CHROM. 3658

CHROMATOGRAPHY OF SOME CYSTINE PEPTIDES AND FORMATION OF MIXED DISULFIDES

JAMES J. RICHTER* AND ARTHUR WAINER
Department of Biochemistry, Bowman Gray School of Medicine, Wake Forest University, Winston-Salem, N.C. (U.S.A.)

(Received June 20th, 1968)

SUMMARY

Chromatographic systems for cystine peptides were established and mixed disulfide formation was studied using a Technicon Amino Acid Analyzer. The ninhydrin molar color yield of peptides with N-terminal cystine was increased by splitting the disulfide bond with sodium bisulfite.

Cystine peptides were prepared in the sulfhydryl form by reducing the disulfide bonds with dithiothreitol. The reduced peptides were separated from dithiothreitol on a small column of Dowex 50W X4 in the H^+ form. The sulfhydryl peptides were mixed together and allowed to oxidize completely. The oxidized products were analyzed in the chromatographic system. The formation of symmetrical and mixed disulfides from the sulfhydryl compounds appeared to occur randomly.

INTRODUCTION

The biological significance of disulfide bonds and sulfhydryl groups has two main aspects. Disulfide bonds play an important role in maintaining the conformation of proteins[1]. Sulfhydryl-containing compounds have been shown to provide protection for a variety of organisms against radiation-induced damage[2]. While studying the mechanism of the protective properties of sulfhydryl compounds, ELDJARN AND PIHL[3,4] showed the formation of some mixed disulfides. Recently, VAN RENSBURG AND SWANEPOEL[5] showed that when bisulfite (HSO_3^-) ruptures mixed disulfides, steric factors sometimes cause the SO_3^{2-} group to attach preferentially to one of the two resulting thiol compounds. Further study of mixed disulfides has been limited by the techniques available. This paper reports the use of ion-exchange chromatography to study disulfide formation from cysteine-containing peptides.

The work consisted of three main problems. First, a chromatographic system for analyzing the amounts of peptides had to be established. Because the commercially

* From a thesis submitted to the Graduate Faculty of Wake Forest University in partial fulfillment of the requirements for the degree of Master of Science.

available compounds were symmetrical disulfides, the second problem was to devise a method of reducing the disulfides to isolate the peptides in their sulfhydryl form. The third phase of the work was the actual formation of mixed disulfides from the reduced peptides and the chromatographic analysis of the products. Because the mixed disulfides formed were to consist of two peptides of differing amino acid composition, we were curious to see if steric factors would favor or hinder any particular disulfides from forming.

MATERIALS AND METHODS

Cystine-containing peptides were obtained from Cyclo Chemical Corp. (Los Angeles, Calif.). Dithiothreitol (DTT) and reduced glutathione (GSH) were obtained from Calbiochem Company (Los Angeles, Calif.).

A Technicon Amino Acid Analyzer was used with a 125 × 0.6 cm column of Chromobeads Type-A cation exchange resin in the sodium form (Technicon Corp., Ardsley, N.Y.). The column was maintained at 60° by a jacketed circulating water bath. Eluting buffers were pumped through the column at 0.6 ml per min. Ninhydrin reagent was obtained from Pierce Chemical Company (Rockford, Ill.). 0.01 *N* NaCN (0.2 ml/75 ml buffer) was added to the eluting buffers to reduce the ninhydrin, as described by ROSEN *et al.*[6]. Ninhydrin color of the effluent was measured at 570 mμ.

The first problem was to adapt the amino acid analyzer for the chromatography

TABLE I

COMPOSITION OF BUFFER GRADIENTS FOR PEPTIDE ELUTION

The pH of the solution in chamber 1 for Group I peptides was 4.5. The stock solutions of citrate buffer, pH 3.10, pH 5.10, and pH 10.50, were prepared as directed by the Technicon Corp.[7]. The stock solutions of buffers used for Group II peptides contained 10 ml Brij 35 per liter (Pierce Chemical Co.).

