4°C with an 8 litre linear gradient of 0–0.4M-NaCl in 0.1M-citric acid dissolved in 5M-urea, pH 2.8 (flow rate, 200 ml/h; fraction volume, 20 ml).

visually on an arbitrary six-point scale, taking into account the nature of each amino acid. Positive identification of an amino acid residue is only reported when all the following standards are met. (1) The yield of only one amino acid derivative must increase markedly. (2) The yield must show the expected decrease on the subsequent step, except for repeating amino acids, where the yield will stay high until the step after the last residue of the repetition. (3) The evidence obtained from gas chromatography and t.l.c. experiments must correspond. (4) No conflict must exist between the sequence results and other evidence.

Positive identification of amino acid residues in sequencer experiments is only possible if the levels of background contamination of amino acid derivatives stay constant or change only gradually. For this reason only peptides free of detectable impurities can be analysed.

Nomenclature

Proteins. A two-digit Arabic number is used to identify the proteins. The first digit indicates the elution position on DEAE-cellulose chromatography, and the second digit the position on subsequent chromatography on cellulose phosphate.

Peptides. Capital letters are used to indicate enzymic digestions: T, L, C, P and Q for trypsin, thermolysin, chymotrypsin, pepsin and papain respectively. Arabic numerals following the capital letters indicate sequential numbering of the peptides.

Results

Isolation of protein M1.2 from mohair SCMKB

Total mohair SCMKB was chromatographed on DEAE-cellulose and the elution pattern obtained is shown in Fig. 1(a). Fraction M1 was rerun to eliminate cross contamination and then chromatographed on cellulose phosphate. The components were isolated as shown in Fig. 1(b), and protein M1.2 was purified by rechromatography on cellulose phosphate.

The amino acid composition of protein M1.2 is given in Table 1. The protein was found to be devoid of tryptophan and methionine.

Peptides from tryptic digestion

Purification of the tryptic peptides. The elution pattern of the tryptic peptides of protein M1.2 is shown in Fig. 2. The major peptides were pure and their amino acid compositions are given in Table 1. Peptide T6 corresponds to peptide T5 but contains an extra carboxymethylcysteine residue. The aggregate composition of the tryptic peptides T1–T4 and T6 equals the composition of protein M1.2, as shown in Table 1.

Sequence of peptide T1. Since Edman degradation of peptide T1 gave no result, the peptide was further digested with papain. Paper electrophoresis at pH 4.5 was used to separate the peptide mixture and two peptides, T1Q1 and T1Q2, were obtained. Their mobilities relative to lysine and their amino acid

Table 1. Amino acid composition of protein M1.2 and its tryptic peptides

Protein or peptide ...	Amino acid composition (mol of residue/mol)							Total T1–T4+T6
	M1.2	T1	T2	T3	T4	T5	T6	
Lys	1.09			0.92				1
His	2.93				1.01	1.71	1.77	3
Arg	4.07	1.06	0.96	1.03		1.08	1.13	4
CmCys	13.74	2.03	2.02	1.93	3.78	3.02	3.87	14
Asp	7.89			1.05	3.04	4.01	3.98	8
Thr	9.70			3.10	2.01	4.75	4.82	10
Ser	8.98		1.15	2.03	2.88	2.91	3.18	9
Glu	4.18				1.02	3.11	2.81	4
Pro	13.31	1.08		1.05	5.02	6.18	6.30	13
Gly	5.05			1.10	1.11	3.00	2.90	5
Ala	3.81	1.87		1.01		1.11	1.07	4
Val	4.76		0.93		1.80	1.86	1.66	5
Ile	3.89			0.88	0.95	1.93	1.93	4
Leu	7.09				3.04	3.90	4.01	7
Tyr	2.23				0.98	1.07	1.04	2
Phe	4.00			1.04	0.96	1.98	2.18	4
Yield (μ mol)		20	12	17	19	11	18	

compositions were as follows: peptide T1Q1 (yield 80%, mobility -1.33) CmCys 0.99, Ala 1.01; peptide T1Q2 (yield 55%, mobility -0.06) Arg 0.99, CmCys 0.96, Pro 1.04, Ala 1.01.

