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A novel method for preparation of soluble α-keratin proteins in a high yield

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Wool keratin fibers were solubilized in water without any degradation of wool keratin proteins by the derivatization of disulfide (SS) bonds of cystine residues to carboxymethyl alanyl disulfide (CMAD) groups. After the reduction of wool with 0.2*M* thioglycolic acid (TGA) in 8*M* urea at pH10 for 48h at 25°C, 0.2*M* dithiodiglycolic acid (DTDG) was added to the solution containing swollen wool fibers and gently stirred for 24h, and then adjusted to pH7.0 with acetic acid, and finally 0.2*M* sodium bromate was added slowly to the neutral solution, and then the solution was filtered to remove residues after standing for 24h. The filterate containing soluble CMAD wool keratin proteins was fractionated using Sephadex G25, and the fractions were freeze-dried before weighing. The yield obtained was 95.2% on the basis of dry wool fiber. The yield was found to be functions not only of the concentrations of TGA and DTDG, but also the time of reduction and oxidation and pH as well. SDS-gel electrophoresis patterns of CMAD wool keratin proteins prepared below pH 10 were very similar to the wool protein derivatives. FT-IR showed that the soluble wool keratin proteins contain considerable amount of carboxyl groups associated with the CMAD side chain.
Key words: Wool, TGA, Soluble keratin protein, SDS PAGE-electrophoresis, FT-IR

INTRODUCTION

From the viewpoint of the use of renewable resources for technological application, practical interest that has been received much attention lately is the industrial utilization of the proteins from waste wool. It might be necessary to convert them to the products of commercial interest, including fibers, films, plastics, coatings and adhesives. With respect to the production of proteins from wool, several methods have been published [1,2]. A notable method for preparation of α-keratin protein has been known as the so-called "Shindai method" [1]. This process consists of the extraction of the wool and the related fiber materials with a mixed solution composed of concentrated thiourea and urea containing 2-mercaptoethanol. The yield of protein from wool fiber has been reported to be 85% based on dry fiber sample [1]. One of the authors has been reported a method for solubilization of wool proteins by derivatization of cystine residues to carboxymethyl alanyl disulfide (CMAD), and the yield of protein was about 60% or more at the conditions of 8*M* urea at pH11 and at room temperature[2]. In the present paper, we describe the recent advance for the preparation of water soluble CMAD wool keratin proteins at a milder condition of 8*M* urea and at pH 10, and the results obtained for characterization of the CMAD proteins using gel electrophoresis and FT-IR.

EXPERIMENTAL

1. Materials

The fine Merino wool fibers were purified by washing with a neutral detergent and subsequently with water and followed by air-drying. Thioglycolic acid (TGA) used as reducing agent and dithiodiglycolic acid (DTDG) were used as received. Sodium bromate used was special reagent grade.

2. Preparation of CMAD wool keratin proteins

The purified wool fibers (0.2g) taken into an Erlenmeyer flask (100mL) were stirred in a 10mL of a given concentration of TGA aqueous solution adjusted to a given pH with NaOH aqueous solution at 25°C for a given time. In this reduction process, liquor to wool ratio was 50: 1. After the addition of 0.91g urea corresponding to 8M concentration, the solution was stirred for further 24h. The pH was exactly adjusted to 7.0 with acetic acid, and 0.61g of $NaBrO_3$ corresponding to 0.2*M* concentration was added to the reaction solution. The solution was stirred at 25°C for 24h, and then filtered by a filter paper to remove the residues in solution. The residues were washed thoroughly with water and freeze-dried and then weighed. The filterate containing soluble CMAD wool keratin proteins was concentrated at room temperature by using a vacuum evaporator until about 20 to 30mL and fractionated using Sephadex G25. The fractions were collected and then freeze-dried before weighing. Here, the yield of CMAD wool keratin

protein was defined by the weight percentage of the collected and freeze-dried proteins based on the weight of starting wool fibers.

RESULTS

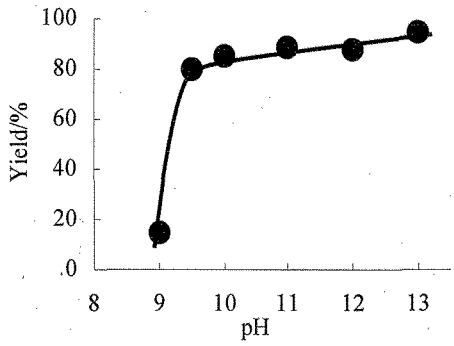

Fig. 1 Effect of pH on yield of CMAD wool keratin proteins.
Reaction condition: 0.2*M* TGA, 25℃, 48h; 0.2*M* DTDG.

