In situ crosslinking of polypeptides*

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Since the insolubility of crosslinked polymers precludes the fabrication of devices, a method has been developed by which the crosslinking can be carried out in the solid state. The crosslinking agent is initially chemically bound to the polymer, which is then brought into the desired shape or form, prior to crosslinking. The crosslinking agent is then activated to provide the desired stability of the device. Initially lysine/leucine copolymers have been crosslinked by reacting: 1–10% of the ϵ-amino groups with phenacylthioglycolic acid anhydride in DMF to give the corresponding ϵ-phenacylthioglycoloyl derivatives. After the removal of the phenacyl groups, free thiol groups are formed. Films of the thioglycoloyl polymers were obtained by casting from aqueous acetic acid onto glass plates. The dry films were exposed to ammonia gas, I_2 vapours and then ammonia gas again. The films obtained were insoluble and swelled to give clear, transparent films. The ease of preparation of the phenacylthio derivatives and selectivity of deprotection by zinc dust in aqueous acetic acid indicate the usefulness of this group for the protection of the thiol function.

INTRODUCTION

Aggregation of insoluble biopolymers can be attributed to a number of factors, such as crystallization, hydrogen bond formation, ionic interactions, Van der Waal's forces, covalent crosslinks, etc.

In native proteins various types of inter- and intra-chain crosslinks are known including desmosine and isodesmosine links in elastin, lysyl crosslinks in collagen and a variety of sulphur bridges in globular and fibrous proteins. α-Keratins, the widely occurring protective hairy covering of vertebrates, are in essence crosslinked proteins. Correlation of the mechanical properties and cystine content of various keratins suggests that the main process in keratinization is the formation of disulphide crosslinks[1].

Based on the above observations, and the fact that the disulphide bond occurs widely in living organisms, we propose that disulphide crosslinking of appropriate polymers by means of disulphide bridges may provide an interesting class of biocompatible materials. This is a preliminary report on the chemical aspects of a new synthesis which allows easy manipulation of the material in question (in this case copolypeptides) such that appropriate shape and form of the crosslinked material may be easily attained.

EXPERIMENTAL

Two random copolypeptides both containing L-lysine and L-leucine (molar ratio 4:1 and 1:1) were crosslinked. 1, 5 and 10% of the ϵ-amino sidechains of the Lys residues were converted to the thioglycoloyl derivatives in the following manner: the polypeptides were reacted with phenacylthioglycolic acid anhydride. The phenacyl group was subsequently removed. Films were made of the resulting thioglycoloyl peptides by means of casting from dilute acetic acid solution. The crosslinking was carried out in the solid state by means of mild oxidation of the thiol groups to disulphide links. The reaction scheme is summarized in *Figure 1*.

The polymers were provided by Dr J. M. Anderson and his coworkers: Drs Deshmane, Hayashi and Sederal. The reagents and intermediates were synthesised according to conventional methods of organic chemistry using thioglycolic acid (J. T. Baker) and α-bromoacetophenone (Eastman Kodak Co.). The other commercially available solvents and chemicals were purchased from Fisher Scientific Co.

For preparative dialysis, regenerated cellulose dialysis tubes (Sargent-Welch) were used.

The reactions were monitored by t.l.c. on standard precoated Silica Gel 60 F-254 plates (E. Merck) in the developing solvent chloroform–dioxane–acetic acid (9:1:0.5). The chromatographic zones were located by means of the u.v. absorbance of the phenacyl groups quenching the fluorescence of the t.l.c. plates.

Capillary melting points were measured, and are given uncorrected.

The amino-acid composition was analysed in a Durrum Amino Acid Analyzer (Durrum Chemical Co., Palo Alto, Ca. 94303, USA).

The elemental analysis was carried out by the Baron Consulting Co. (Orange, Conn. 06477, USA).