Autograd chamber No.	*Group I peptides*			*Group II peptides*		
	ml sodium citrate buffer			*ml sodium citrate buffer*		
	pH 3.10	*pH 5.10*	*pH 10.50*	*pH 3.10*	*pH 5.10*	*pH 10.50*
1	20	70		45		
2	20	70		45		
3		90		30	15	
4		40	50	10	35	
5			90		45	
6			90		20	25
7			90			45
8						45
9						45

of the peptides. Technicon Corp. recommended a gradient of pH 3.10–pH 10.50 for the eluting buffers using their nine-chamber Autograd device[7] for peptide elution. This gradient was modified in two ways for the chromatography of the cystine peptides as shown in Table I.

The peptides used were divided into two groups because of their chromatographic behavior and color yield with ninhydrin. L-Cystinyl-bis-(L-alanine) $(Cys\text{-}Ala)_2$, L-cystinyl-bis-(L-valine) $(Cys\text{-}Val)_2$ and L-cystinyl-bis-(L-leucine) $(Cys\text{-}Leu)_2$ made up

Group I. Group II consisted of bis-(L-alanyl-L-cysteinyl-L-tyrosine) $(Ala\text{-}Cys\text{-}Tyr)_2$, glycylglycyl-bis-cystine $(Gly\text{-}Cys)_2$, and oxidized glutathione (GSSG).

The elution gradient pH 4.5–pH 10.50 was used for the chromatography of Group I peptides. The molar color yields of the cystine dipeptides (Group I) were very low. Whereas 0.1–0.5 μmoles of most amino acids give sufficient color for automated analysis, 10 to 20 times these amounts of cystine dipeptides were necessary to give comparable color values. It is interesting to note that the amino acid cysteine has a ninhydrin color yield only 6% that of norleucine, while the color yield of cystine is about 50% that of norleucine[8]. Other dipeptides have ninhydrin color yields similar to those of amino acids[9,10].

The ninhydrin color yields of Group I peptides were increased by splitting the disulfide with $NaHSO_3$. The column effluent was mixed with $NaHSO_3$ in the analytical portion of the amino acid analyzer. $NaHSO_3$ (10 mg/ml) was dissolved in 4 *N* sodium acetate buffer pH 5.5 and pumped into the system at 0.1 ml per min (Fig. 1). After mixing with $NaHSO_3$, the effluent was mixed with ninhydrin solution in the normal manner. Absorption spectra of ninhydrin–peptide solutions showed peaks at 405 mμ and 570 mμ. Both peaks were approximately doubled if the peptide was reacted with bisulfite before ninhydrin color development. Since the amino acid analyzer is normally

