Grafting onto wool: 8. Amino-acid compositions of various fractions of wool graft copolymers synthesised by methanol–benzoyl peroxide system

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The amino-acid composition of the mother protein in the graft copolymer synthesised by the methanol–benzoyl peroxide initiating system was investigated. True graft copolymers were successfully separated from the grafted fibres by applying a fractional precipitation method. Analyses showed that the mother protein contains less cystine, threonine, serine, proline but more lysine, aspartic acid, glutamic acid, alanine and leucine than wool. The amino-acid compositions of any of the fractions obtained were almost identical to that of the low-sulphur *S*-carboxymethylkerateine protein which is considered to originate in crystalline microfibrils. On the formation of grafting centres in the protein chains in the complex structure of wool, such a selectivity has also been observed for the case of lithium bromide and peroxydisulphate redox initiating system. It was suggested, however, that there are two different types of molecule in the low sulphur protein components involved in these two graft copolymers.

INTRODUCTION

Electron microscopy[1–4] and X-ray diffraction studies[3–5] have been used to assess changes in the structure of keratin produced by grafting processes. The location of the polymer in the wool structure could be varied by the nature of monomer used and greatly influenced by the conditions used for the graft copolymerization[6]. It has been reported that a large amount of polymer introduced using a LiBr–$K_2S_2O_8$ initiating system is localized within the regions of the crystalline microfibrils[4], and as a result, disruption and randomization of the α-helical sections occurs[5]. A decrease in the quantity of α-crystallites of a similar order of magnitude has been found for fibres grafted with MMA by initiating with benzoyl peroxide in methanol[7].

It has been found that the mother protein in the graft copolymers is similar to the low-sulphur fraction separated from *S*-carboxymethylkerateine[8]. Further detailed studies are needed to characterize the mother protein in the graft copolymer prepared by different initiating systems.

EXPERIMENTAL

Materials

The tops of fine Australian Merino wool fibres were purified by Soxhlet extraction with acetone for 24 h, washed with cold water and then air dried. Benzoyl peroxide (BPO), thioglycollic acid (TGA), 1-fluoro-2,4-dinitrobenzene (FDNB), 99% formic acid, and 30% hydrogen peroxide were special reagent grade. Purified methyl methacrylate (MMA) was the monomer used. Reagents used for amino-acid analysis were analytical reagent grade.

The wool fibres (1 g) were treated with a solution containing 1.0 g BPO, 8 g MMA, and 91.0 g MeOH, and followed by extraction with acetone to remove a large amount of homopolymer formed in the grafting system.

Separation of true graft copolymers

The grafted fibres were dissolved in an aqueous solution containing 11.0 M TGA and 0.5 M acetic acid at 60°C for 24 h with a liquor to wool ratio 20:1, and then filtered through a G3 glass funnel under mild suction to remove small amounts of undissolved materials. Throughout the test, a few percent by wt of wool used remained undissolved in the solution. The viscous and clear solution obtained was then used for the preparation of true graft copolymers.

Twice the volume of distilled water was added to the solution drop by drop under mild stirred conditions at room temperature. The solution containing precipitates of hydrophobic graft copolymers was allowed to stand over night. The precipitates representing the first fraction were collected by centrifuging, they were then washed with a large excess of water, and dried. The first fractions were again dissolved and the operation was repeated to obtain the second fraction from the same volume of the solution as used in the first precipitation. Furthermore, three cycles of the same operation were performed. It has been confirmed that under the same procedure as for the fractionation of grafted fibres, no protein materials were precipitated from the dissolved solution of the native wool fibres[8].

Oxidation of protein by performic acid

Approximately 200 mg of protein in the wool graft copolymers were treated at −10°C for 4 h with a performic acid solution prepared by adding a solution containing 5 ml formic acid and 1 ml methanol to the solution mixture containing 9.5 ml formic acid and 0.5 ml of 30% hydrogen peroxide, which had been allowed to stand at 25°C for 2 h. The graft copolymers in the solution became transparent and gel-like during the treatment. After the reaction, the solution was diluted with 350 ml distilled water at 0°C, and then freeze-dried.