The ninhydrin-negative peptide, T1Q1, was subjected to partial acid hydrolysis. Paper electrophoresis of the hydrolysate together with known standards was carried out at pH 4.5. The hydrolysate yielded five spots and their mobilities (m), relative to aspartic acid, corresponded to those of peptide T1Q1 ($m = 1.15$), acetylalanine ($m = 0.99$), carboxy-

methylcysteine ($m = 0.93$) and alanine ($m = 0.02$). The fifth spot ($m = 0.69$) was presumed to be alanyl-carboxymethylcysteine. The sequence of peptide T1Q1 is therefore acetyl-Ala-CmCys.

Peptide T1Q2 was subjected to Edman degradation which gave the sequence, CmCys-Ala-Pro-Arg. The complete sequence of peptide T1 is therefore acetyl-Ala-CmCys-CmCys-Ala-Pro-Arg.

Sequence of peptide T2. The sequence of peptide T2 was determined by Edman degradation to be CmCys-CmCys-Ser-Val-Arg.

Fig. 2. Fractionation of tryptic peptides of protein M1.2

Elution pattern of tryptic peptides of protein M1.2 obtained with a Beckman Spectrochrom analyser. Chromatography was performed on a DEAE-cellulose column ($150\text{cm} \times 1.9\text{cm}$) at 20°C with a 8 litre linear gradient of $0.015\text{--}0.30\text{M-NH}_4\text{HCO}_3$ (flow rate 100ml/h ; fraction volume, 10ml).

Fig. 3. Fractionation of the thermolysin peptides of tryptic peptide T3

Elution pattern of the thermolysin peptides of tryptic peptide T3 obtained with a Beckman Spectrochrom analyser. Chromatography was performed on a DEAE-cellulose column ($150\text{cm} \times 0.9\text{cm}$) at 20°C with a 2 litre linear gradient of $0.015\text{--}0.30\text{M-NH}_4\text{HCO}_3$ (flow rate 50ml/h ; fraction volume, 5ml).

Sequence of peptide T3. Thermolysin digestion was carried out on peptide T3, and chromatography of the peptide mixture on DEAE-cellulose gave the elution pattern shown in Fig. 3. The peptides were pure, and their amino acid compositions are given in Table 2. The sequence results for peptide T3 and for its thermolysin peptides are given in Fig. 4.

Sequence of T4. The Beckman Sequencer was used to determine the complete amino acid sequence of peptide T4 as given in Fig. 5. To confirm this sequence, peptide T4 was digested with thermolysin. The peptides released were chromatographed on DEAE-cellulose and the elution pattern shown in Fig. 6 was obtained. Peptides T4L2 and T4L3 were eluted together, and were separated by paper chromatography in BAW solvent, giving R_F values of 0.62 and 0.41 respectively. The amino acid compositions

Table 2. Amino acid compositions of the thermolysin peptides of peptide T3

Peptide ...	Amino acid composition (mol of residue/mol)			
	T3L1	T3L2	T3L3	T3L4
Lys		1.00	1.01	
Arg				0.96
CmCys		0.90	0.91	1.04
Asp		1.00	1.03	
Thr	3.01			
Ser		2.02	1.99	
Pro	0.97			
Gly	1.01			
Ala	0.98			
Ile		0.71	0.82	
Phe			0.97

Fig. 4. Amino acid sequence of peptide T3 and its thermolysin peptides

Arrows indicate positive identification of residues by sequential analysis.

Fig. 5. Amino acid sequence of peptide T4 and its thermolysin peptides

The complete amino acid sequence of peptide T4 was determined in the Beckman Sequencer.

Fig. 8. Fractionation of the chymotryptic peptides of tryptic peptide T5

DEAE-cellulose was used with a Beckman Spectrochrom analyser. Conditions are as given for Fig 3.

Table 4. Amino acid composition of the chymotryptic peptides of peptide T5

Peptide ...	Amino acid composition (mol of residue/mol)					
	T5C1	T5C2	T5C3	T5C4	T5C5	T5C6
His	0.91		0.96	0.94		
Arg						0.96
CmCys		1.00				2.07
Asp	1.09		1.92	1.16		1.04
Thr	1.10	1.05	1.13	0.97	2.01	
Ser			2.95	2.00		
Glu						2.96
Pro	1.17	0.91	2.02	1.88		2.07
Gly			2.00	1.16		1.01
Ala						0.99
Val	0.80	0.99				
Ile			0.94			0.94
Leu			3.57	2.00		
Tyr	0.93					
Phe		1.00			0.99	

of the pure thermolysin peptides are given in Table 3. Their sequence results and alignment with peptide T4 are given in Fig. 5.