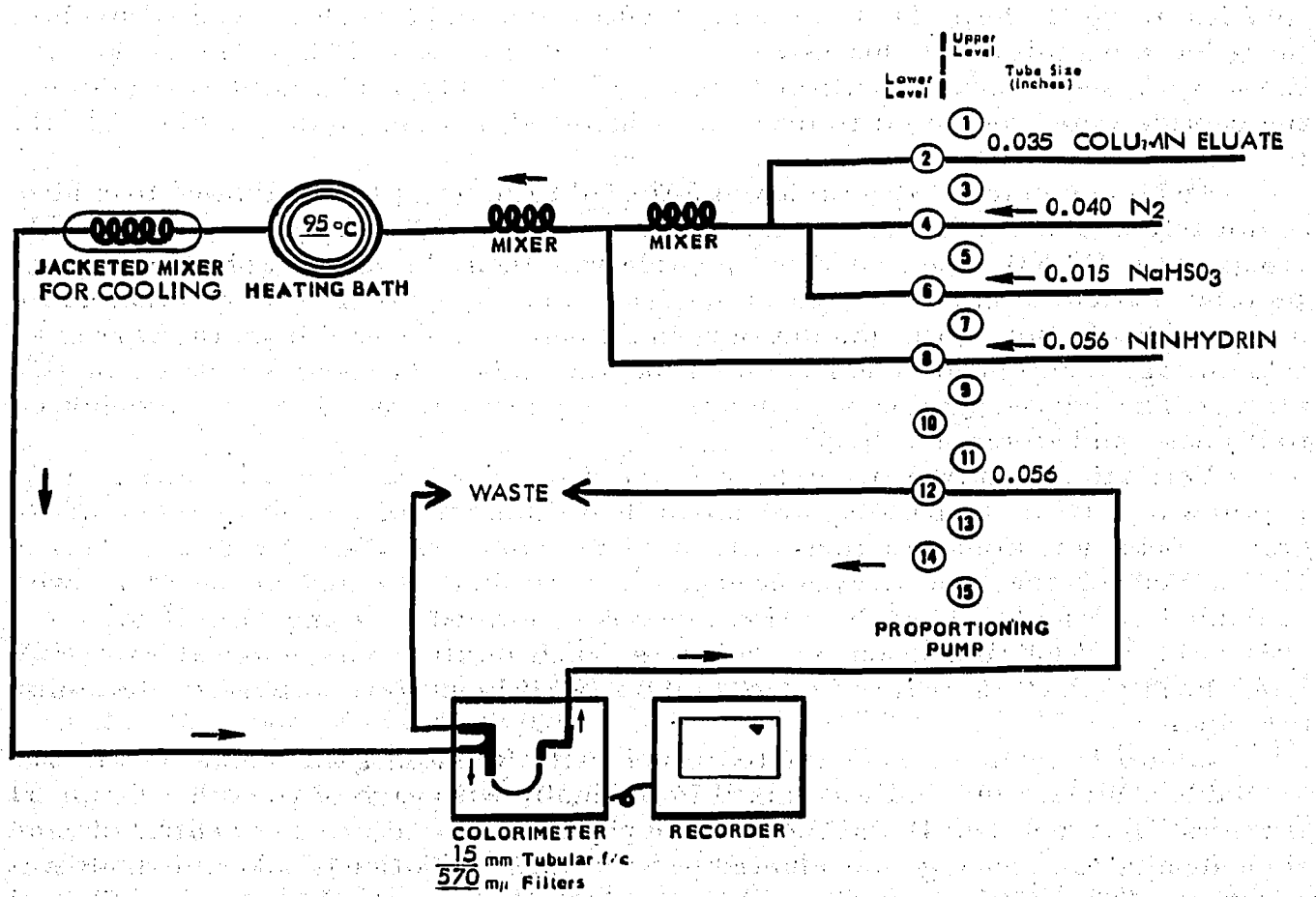

Fig. 1. Manifold arrangement of the Technicon Amino Acid Analyzer for the analysis of Group I peptides. Group II peptides were analyzed in the same system but without the addition of $NaHSO_3$.

equipped with a 570 mμ filter, we used this wavelength to measure color intensity.

A typical chromatogram of Group I peptides was carried out as follows. The column was washed with 0.2 *N* NaOH for 30 min and then regenerated with buffer of the same pH as at the start of the gradient (pH 4.5 for Group I). The regenerating buffer contained 0.01 *N* NaCN (0.2 ml/75 ml buffer). After the column had been equilibrated for 1.5 h, the sample was applied and the pH gradient elution started. A chromatogram of all Group I peptides was complete in 5 h. The mixed disulfide formed from a pair of dipeptides emerged between the elution peaks for each of the respective symmetrical disulfides.

Group II peptides, GSSG, $(Gly\text{-}Cys)_2$, and $(Ala\text{-}Cys\text{-}Tyr)_2$ had color yields close to those of amino acids and required a different pH gradient elution pattern. The buffer gradient for elution of Group II peptides was pH 3.10–pH 10.50 (Table I). Group II peptides gave much greater ninhydrin color yields than those of Group I and sulfitolysis of Group II peptides did not increase their color yields. Therefore, it was not necessary to react Group II peptides with $NaHSO_3$ in the amino acid analyzer.