Phenacylthioglycolic acid (I)

The synthesis was carried out according to the method of Holmberg[2]. NaOH (16 g, 0.4 mol) and thioglycolic acid (18.4 g, 0.2 mol) were dissolved in 110 ml of water under cooling in an ice bath. α-Bromoacetophenone (40 g, 0.2 mol) was added, and the mixture was vigorously stirred for 30 min under continued cooling. The solution was allowed to stand overnight at room temperature. Acidification with HCl resulted in an oily precipitate, which was extracted with ethyl acetate. The organic layer was separated and dried over $MgSO_4$. After removal of the solvent *in vacuo* a solid residue was obtained. Recrystallization from benzene gave the pure product: yield, 28.0 g (67% of the calculated); m.p., 98°–100°C (literature[2], 100°–102°C); R_f, 0.33.

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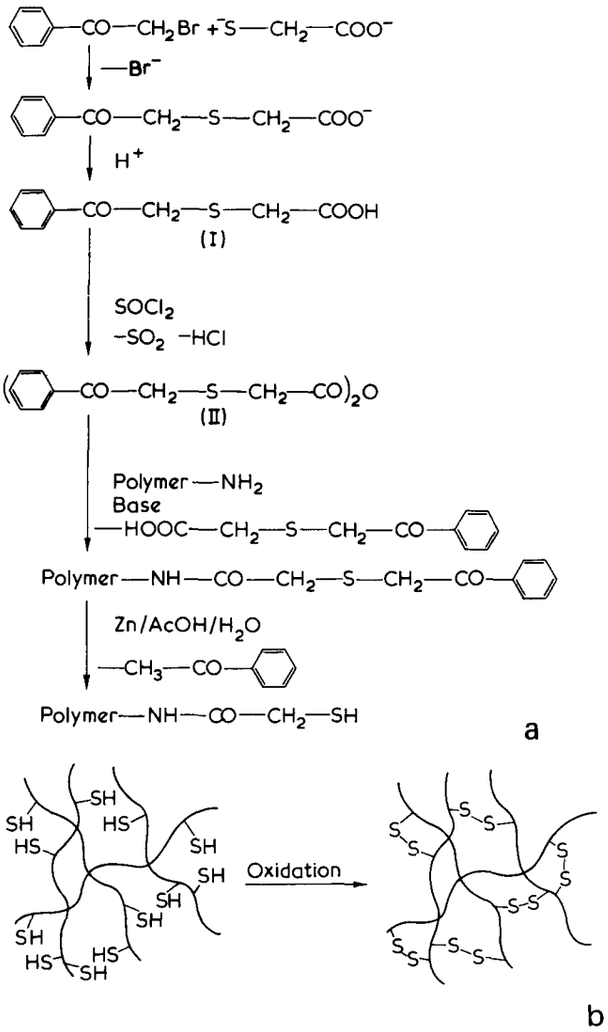

Figure 1 Reaction scheme for the synthesis of thioglycoloyl polypeptides through the S-protected carboxy activated acid (a) and for the crosslinking (b)

Phenacylthioglycolic anhydride (II)

21 g (0.1 mol) of I was dissolved in benzene (200 ml). Thionyl chloride (7.1 g, 0.06 mol) was added to the gently warmed solution. After the HCl development had ceased, the volatiles were evaporated *in vacuo*. The residue was recrystallized from benzene: yield, 1.0 g (56% of the calculated). A substantial amount of the product was detected in the filtrate. However, no attempt has been made to isolate the secondary crop. M.p. : 92°–94°C (uncorr.). Elemental analysis: calculated: C, 59.5%; H, 4.5%; S, 15.9%; found: C, 58.4%, H, 4.6%; Cl, trace: S, 15.5%.

Phenacylthioglycoloyl polymers

1% Acylation. The hydrobromides of poly(L-Lys-L-Leu) of ratio 4:1 (0.237 g equivalent 1 mmol of L-Lys) and poly-(L-Lys-L-Leu) of ratio 1:1 (0.322 g equivalent 1 mmol L-Lys) were reacted with4.0 mg (0.01 mmol) of II.

5% Acylation. The same amounts of the polymers as given under '2% acylation' were reacted with 20.0 mg (0.05 mmol) of II.

10% acylation. The same amount of the polymers as given under '2% acylation' were reacted with 40.2 mg (0.1 mmol) of II.