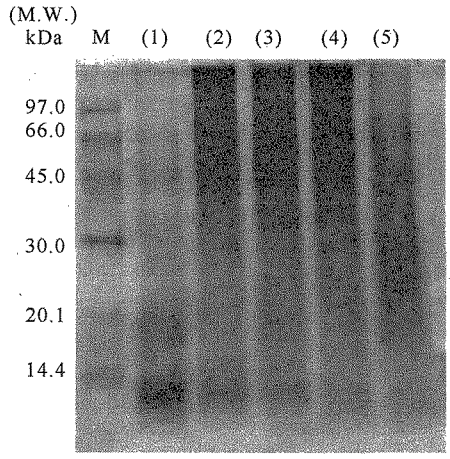

Fig.2 SDS-PAGE electrophoresis patterns of CMAD wool keratin proteins provided at different pH.
Each lane number denotes the corresponding pH: (1)9, (2)9.5, (3)10, (4)11, (5)12, and M, marker. The reaction conditions were the same as described in Fig.1.

Fig. 1 shows the effect of pH on the yield of CMAD wool keratin protein. The yield is abruptly increased from 14.9% at pH 9.0 to 80.3% at pH 9.5 and then gradually increased thereafter. At pH 13.0 that the maximum pH examined in this experiment, the amount of solubilized protein reaches to around 94.9%, which corresponds to all of the proteins in fiber.

Fig. 2 shows the electrophoresis patterns of

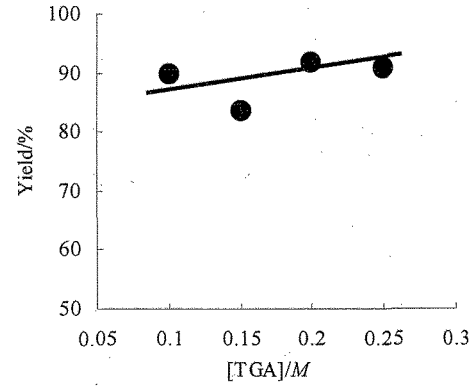

Fig. 3 Effect of concentration of TGA on yield of CMAD wool keratin protein.
Reaction conditions: TGA variable, pH10, 25℃, 48h; 0.2*M* DTDG.

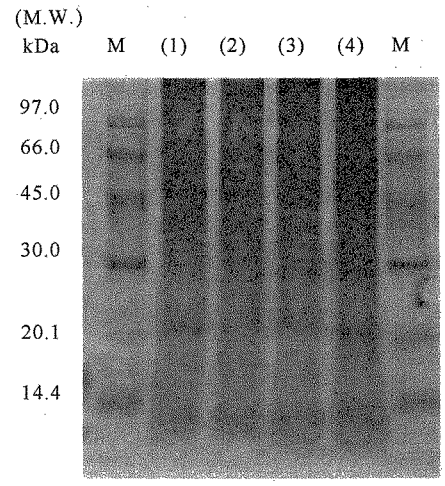

Fig. 4 SDS-PAGE electrophoresis patterns of CMAD wool keratin proteins provided by different molar concentration of TGA.
Each lane number denotes the concentration of TGA: (1)0.1, (2)0.15, (3)0.2, (4)0.25 and M, marker. The reaction conditions were the same as described in Fig.3.

CMAD wool keratin protein obtained by the reducing system at different pH. The CMAD keratin prepared in lower pH system showed well separated clear bands. The molecular weights of about 50,000 and 65,000, which may be associated with the low-sulfur component proteins were clearly delineated in the pH range from 9 to 11, but above pH12 the patterns smeared.

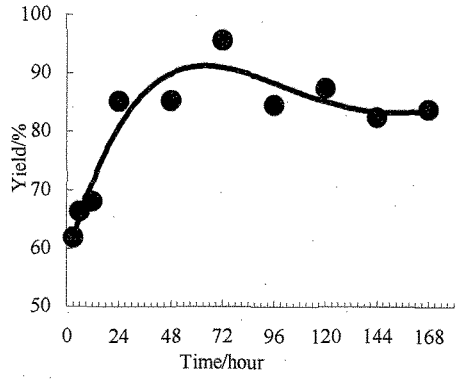

Fig. 5 Effect of reduction time with TGA on yield of CMAD wool keratin proteins.
Reaction conditions: 0.2*M* TGA, pH10, 25℃; 0.2*M* DTDG.

