A NEW, IMPROVED TECHNIQUE FOR AUTOMATED SEQUENCING OF NON-POLAR PEPTIDES*

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SUMMARY

A method for the automated sequential degradation of non-polar peptides is reported. Both the polarity and film-forming properties of these peptides are increased by the attachment of 2-amino-1,5 napthalene disulfonic acid to the C-terminal residue via a water-soluble carbodiimide. The sequences of three individual peptides modified by the procedure are reported with high yields while these same peptides could not be successfully sequenced if no modification was made.

With the development of automated techniques (1,2), the sequential degradation of proteins has been greatly facilitated. Nevertheless, while automation can be applied successfully to most proteins, peptide sequential degradation has presented problems due primarily to extreme losses of the residual peptide during extraction procedures (3). This problem is magnified several-fold when dealing with peptides derived from elastin since the latter protein is composed primarily of non-polar amino acids (4).

To overcome such difficulties two general approaches have been taken to improve peptide analyses. One of these involves sequencing in a heterogeneous phase by the attachment of the peptide to an insoluble matrix (2,5). The other technique, utilizing the classical homogeneous phase approach of Edman and Begg (1), involves modification of the physical properties of tryptic peptides to render them less extractable (3). This method has also been extended to cysteine-containing peptides (6) by conversion of

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the cysteine residue to an S-(β-aminoethyl) cysteine with ethylenimine (7).

Recently, we have described a method for the modification and eventual sequencing of the tetrapeptide Gly-Val-Pro-Gly. A hydrophilic group was incorporated onto the C-terminal glycine residue employing a water soluble carbodiimide in a manner analogous to peptide synthesis (8).

This communication extends those observations and presents a simplified technique for the automated sequencing of hydrophobic peptides through the attachment of a highly specific polar reagent to the C-terminal amino acid residue.

EXPERIMENTAL PROCEDURE AND RESULTS

Gly-Phe-Phe was purchased from the Mann Research Laboratories. Peptide C16c was isolated from a chymotryptic digest of tropoelastin* and Gly-Val-Pro-Gly was obtained from an elastin digest (9). N-ethyl,N'-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC) and 2-amino-1,5 napthalenedisulfonic acid (ANS) were purchased from the Ott Chemical Co. and Aldrich Chemical Co., respectively.

Sequencing was performed on a Beckman Automated Sequencer Model 890C using peptide program #071472. All fractions were converted to the phenylthiohydantoin (PTH)-amino acids in the usual manner (1) and extracted into ethyl acetate. PTH derivatives were identified on a Beckman Model 65 gas chromatograph using the system of Pisano and Bronzert (10). Identification of the C-terminal residue was made by hydrolyzing the aqueous layer with 6 N HCl at 140°C for 24 hours in sealed, evacuated tubes flushed with nitrogen. Subsequent analyses were performed on a Jeolco Model 5AH automated amino acid analyzer.

One equivalent of lyophilized peptide (0.050 to 0.300 umoles) was reacted with two equivalents each of EDC and ANS in 200 ul of water previously adjusted to pH 4.0 with 0.01 N HCl and the reaction mixture was stirred for 4 hours at room temperature. At this time, the mixture was diluted with 400 ul of 10% acetic acid and transferred directly to the sequencer cup. The sample

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*Details of peptide purification are in preparation. Amino acid analysis indicated 6 Gly, 1 Ala, 2 Val, 1 Pro, 1 Ile, 1 Thr, 1 Phe, 1 Arg.

TABLE I

SEQUENTIAL ANALYSES OF PEPTIDES*

Peptide Gly-Phe-Phe

STEP	1	2	3	4	5	6	7	8	9	10	11	12	13	14
	Gly	Phe	Phe											
Unreacted	76	1	0											
Modified	70	44	16[1]											

Peptide Gly-Val-Pro-Gly

	Gly	Val	Pro	Gly
Unreacted	80	5	0	0
Modified	90	44	16	8[1]

Peptide C16c

	Gly	Ala	Arg[2]	Gly	Gly	Val	Gly	Val	Gly	Gly	Leu	Pro	Thr	Phe[2]
Modified**	72	80	-	60	30	28	18	16	3	6	1.7	1.3	0.9	-

*Expressed as percent Recovery of PTH Amino Acids

**Not sequenced without modification

1. Determined by amino acid analyses of aqueous layer

2. Determined by subtraction

was dried using the Beckman sample application routine #02772 and the peptide program begun.

The results of the sequence analyses of peptides Gly-Gly-Phe, Gly-Val-Pro-Gly and C16C, which were determined by this procedure, are given in Table 1.

DISCUSSION

The effectiveness of the modification procedure is illustrated by comparing the yields obtained from the unreacted and reacted peptides Gly-Phe-Phe and Gly-Val-Pro-Gly. As seen in Table 1, the loss of unreacted peptide is very drastic and would not permit complete sequence analysis. In fact, the third sequencing step of both peptides showed a recovery of less than 0.1% of PTH-amino acids.

As stated in the introduction, we have successfully sequenced the peptide Gly-Val-Pro-Gly through attachment of aminomethanesulfonic acid to the C-terminal glycine residue (9). However, this reaction necessitated prior modification of the N-terminal amino group since coupling was performed at pH 9.5, which is above the pK of the α-amino group. Our present technique offers several advantages over the previously reported one. By performing the reaction at 4.0, the primary amino groups of the peptide (N-terminal and ε-amino groups) are in the pronated form while the aromatic amino group of ANS, which has a pK lower than 2.5, is essentially uncharged. Under these conditions, only the latter can serve as the nucleophile. The non-reactivity of the α-amino group is evident by the absence of polymerization in the sequencing of peptides Gly-Phe-Phe and Gly-Val-Pro-Gly. In both cases, the sequence analyses was carried out one step beyond the actual length of the peptide and no PTH-amino acid was detected.

In order to determine the C-terminal residues, the aqueous layer of the sequencer fraction had to be hydrolyzed. Apparently the amide linkage formed between the C-terminal carboxyl group and the reagent is not succeptible to cleavage by heptafluorobutyric acid under conditions present in the sequencer cup.

As previously reported by Braunitzer (11) napthalene derivatives enhance the film forming characteristics of peptides. Since the automated method of Edman and Begg depends on the quality of film in the cup, the strongly polar naphthalene derivative serves a dual purpose of increasing both the quality of film and the polarity of the peptide.

We have designed the method for application to small amounts of material ($<$ 500 nm). For that reason, we have purposely omitted rigorous procedure for removal of excess reagent which may result in peptide loss. It should be noted that the concentrations of reagents used present no interference with the normal sequencing procedure. If large quantities of sample are to be used, it may be necessary to separate the derivatized peptide from the reagent prior to application to the sequencer.

In conclusion, we feel that the technique reported will be a valuable tool for sequencing elastin derived peptides. A broader use of the method is anticipated because of its direct application to the classical principles of homogenous-phase sequencing (1). For example, sequencing of chymotryptic peptides is now possible regardless of peptide length or charge. Since the carbodiimide involves concomitant activation of the γ and β carboxyls of glutamic and aspartic acids, incorporation of ANS into these groups will further increase the hydrophilicity of the sample. Identification of the acidic amino acids can then be made by amino acid analyses after hydrolysis of the aqueous layer or by simple substraction depending on the size and composition of the peptide.

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