Amino-acid analyses

Amino-acid analyses were carried out using an Hitachi Automatic Amino-Acid Analyser, Model KLA-5 on hydrolysates (6 N HCl, 110°C, *in vacuo*) of the copolymer fraction treated with performic acid.

Isolation and dinitrophenylation of branch polymer

Fractionated graft copolymers were treated with 6 N HCl at 100°C for 24 h using a two-step digestion method to isolate the branch polymers from the graft copolymers. The digestion residues were treated with FDNB and then purified to obtain dinitrophenylated polymer (DNP-polymer) as described previously[9].

Determination of N-DNP end groups

The absorption spectra of DNP-polymers were measured in ethyl acetate using an Hitachi spectrophotometer, Model 124. For the estimation of the number of *N*-DNP-amino-acid end groups, $\epsilon_m = 1.70 \times 10^4$ was used as the molar extinction coefficient at 340 nm[9]. The number-average molecular weight of DNP-polymer was obtained by osmometry in toluene with membrane 0–8 at 30°C with the use of a Hewlett Packard high speed membrane osmometer, Model 502. From the u.v. spectroscopic analysis of the number of *N*-DNP-amino-acid end groups and the measurement of the average molecular weight of the DNP-polymer, the number of *N*-DNP-amino-acid end groups linked to the isolated polymers was calculated.

RESULTS AND DISCUSSION

Number-average molecular weight of mother protein

With the fractional precipitation method applied to the separation of true graft copolymers from the grafted fibres, nearly complete fractions could be obtained by repeating the three fractional operation on a liquor to wool ratio of 20:1. For the copolymer system with a high level of graft ing and with a high molecular weight branch polymer. successful separation could be attained by only one fractional operation in a higher liquor ratio such as 100:1. When the number-average molecular weight of branch polymers is less than 10^5 the copolymer in the precipitated phase tends to be finely dispersed in the turbid solution, and the copolymer yield is significantly lowered[8]. Under such conditions, considerable difficulties arise in collecting the precipitates from the solution in a lower concentration of the copolymer. Repeating the fractional operation was examined for the present copolymer systems with a liquor to wool ratio of 20:1.

Assuming that there is one grafting site per polypeptide chain, the number-average molecular weight of keratin protein incorporated in the graft copolymer, $\bar{M}_{n,k}$ can be represented by the following equation:

$$\bar{M}_{n,k} = (X - 1)\epsilon_m / OD_a \quad (1)$$

where X is the weight ratio of the fractionated material to the acid digestion residue, ϵ_m is the molar extinction coefficient of DNP-amino-acid L-methionine at 340 nm in ethyl acetate[9], OD_a is the optical density of DNP-end groups in 0.1% w/v polymer solution at 340 nm.

The results are shown in *Table 1*. With the fraction III prepared from the grafted fibres with relatively higher extent of grafting, the $\bar{M}_{n,k}$ values are quite similar to the order of magnitude of the limiting value of approximately 24 000 obtained by successive fractionation for the graft copolymers synthesised by the $LiBr–K_2S_2O_8$ redox system[8]. On the other hand, it is noted that for the fraction III from the grafted fibres with the lowest level of grafting tested in these experiments (36.0% MMA), a considerably larger value is observed with respect to the molecular weight of keratin protein.