Sequence of peptide T5. The partial amino acid sequence of peptide T5 was determined by using the Beckman Sequencer, and positive identifications are indicated in Fig. 7. To complete the sequence, peptide T5 was digested with chymotrypsin. The peptides were chromatographed on DEAE-cellulose, and the elution pattern is shown in Fig. 8. Peptides T5C3 and T5C4 were separated by paper chromatography in BAW ($R_F = 0.36$ and 0.24 respectively). The amino acid compositions of the pure chymotryptic peptides

Vol. 145

Fig. 9. Fractionation of the peptic peptides of protein M1.2

DEAE-cellulose was used with a Beckman Spectrochrom analyser. Conditions are as given for Fig. 2.

Table 5. Amino acid composition of four peptic peptides of protein M1.2

Peptide ...	Amino acid composition (mol of residue/mol)			
	P1	P2	P3	P4
His		0.76		
Arg	1.88			1.01
CmCys	4.09	1.91	1.11	1.93
Asp		3.04		0.97
Thr	3.16	2.03		
Ser	1.10	0.94		
Glu			2.02	1.00
Pro	1.85	4.09	1.02	1.05
Gly	1.22		1.13	
Ala	3.00			1.03
Val	0.97	2.78		
Ile			0.88	
Tyr		1.88		
Phe		0.99	0.84	
R_F^*	0.29	0.33	0.44	0.09

* Chromatography in solvent BAW.

are given in Table 4. Their sequence results and alignment with peptide T5 are given in Fig. 7.

Peptides from peptic digestion

In order to align the tryptic peptides and to complete the amino acid sequence of protein M1.2, a peptic digestion was carried out. The peptides were

IIIB2	5	10	15	20
IIIB3	-Pro-Arg-	-Arg-	Gly-	
IIIB4	-Arg-Leu-	-Pro-	Ser-	
IIIB4	-Arg-Leu-	-Pro-	Ser-	
M1.2	Acetyl-Ala-Cys-Cys-Ala-Pro-Arg-Cys-Cys-Ser-Val-Arg-Thr-Gly-Pro-Ala-Thr-Thr-Ile-Cys-Ser-Ser-Asp-Lys-Phe-			
	T1	T2	T3	
		P1		
IIIB2	25	30	35	40
IIIB3			-Asn-Ile-Ser-	-Cys-
IIIB4			-Thr-Val-Trp-	-Cys-
IIIB4			-Thr-Val-Trp-	-Cys-
M1.2	Cys-Arg-Cys-Gly-Val-Cys-Leu-Pro-Ser-Thr-Cys-Pro-His-Asp-Ile-Ser-Leu-Leu-Gln-Pro-Thr-Phe-Cys-Asp-			
			T4	
IIIB2	50	55	60	65
IIIB3	-Ser-	-Val-	-Cys-Val-Tyr-	-Asp-Thr-Tyr-
IIIB4	-Arg-	-Pro-	-Tyr-His-Val-	-Gln-Pro-Ser-
IIIB4	-Arg-	-Pro-	-Cys-His-Ile-	-Gln-Pro-Ser-
M1.2	Asn-Ser-Pro-Val-Pro-Tyr-His-Val-Pro-Asp-Thr-Tyr-Val-Pro-Thr-Cys-Phe-Leu-Leu-Asn-Ser-Ser-His-Pro-Thr-			
			P2	
IIIB2	75	80	85	90
IIIB3	-Ser-Gly-	-Phe-Ile-	-Pro-Gly-	-Asn-Val-
IIIB4	-Glu-Ser-	-Tyr-Thr-	-Ser-Ser-	-Pro-
IIIB4	-Glu-Ser-	-Tyr-Thr-	-Pro-Ser-	-Pro-
M1.2	Pro-Gly-Leu-Ser-Gly-Ile-Asn-Leu-Thr-Thr-Phe-Ile-Gln-Pro-Gly-Cys-Glu-Asn-Ala-Cys-Glu-Pro-Arg-Cys-			
	T6			
	T5		P3	P4