The cystine peptides were prepared in the sulfhydryl form by reducing the disulfide bonds with dithiothreitol[11] at neutral pH. After 15 min, reduction of the peptides was complete and the pH was lowered to 2 to prevent reoxidation. The reduced peptides were separated from DTT on a 3.0 × 0.5 cm column of Dowex 50W X4 in the H^+ form. DTT was eluted with water and 6 *N* HCl was used to elute the reduced peptides. The eluates were neutralized and assayed for –SH content with ELLMAN's reagent (dithiobisnitrobenzoate, DTNB)[12]. The acid fractions containing the peptide were lyophilized to dryness, redissolved in water, and lyophilized again to remove excess HCl.

An aliquot of reduced peptide was adjusted to neutral pH and allowed to oxidize completely overnight. Complete oxidation was confirmed by a negative test for –SH groups with DTNB. The reoxidized peptide was then chromatographed in the appropriate system. From the ELLMAN test of the reduced peptide and the chromatogram of a reoxidized aliquot, the amount of reduced peptide recovered from the Dowex 50 column was determined. Each Group I peptide was reduced and isolated in this manner. The reduced peptides were divided into equal fractions (10 μmoles), lyophilized to dryness, and stored in a freezer.

Formation of mixed disulfides was the third problem. Group I and Group II peptides were treated separately because of their different color yields and chromatographic behavior. Equal fractions of Group I peptides were dissolved in 1.0 ml 0.1 *N* HCl, mixed together, and the pH adjusted to neutrality. The mixtures were allowed to oxidize overnight. Complete oxidation was confirmed by using DTNB to detect –SH groups. When oxidation was complete, each mixture was acidified with HCl, lyophilized to dryness, redissolved in 1 ml 0.1 *N* HCl, and then applied to the amino acid analyzer.

Group II peptides were treated differently. Reduced glutathione (GSH) was available commercially and was mixed individually with each of the other Group II peptides. Mixtures of GSH and the oxidized peptides were adjusted to neutral pH and thiol–disulfide interchange was allowed to occur until oxidation of all compounds was complete. The mixtures were acidified, lyophilized, redissolved in 0.1 *N* HCl, and chromatographed.

The areas under the ninhydrin peaks of all chromatograms were integrated with

a Technicon Integrator/Calculator. The amount of symmetrical disulfides were calculated based on color yields previously determined from known amounts of oxidized peptides.

RESULTS

The formation of mixed and symmetrical disulfides from reduced peptides appeared to occur randomly. Table II presents the amount of the various disulfides

TABLE II

PERCENT TOTAL S AS SYMMETRICAL OR MIXED DISULFIDES AFTER OXIDATION OF GROUP I PEPTIDES

Reduced peptides mixed together	*Oxidation products*					
	1 (Cys-Ala)$_2$	*2* Cys–Ala | Cys–Val	*3* (Cys–Val)$_2$	*4* Cys–Ala | Cys–Leu	*5* Cys–Val | Cys–Leu	*6* (Cys–Leu)$_2$
Cys–Ala + Cys–Val	23.4 24.1 (25) 23.6	47.8 49.7 (50) 51.4	28.8 25.6 (25) 26.0			
Cys–Ala + Cys–Leu	20.8 21.1 (25)			53.4 56.2 (50)		25.8 22.7 (25)
Cys–Val + Cys–Leu			22.9 22.9 (25) 19.2		50.5 51.1 (50) 52.2	26.6 26.0 (25) 28.6
Cys–Ala + Cys–Val + Cys–Leu	11.1 12.5 (11.1)	(22.2)	9.87 12.0 (11.1)	(22.2)	(22.2)	11.6 12.2 (11.1)

Numbers in parentheses are theoretical values for random combination. Experimental values for mixed disulfides were determined as the difference between 100% and the symmetrical disulfide values. A typical chromatogram is given in Fig. 2. Buffer gradients for elution are given in Table I. Other details of the chromatographic system are given in the text.

formed in each experiment with Group I peptides. The amount of a given sulfhydryl peptide (RSH) not represented in the symmetrical disulfide form (RSSR) was assumed to exist as half of a mixed disulfide (RSSR'). Thus, when RSH and R'SH were mixed in equal amounts and allowed to oxidize completely, the products were RSSR, R'SSR, and R'SSR', where R and R' designate peptides of differing amino acid composition. One would expect 50% of the total sulfur to appear as the mixed disulfide (RSSR') and 25% to appear in each of the two symmetrical disulfides, if RSH and R'SH combined randomly. As can be seen in Table II, the experimental results approach the theoretical values for random formation of disulfides.