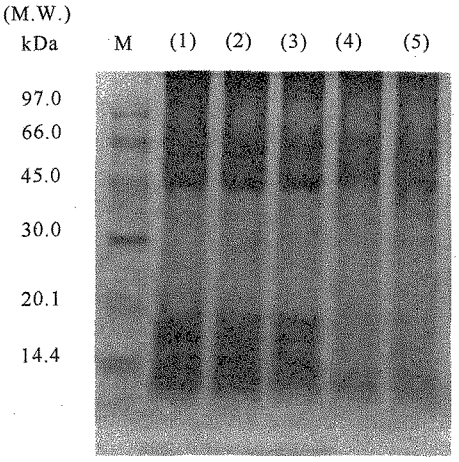

Fig. 6 SDS-PAGE electrophoresis patterns of CMAD wool keratin proteins provided at different reduction time.
Each lane number denotes the reduction time: (1)3, (2)6, (3)12, (4)24, (5)48, M, marker. The reaction conditions were the same as described in Fig.5.

Fig. 3 shows the effect of TGA concentration on the yield of CMAD wool keratin protein. The yield tends to increase with increase of the concentration of TGA in the range of 0.1 to 0.25*M* at pH 10.

Fig. 4 shows the electrophoresis patterns of CMAD wool keratin proteins obtained by the reducing systems with different concentration of TGA at pH 10. An approximately similar pattern was obtained for all of the samples.

Fig. 5 shows the effect of reduction time by TGA on the yield of CMAD wool keratin protein. The yield was steeply increased until 24h and then increased gradually up to 72h. When the amount reached to about 95%, the value tended to decrease slightly thereafter. It is important to note that the yields are over the values of 85%, which corresponds to the total amount of keratin proteins in wool.

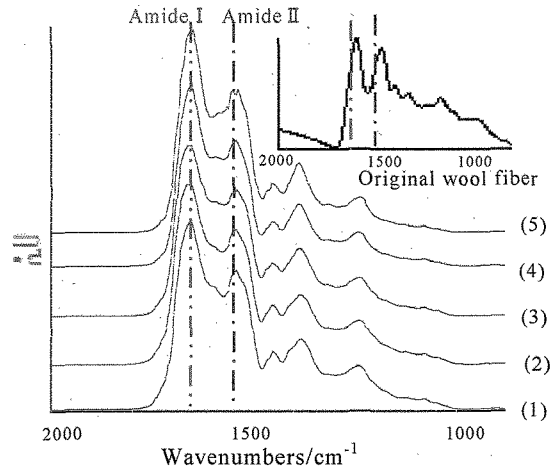

Fig. 7 FT-IR spectra of various CMAD wool keratin proteins provided at different reduction time.
The figures for the number designated on each spectrum denote the reduction time in h :(1)3, (2)12, (3)24, (4)72, (5)168, and original wool fiber. The reaction conditions were the same as described in Fig.5.

Fig. 6 shows the electrophoresis patterns of CMAD wool keratin proteins provided at different reduction time by TGA. The patterns were clearly delineated within the range of the reduction time from 3 to 48h, and the pattern tended to smear. This suggests that a degradative Reaction occurs during the derivatization reactions of keratin protein.

Fig. 7 shows the FT-IR spectra of various CMAD wool keratin proteins prepared at different reduction time and the original wool fiber. The latter spectrum is shown on the upper right side in the Figure. FT-IR spectrum of the original wool sample showed characteristic of keratin fiber features, i.e., Amide I (~1650 cm^{-1}), Amide II (~1540 cm^{-1}) and Amide III (~1235 cm^{-1}). The CMAD wool proteins showed significant changes on the spectra. Intensity and width of the peak corresponding to Amide I increased by the CMAD treatments and the envelope became unsymmetrical. As the anti-symmetry stretching vibration of the carboxylate shows absorption from 1630 to 1560 cm^{-1}, the variation of peak shape should be due to the overlapping of the band of carboxyl groups of CMAD keratin proteins on that of Amide I.

DISCUSSION

In our solubilization treatment of wool keratin fiber, three step reactions are included: reduction, forced reverse reaction, and oxidation. Major chemical reactions occurring in each step of the present treatment are represented by the following equations, eqs. (1)-(6).