Fig. 10. Complete amino acid sequence of protein M1.2

Only those parts of the amino acid sequences of wool proteins IIIB2 (Haylett & Swart, 1969), IIIB3 (Haylett *et al.*, 1971a) and IIIB4 (Swart & Haylett, 1971) where differences occur, are given. Tryptic (T) and peptic (P) peptides of protein M1.2 are indicated. Differences between proteins M1.2 and IIIB2 are shown underlined.

initially separated by chromatography on DEAE-cellulose and the elution pattern is given in Fig. 9. Owing to partial cleavage a large number of peptic peptides were obtained, and only those necessary to complete the amino acid sequence of protein M1.2 are indicated.

Peptides P1–P4 were purified by paper chromatography in BAW solvent, and their R_F values and amino acid compositions are given in Table 5.

Alignment of tryptic peptides

Peptide P1 gave no result on Edman degradation and was assumed to have a blocked *N*-terminus. Its amino acid composition aligns the first three tryptic peptides from the *N*-terminal of protein M1.2 in order T1–T2–T3.

The composition of peptide P2 connects peptides T4 and T5, and peptide P3 confirms the alignment of the chymotryptic peptides of tryptic peptide T5.

The complete amino acid sequence of peptide P4 was determined by Edman degradation to be Asn-Ala-CmCys-Glu-Pro-Arg-CmCys. The sequence of peptide P4 completes the amino acid sequence of peptide T5C6 (see Fig. 7), but contains an additional carboxymethylcysteinyl residue at its *C*-terminus. This proves that peptide T5 originated from peptide T6 by loss of the *C*-terminal carboxymethylcysteine.

Amino acid sequence of protein M1.2

Twenty-nine residues of protein M1.2 were allocated as the result of single non-automatic degradation experiments. In all cases, however, high yields of phenylthiohydantoins were obtained and these allocations are unambiguous. The results reported for seven residues (36–38, 40, 41, 71 and 72, Fig. 10) were obtained only from duplicate sequencer experiments. The complete amino acid sequence of protein M1.2 has therefore been elucidated and is given in Fig. 10.

Discussion

Digestion of protein M1.2 with trypsin gave an unusual cleavage of a tyrosylhistidyl bond (residues 55 and 56, Fig. 10). As for wool protein IIIB2 (Haylett & Swart, 1969), protein M1.2 was not cleaved after the lysine residue in position 23, and was only partially cleaved after the arginine residue in the penultimate position.

Protein M1.2 is the first mohair protein of which the complete amino acid sequence has been determined. It consists of 97 residues and has acetylalanine and carboxymethylcysteine as *N*- and *C*-

terminal residues respectively. The calculated molecular weight of the protein is 11206.

Protein M1.2 is clearly homologous to the wool proteins from group IIIB, which also have molecular weights of about 11000 (Haylett *et al.*, 1971b). The *S*-carboxymethylated proteins have net charges of –13, –15, –16 and –17 for proteins M1.2, IIIB2, IIIB3 and IIIB4 respectively. In the native fibres these proteins were slightly basic in character and carried the same net charge.

Protein M1.2 is closely related to wool protein IIIB2, differing in only six positions, but differs from proteins IIIB3 and IIIB4 in 26 and 27 positions respectively. According to the genetic code, the six differences between proteins M1.2 and IIIB2 can be ascribed to four single and two double nucleotide base changes. Five of these eight base changes occur between three consecutive amino acid residues in proteins IIIB2 (–CmCys-Val-Tyr–)⁵⁵ and M1.2 (–Tyr-His-Val–)⁵⁷. This triplet is, however, identical in proteins M1.2 and IIIB3, and differs from them by only two point mutations in protein IIIB4 (–CmCys-His-Ile–)⁵⁵. An unusual phylogenetic relationship therefore exists between these four proteins. A probable explanation is that in wool protein IIIB2 this triplet has mutated as a single event simultaneously altering several nucleotide bases. It is clear, however, that the IIIB proteins diverged before the divergence of sheep and goats.

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