Amino-acid composition

Results of the amino-acid composition of various fractions are shown in *Table 2*. Compared with the amino-acid composition of the wool, the control wool contains considerably low concentrations of cysteic acid and tyrosine, but a high concentration of serine. However, whole wool from the graft copolymer with 44.1% MMA contains a similar quantity of cysteic acid as in wool and contains a trace of tyrosine, less phenylalanine and more serine than wool. It seems likely that the production of new serine is made up from the consumption of the other specific amino-acids in wool. Significant decreases in the content of tyrosine and phenylalanine are probably due to the specific interactions of primary radicals with these two amino-acid residues. Comparing the amino-acid contents in the various fractions with those of wool it is noted that there are decreases in the content of cysteic acid, threonine, serine, proline, glycine, tyrosine and phenylalanine and increases in lysine, aspartic acid, glutamic acid, alanine and leucine. By repeating the fractionation, the concentrations of proline, serine and glycine are gradually decreased while the concentrations of tyrosine and glutamic acid are considerably increased, and the concentrations of many other amino-acids such as cysteic acid, lysine, histidine, arginine, aspartic acid, threonine, alanine, valine, isoleucine, leucine and phenylalanine remain almost unchanged. It particularly interesting to find that the amino-acid compositions of any of the fractions obtained are almost identical to that of the low sulphur *S*-carboxymethylkerateine fraction (SCMKA) from wool reported by Thompson and O'Donnell[10]. Characteristic differences are also observed between the analyses of proline in the fractions obtained from the grafted samples with different polymer content. This can be seen for fraction III, where the proline content of the fraction from the grafted sample at the lowest level of grafting (36.0% polymer content) is clearly different from the other two fractions obtained from the grafted samples containing relatively large amounts of polymer. The latter fractions show no distinguishing differences in amino-acid composition as a whole. It is noteworthy that there is a striking similarity between the fraction III obtained from the grafted sample of 36.0% polymer content and fraction II from both the 44.1% and the 45.3% grafted samples. It is clear that frac-

Table 1 Number-average molecular weight of mother protein

Graft-on (%)	Fraction	$(X-1)$[a]	OD_a[b]	$\bar{M}_{n,k} \times 10^{-4}$
36.0	III	0.333	0.173	3.27
44.1	II	0.355	0.232	2.60
44.1	III	0.277	0.220	2.14
52.7	III	0.292	0.205	2.42

[a]Ratio of the weight of keratin protein (w_k) to that of grafted polymer (w_p) in fractional precipitates. [b]Optical density of DNP-amino-acid end groups in 0.1% w/v DNP-polymer solution of ethyl acetate

Table 2 Amino-acid compositions of proteins incorporated in various copolymer fractions (residues per 1000 residues)

Amino-acid	Wool	Control[a]	Unreduced wool at various extents of grafting (%)								Reduced wool at various extents of grafting (%)	
			Grafted wool	60–I		20–I	20–II		20–III		20–III	
			44.1	41.3	52.3	52.5	44.1	45.3	36.0[b]	44.1	52.7	100.1[c]	111.6[d]
Lys	28	25	26	38	32	37	35	37	38	33	34	45	40
His	8	6	6	7	8	tr	7	7	8	7	7	6	4
Arg	64	76	77	54	61	57	58	67	58	69	62	58	61
$CySO_3H$	90	76	87	52	50	54	48	59	57	53	55	71	61
Asp	67	69	70	88	94	76	96	87	88	91	93	70	75
Thr	71	69	70	57	52	57	54	47	53	51	45	60	61
Ser	97	118	120	91	82	92	82	73	83	72	74	100	92
Glu	120	134	134	161	168	166	179	198	179	191	206	143	178
Pro	77	75	76	44	26	51	34	31	36	21	25	58	50
Gly	82	86	89	71	74	86	61	63	63	62	58	99	72
Ala	58	58	59	79	82	89	85	76	76	84	79	81	79
Val	60	53	60	64	57	65	68	64	58	69	64	57	58
Met	tr	tr	tr	tr	5	tr	tr	tr	4	4	tr	tr	tr
Ileu	39	32	34	39	35	40	41	39	37	37	37	29	40
Leu	80	79	81	100	115	115	114	120	109	113	119	94	109
Tyr	33	20	tr	29	35	tr	13	11	28	24	23	7	4
Phe	26	24	11	26	24	15	25	21	25	19	19	22	16

[a]Treated with the same conditions of grafting as in the absence of monomer; [b]time of grafting: 2 h; [c]thiol content of reduced fibres: 86 μmol/g; [d]thiol content of reduced fibres: 128 μmol/g

tionation of the grafted fibres containing small amounts of polymer is still incomplete after three cycles of such fractional operations. This indicates that the amounts of grafted polymer itself are directly connected with the ease in the separation of true graft copolymers from the whole polymer system. During the dissolution of the fractionation processes, the regular aggregates of proteins in wool are probably dispersed individually into the solution with the help of grafted branches. From X-ray diffraction[5] and electron microscopy[4] data, it has been proved that dissociation of regular aggregates in microfibrils was greatly accelerated by the deposited polymer over about 40% at the levels of grafting. For complete fractional separation, these structural effects of the grafted polymer present in the copolymer system could be expected to be enhanced when a large amount of polymer was deposited. This postulation is supported by the experimental finding with respect to the calculated molecular weight of keratin protein discussed above. It appears, therefore, that the proteins in fraction III obtained from the grafted fibres with 36.0% polymer content are composed of aggregated components of the low sulphur protein, one being the mother proteins incorporated in graft copolymers and the other the free molecules which contain a much higher concentration of proline than the mother proteins.