Similarly, when all three sulfhydryl peptides were oxidized together, each of the resulting symmetrical disulfides contained close to the theoretical 11.1% (1/9) of the total sulfur (Table II). Fig. 2 is a typical chromatogram of Group I peptides. It is

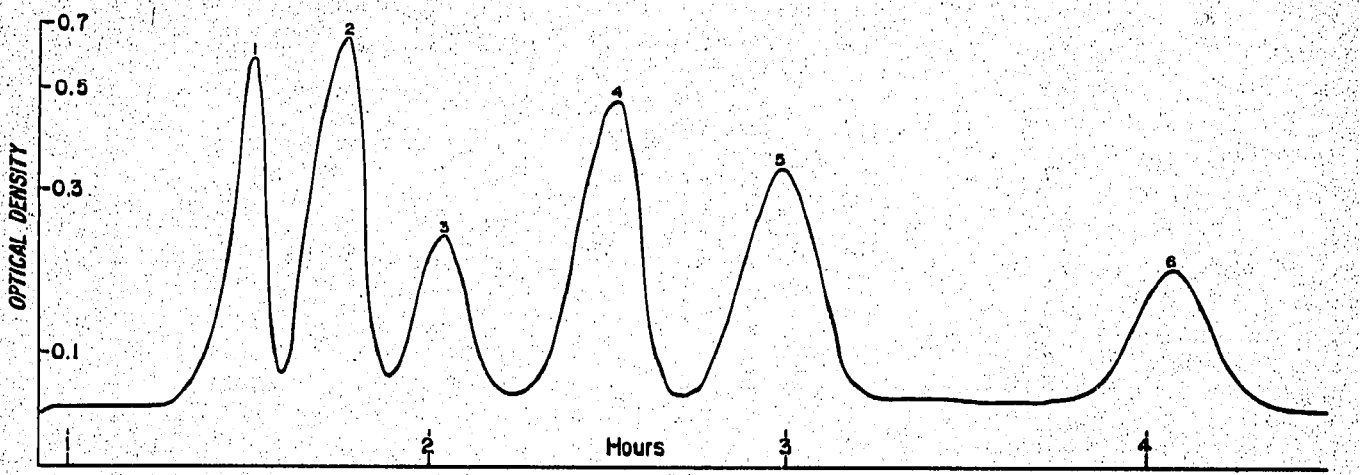

Fig. 2. A typical chromatogram of Group I peptides. The peaks are identified by the numbers given in Table II.

one of the experiments in which three reduced peptides oxidized to form three symmetrical and three unsymmetrical disulfides. When only two reduced peptides were oxidized together, the chromatograms were similar to Fig. 2 with peaks in the appropriate positions.

Since the interaction among Group I peptides appeared to occur randomly, the amino acid sequences of these peptides apparently have no influence on the formation of mixed disulfides.

The results of experiments with Group II peptides are presented in Table III.

TABLE III

PERCENT TOTAL S AS SYMMETRICAL OR MIXED DISULFIDES AFTER OXIDATION OF GROUP II PEPTIDES

Numbers in parentheses are theoretical values for random combination. Experimental values for mixed disulfides were determined as the difference between 100% and the symmetrical disulfide values. Buffer gradients for elution are given in Table I. Other details of the chromatographic systems are given in the text.