Reduction step:

$$K\text{-}S\text{-}S\text{-}K + R\text{-}SH \leftrightarrows K\text{-}SH + R\text{-}S\text{-}S\text{-}K \quad (1)$$
$$R\text{-}SH + R\text{-}S\text{-}S\text{-}K \leftrightarrows K\text{-}SH + R\text{-}S\text{-}S\text{-}R \quad (2)$$

where K means the keratin chain, K-S-S-K is the cystine (CyS) residue in the chain, R-SH is the reducing agent of TGA, K-SH is the cysteine (CySH) residue formed by reduction, R-S-S-K the mixed disulfide, namely, carboxymethyl alanyl disulfide (CMAD) group, and R-S-S-R is the oxidized form of TGA, i.e., DTDG. The eqs. (1) and (2) seem to predominate this reducing system, since an extremely large amount of TGA is used for the reduction of SS linkages in wool, which contains 400 μmol/g wool.

Reverse reaction step:

$$K\text{-}SH + R\text{-}S\text{-}S\text{-}R(\text{excess}) \leftrightarrows K\text{-}S\text{-}S\text{-}R + R\text{-}SH \quad (3)$$

Eq.(3) is the reverse reaction of eq.(2), and the reaction proceeds at the condition of a higher concentration of DTDG, which may occur in the addition of an excess DTDG. Eq.(3) is of course coupled with eq.(1), producing cystine cross-links. However, eq.(1) does not occur explicitly, since almost all of the keratin proteins in wool become soluble without formation of gel-like products resulting from three dimensional cystine cross-linked network. It is appropriate to consider that intra-molecular SS cross-links may be more easily formed than inter-molecular linkages under a swollen state of the fibers containing carboxylate ions of the mixed disulfide groups incorporated by eq.(3).

Oxidation step:

$$R\text{-}SH + K\text{-}SH \rightarrow R\text{-}S\text{-}S\text{-}K \quad (4)$$
$$R\text{-}SH + R\text{-}SH \rightarrow R\text{-}S\text{-}S\text{-}R \quad (5)$$
$$K\text{-}SH + K\text{-}SH \rightarrow K\text{-}S\text{-}S\text{-}K \quad (6)$$

The reactions expressed by eqs.(4) and (5) predominate in the oxidation step, as clearly indicated by the previous discussion. Eq.(6) is likely to be comparatively minor reaction for the case of sodium bromate as a milder oxidizing agent [3].

As revealed in Figs. 2, 4, 6, together with Figs. 1, 3 and 5, the CMAD proteins synthesized in high yield from wool fiber without degradative hydrolysis of the protein molecules are soluble in an alkaline pH according to eq.(7).

$$K\text{-}S\text{-}S\text{-}CH_2\text{-}COOH \leftrightarrows K\text{-}S\text{-}S\text{-}CH_2\text{-}COO^- + H^+ \quad (7)$$

As demonstrated FT-IR spectra shown in Fig. 7, the side chains of S-carboxymethyl alanyl disulfide group are inserted into the protein molecules according to the reactions represented by eqs.(3) and (4). The reaction rate of eq.(5) may be higher than eq.(6), since the collision frequency between the small molecules as eq.(5) is expected to be more higher than between the side chain molecules incorporated in a high molecular weight of polymer as represented by eq.(6). And further the reactions of eq.(6) may occur to form intra-molecular SS bonds as already discussed on the formation of SS linkages in the reverse reactions of eq.(3).

CONCLUSION

1. Solubilization treatment consists of three processes: i) reduction of wool with thioglycolic acid (TGA), ii) its reverse reaction proceeding by the addition of oxidized form of TGA, i.e., DTDG, and iii) final oxidation reaction process.
2. Water soluble α-keratin proteins were successfully synthesized by applying the above reduction and oxidation method for derivatization of cross-linked cystine residues to S-carboxymethy alanyl disulfide (CMAD) groups.
3. The yield defined as weight percentage of CMAD protein on the basis of starting materials was found to be functions not only of the concentrations of TGA and DTDG, but also the time of reduction and oxidation, and pH as well.
4. The yield of soluble CMAD protein reached to about 90% and no hydrolytic scission of wool keratin molecules was found.
5. The CMAD proteins could be easily converted into initial keratin structure cross-linked with disulfide bonds by reduction and oxidation treatments.

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