When a high liquor ratio (60:1) was used for the fractionation, the amino-acid composition of protein in fraction I of the grafted sample with 52.3% polymer content is very similar to that of the fraction III obtained from various grafts using the liquor to wool ratio of 20:1. This suggests that a successful separation of true graft copolymers was nearly completed after one fractional operation with such a high liquor ratio. However, the amino-acid analyses for fraction I of the 41.3% grafted sample clearly show that aggregated free molecules were involved in the fractional precipitates.

Irrespective of a serious decrease of tyrosine content in the grafted fibres, considerable amounts of tyrosyl residues are included in the separated graft copolymer without suffering from chemical attack by the primary radicals produced in the system. It may be supposed that the weight fraction of the mother protein molecules to the whole keratin molecules is considerably smaller, and that the tyrosyl residues present in the mother protein within the fibres are protected from the chemical attack by the tyrosyl sites in a specific sequential conformation of the protein molecule in addition to the steric effects such as an entrappment of the sites into the crystal in microfibril or occlusion within the cystine rich region in the matrix where the non-helical tails of constituent protein of the microfibrils are considered to be split over the linked with high sulphur keratin molecules[11].

The results of amino-acid analyses of the fractions III for reduced fibres at high grafting yields are shown in the last two columns of *Table 2*. As compared to fraction III for non-reduced fibres,considerably higher concentrations of cysteic acid, threonine, proline, serine and glycine and lower concentrations of aspartic acid, glutamic acid, and tyrosine are observed for each fraction III of both reduced fibres prepared by varying the cystine content. These results imply that different types of protein molecules were involved in the true graft copolymer of reduced wool, and support the idea that the increase in the thiol groups produced by the reduction of cystine disulphide bonds is attributed to the increase in the number of grafting sites[9]. The range of the amino-acid compositions analysed for four fractions from the grafted fibres prepared by using a LiBr–$K_2S_2O_8$ initiating system are shown in *Table 3*. No dramatic changes in the analyses between the fractions of the two different initiating systems are found. Minor yet characteristic changes are observed in the contents of the specific amino-acids. The group of BPO fractions contains less lysine, glutamic acid and leucine, but more cysteic acid, glycine and methionine than the group of the LiBr–$K_2S_2O_8$ fractions. Major differences are in the cysteic acid and glycine contents between the two groups.

Since these differences may be related to the fact that cystine and glycine do not favour helix formation, it is probable that there are, at least, two types of protein molecules with different helix content originating from the low

Table 3 Amino-acid compositions of various fractions from wool (residues per 1000 residues)

Amino-acid	Composition range in true graft copolymer fractions		Low sulphur fraction[b]	High sulphur fraction[b]	SCMKA Component[c]		Helical section Ch.C[d]
	$LiBr–K_2S_2O_8$[a]	BPO			7	8	
Lys	38–55	32–34	41	7	44	31	62
His	7–9	7–8	7	9	5	6	8
Arg	62–65	61–69	73	67	75	78	63
$CySO_3H$	31–37	50–55	68	179	62	60	35
Asp	83–94	91–94	81	41	90	108	106
Thr	49–52	45–52	44	104	44	53	40
Ser	72–75	72–82	73	119	83	76	63
Glu	189–228	168–206	141	64	156	182	214
Pro	20–24	21–26	42	136	29	37	14
Gly	46–52	58–74	88	54	74	42	32
Ala	82–86	79–84	64	29	78	60	83
Val	62–67	57–69	59	67	64	63	66
Met	tr	tr –5	6	0	6	3	tr
Ileu	40–44	35–37	37	30	39	38	40
Leu	118–138	113–119	103	50	99	118	127
Tyr	24–34	23–35	43	19	29	26	27
Phe	18–24	19–24	30	24	24	21	18

[a]Arai *et al.*[8]; [b]Thompson and O'Donnell[10]; [c]Thompson and O'Donnell[12]; [d]Crewther and Dowling[15]

sulphur fractions which have been said to be a constituent of the microfibril. The low sulphur fraction[10] SCMKA is known to consist of two main types of protein, component 7 and component 8 with a molecular weight of about 51 000 and 45 000, respectively, but with a physical and chemical heterogeneity in composition to some extent[12–14]. Amino-acid analyses show that the BPO fractions are strikingly similar in components 7 and 8 contents, especially component 7.