Peptides mixed together	*Oxidation products*					
	GSSG	*GS* | *Gly–Cys*	*GS* | *Ala–Cys–Tyr*	*(Gly–Cys)₂*	*GS* | *Ala–Cys–Tyr*	*(Ala–Cys–Tyr)₂*
GSH + $(Gly–Cys)_2$	23.5 25.5 (25)	51.9 50.6 (50)		24.6 23.7 (25)		
GSH + $(Ala–Cys–Tyr)_2$	24.8 (25)		52.7 (50)		22.5 (25)	
GSH + $(Gly–Cys)_2$ + $(Ala–Cys–Tyr)_2$	11.2 (11.1)	(22.2)	(22.2)	11.0 (11.1)	(22.2)	8.8 (11.1)

When GSH was mixed with either of the disulfides $(Gly\text{-}Cys)_2$ or $(Ala\text{-}Cys\text{-}Tyr)_2$, thiol–disulfide interchange appeared to occur randomly. Because random interaction was observed in this manner, it was not necessary to oxidize mixtures starting with all peptides in the reduced form.

When equal amounts of GSH and $(Gly\text{-}Cys)_2$ and $(Ala\text{-}Cys\text{-}Tyr)_2$ were mixed and oxidized, it appeared that equilibrium of thiol–disulfide interchanges was not reached. Therefore, the sulfhydryl forms of these peptides were mixed and allowed to oxidize. As can be seen in Table III, random formation of disulfides appeared to occur when all three Group II peptides oxidized in this manner. The tripeptide $(Ala\text{-}Cys\text{-}Tyr)_2$, was considerably less pure than the others. This may account for its greater deviation from the theoretical amount than the others.

DISCUSSION

The ninhydrin color yield of cystine is 50% that of normal amino acids and the color yield of cysteine is only 6%[8]. Ninhydrin color yields of Group I peptides (where cystine was in the N-terminal position) were all very low, similar to cysteine. Group II peptides (where cystine was in the middle or in the C-terminal position) all produced ninhydrin color yields similar to those of normal amino acids. Furthermore, sulfitolysis of N-terminal cystine peptides increased the color yields by a factor of about 2. It is apparent from these observations that the sulfur atoms of cystine have an effect on the reactivity of that compound's amino group toward ninhydrin.

Disulfide formation among the peptides studied appeared to occur randomly. The amino acid sequences did not influence the formation of any particular combination of peptides. These results are consistent with the concept that the amino acid sequences of a protein determines its conformation and the arrangement of disulfides between cysteine residues.

The chromatography of cystine peptides and the methods for preparing them in the reduced form may be of general usefulness in the future study of peptides and disulfides.

ACKNOWLEDGEMENTS

This work was partially supported by a grant (07233) from the NIH. J.J.R. was also the recipient of a National Science Foundation Traineeship. A.W. was a recipient of a Special Fellowship from the NIH.

REFERENCES

1 C. B. Anfinsen and E. Haber, *J. Biol. Chem.*, 236 (1961) 1361.
2 A. Pihl, L. Eldjarn and J. Bremer, *J. Biol. Chem.*, 227 (1957) 339.
3 L. Eldjarn and A. Pihl, *J. Biol. Chem.*, 223 (1956) 341.
4 L. Eldjarn and A. Pihl, *J. Biol. Chem.*, 225 (1957) 510.
5 N. J. van Rensburg and O. A. Swanepoel, *Arch. Biochem. Biophys.*, 118 (1967) 531.
6 H. Rosen, C. Berard and S. Levenson, *Anal. Biochem.*, 4 (1962) 213.
7 *Technical Manual, Peptide Autoanalyzer*, Technicon Instruments Corp., Ardsley, N.Y.
8 A. Wainer, *J. Chromatog.*, 21 (1966) 126.
9 J. S. King Jr. and A. Wainer, *J. Chromatog.*, 24 (1966) 215.
10 S. Yanari, *J. Biol. Chem.*, 220 (1956) 683.
11 W. W. Cleland, *Biochemistry*, 3 (1964) 480.
12 G. L. Ellman, *Arch. Biochem. Biophys.*, 82 (1959) 70.