The polymers were dissolved in about 20 ml of dimethylformamide (DMF). After mixing with II, 0.22 g (~2.2 mol) of triethylamine was added to each solution. The reaction was conducted at ambient temperature. The conversion was monitored by t.l.c. on silica gel plates in the solvent system $CHCl_3$–dioxane–AcOH (9:1:0.5). The following R_f values were observed: phenacylthioglycoloyl polymers, R_f 0.00; unreacted II, R_f 0.90. The disappearance of the II zone indicated the end of the reaction. The modified polymers were then precipitated with ether. The solid products were centrifuged. After discarding the supernatant, the precipitate was suspended in ether, and the suspension was centrifuged again. The products were dried *in vacuo* over concentrated H_2SO_4.

Thioglycoloyl polymers

The removal of the phenacyl group is selective by means of reduction with zinc dust in acetic acid[3]. The phenylthioglycoloyl polymers were dissolved in acetic acid also containing some water. The sample obtained from poly(L-Lys-L-Leu) (1:1) through 10% acylation turned out to be insoluble, so further studies on this material were discontinued.

To the solutions of the other derivatives small portions of zinc dust were added under continuous stirring at about 40°C for two days. The conversion was monitored by t.l.c. on silica gel plates in the developing solvent $CHCl_3$–dioxane –AcOH (9:1:0.5). The disappearance of the fluorescence quenching material at the origin and the appearance of benzophenone (R_f = 0.84) indicated the end of the reaction. The mixtures were diluted with water. The excess of zinc was filtered off. The filtrates were transferred into dialysis tubes. The samples were dialysed for five days against 5% acetic acid. The solutions were finally transferred into round bottom flasks and evaporated to dryness *in vacuo*. The residues were dissolved in 2 ml of 5% acetic acid.

In situ *crosslinking*

Polymer films were cast, from the solutions obtained above, on clean glass surfaces. The dry films were exposed to ammonia gas, then I_2 vapour, and finally, ammonia gas again, every sequence taking about 24 h at room temperature. Insoluble, hydrophilic films resulted which were suitable for biological experiments, such as blood coagulation and implantation studies.

The elemental analysis of the film gave an N:S ratio of 13.02. This result is in good agreement with the theoretical value, which is 13.17 in case of 10% acylation.

DISCUSSION

During the synthesis well elaborated standard methods of organic chemistry were used. The analysis of the intermediates and final products, including t.l.c., elemental and amino-acid analysis, satisfactorily prove the identity of the compounds involved.

The choice of thioglycolic acid rather than cysteine, as crosslinking agent, can be explained from the practical point of view that in the case of cysteine, the introduction of another selective protecting group would be necessary.

The percentage of acylation indicates the degree of crosslinking. Since two thiol groups participate in forming one disulphide bridge (*Figure 1*), the maximal degree of crosslinking can be 0.5, 2.5 and 5% from the 1, 5 and 10% acylated polymers, respectively. A qualitative comparison of the products is in agreement with the observations on keratins[1]

that higher degrees of crosslinking resulted in stiffer, more brittle films. Quantitative measurements are now in progress, to evaluate the mechanical properties of the products under various conditions.

An additional advantage of this method is the ease of manipulation with which the material is brought into the derived shape or form, prior to crosslinking. Then, under very mild conditions, the crosslinking is facilitated to achieve mechanical stability.

Preliminary studies[4] indicate consistent compatability of some of the films with blood platelets. Further research in the biomedical areas will include the studies of toxicity, biodegradation and behaviour under the conditions of implantation.

It is clear, that this method of crosslinking is applicable in every case, that a polymer sidechain can be acylated. This fact along with the mild conditions of crosslinking opens new possibilities for immobilizing biologically active species, such as enzymes or intact cells. These aspects are purely speculative, but nevertheless worthy of investigations.

CONCLUSION

The phenacyl group has proven useful for the protection of mercapto groups. Both its attachment and removal are simple. Using phenacylthioglycolic acid anhydride reagent, lysine containing amino-acid copolymers can easily be converted to the corresponding thioglycoloyl derivatives. After removal of the protecting group polymers containing thiol moieties on their sidechains can be obtained. These polymers can be crosslinked in the solid phase on the basis of the formation of disulphide bond.

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