On the other hand, the group of $LiBr–K_2S_2O_8$ fractions is considerably less in cystine contents than the components 7 and 8, but similar to the helical rich fraction Ch.C obtained by chymotriptic digestion of low sulphur fraction SCMKA[15]. Although significant differences are observed in the contents of glycine, proline, and lysine, these discrepancies may possibly be explained by the fact that lysine is concentrated in highly helical proteins, while glycine and proline contents become greater in non-helical sections of a protein which is readily susceptible to the enzyme. It is difficult to explain, however, why the content of cysteic acid is extremely small with respect to the fraction Ch.C. This is probably due to the following reasons: (a) the formation of grafting sites on cysteine side chains through thiyl radicals[16–18], (b) the formation of monomer additives through thiyl radical as found for styrene by Kharash and his coworkers[19,20], and (3) formation of new thiyl radicals through a homolytic scission of disulphide bonds in cystine occurring in the strained network of wool involving mechanically weak disulphide bonds[21]. However, the possibility that the grafted polymer occurred in the inherent protein chains with extremely small amounts of sulphur groups cannot be ignored, since the net decrease of cysteic acid content for whole grafted fibres is unexpectedly small.

For mother protein chains in the wool graft copolymers, an approximately similar order of magnitude in the number-average molecular weights was found for the two different initiating systems by the calculation assuming one grafting site to one protein chain, but a significant difference between the compositions of amino-acids for both mother proteins, especially of glutamic acid and glycine except cysteic acid is evident. This suggests that there are two types of protein in the low sulphur components involved in these two graft copolymers.

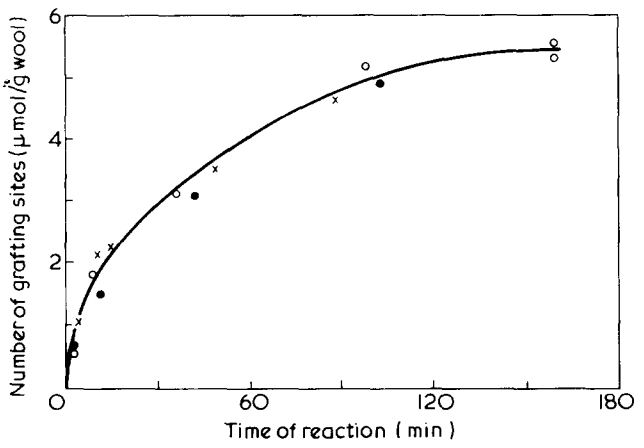

Figure 1 Rate of formation of grafting sites in $LiBr–K_2S_2O_8$ redox system. Composition of reaction liquor: 28.1 g LiBr, 0.2 g $K_2S_2O_8$, 22.9 g butyl carbitol, 45.8 g H_2O, and 5 g MMA (liquor to wool ratio = 100:1). Reaction rate curves were transferred to $t = 0$ along the time axis. Induction periods observed for the reactions at 30°, 40°, and 60°C were, respectively, 20 min, 18 min, and 4 min. Reaction temperatures: ○, 30°; ●, 40°; X, 60°C

Number of grafting sites

The degree of grafting in the $LiBr–K_2S_2O_8$ redox system is mainly dependent on the reaction temperature, time of reaction and the concentration of monomer[15]. However, the values of the number of grafting sites at a given time do not depend on either the concentration of monomer or the reaction temperature as shown in *Figure 1*. This indicates that the magnitude of the activation energy of formation of the grafting sites is nearly zero which is a much lower activation energy than that of the initiation reaction observed for usual redox solution polymerization[6].

Assuming that the number of grafting sites in wool fibres at infinite time of reaction is 6 μmol/g of wool and first order kinetics can be applied, an approximately linear relationship is obtained between $\log[S/(S - s_t)]$ and the time of reaction, as shown in *Figure 2*. This result shows that the initial concentration of thiol groups capable of initiating the chain reaction from the site of radicals is around 6 μmol/g of wool, which corresponds to a value of only ~1/6 of the concentration of free thiol groups present

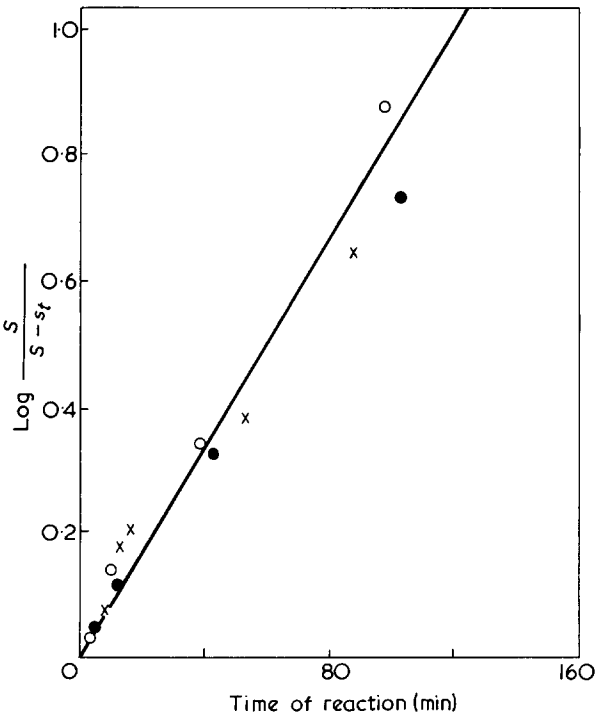

Figure 2 Relationship between $\log[S/(S - s_t)]$ and time of reaction

in native wool fibres. This is likely to be due to a selective formation of grafting sites on the cysteine side chain present in a preferred conformational position in the complex structure of keratin.

By using the following equation[4], a maximum number of grafting sites, n in the 'microfibril-plus-matrix' unit[4,11] can be calculated to be ~10 mol for the LiBr–$K_2S_2O_8$ system, and ~24 mol for the BPO–MeOH system when 15 μmol/g is taken as the number of grafting sites in wool fibres at infinite time of reaction[9]:

$$n = S\rho_w Nv/10^{24} \qquad (2)$$

where ρ_w is the density of wool, 1.33; N is Avogadro's number; v is the unit cell volume, 1.98×10^6 Å^3, and S is the number of grafting sites in wool fibres at infinite time of reaction.

It is of interest that the number of grafting centres generated in the LiBr–$K_2S_2O_8$ initiating system is very close to the number of protofibrils arranged 9 units in a circular ring surrounding a central pair[22]. It might be said, therefore, that on average, one molecule of active cysteine is located in the protofibrillar subunit composed of either two or three α-helical aggregates in a length of 198 Å. Specificity arising on the production of grafting sites may be dependent on the environment around the individual thiol groups[21]. With the BPO initiation, approximately two grafting sites occur selectively in the constituent protein molecules in the protofibrils which may probably be different from the molecules initiating graft copolymerization with LiBr and $K_2S_2O_8$ redox reagents. The relative amounts of component 7 to component 8 in SCMKA has been reported to be approximately 2:1[12]. This ratio is equal to the ratio of the maximum number of grafting sites produced in the 'microfibril-plus-matrix' unit for both grafting systems. It is impossible to assure the assumption introduced for the calculation of the number-average molecular weight of protein chain in the graft copolymers. The average molecular weight of each component 7 and 8 is approximately twice the values obtained by equation (1). If two active sites arise in similar conformational positions in one molecule of the mother protein, the $\bar{M}_{n,k}$ value would be twice as large as that listed in *Table 1*. At present, however, it is an unresolved problem whether the mother protein for both grafting systems belongs to component 7 or component 8, since there is no evidence with respect to the presence of the mother protein composed of homogeneous protein species.

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