SELECTIVE REDUCTION OF PEPTIDE-ESTER GROUPS IN AQUEOUS SOLUTION IV

APPLICATION TO CARBOXYL-TERMINAL DETERMINATION OF PROTEINS

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SUMMARY When protein-ester prepared from protein with HCl-MeOH or triethyloxonium fluoroborate was reduced with sodium borohydride in aqueous solution followed by hydrolysis with 6N HCl, COOH-terminal amino acid was analyzed in good yield as the corresponding amino alcohol. COOH-Terminals of lysozyme, insulin and concanavalin A were analyzed without any appreciable side-reaction. The principal advantage of the reduction method is that Asn, difficult to be determined by usual chemical methods, was easily confirmed as β-amino-γ-butyrolactone.

There are three practical methods for the chemical determination of COOH-terminal amino acid in protein. Although hydrazinolysis is well-known as the most useful method, the determination of Asn or Gln as a COOH-terminal is quite difficult because of the instability of the resultant Asp-β-hydrazide or Glu-γ-hydrazide and of the difficulty of its separation from non-terminal Asp-α-hydrazide or Glu-α-hydrazide (1). The thiohydantoin method for the sequential degradation of peptide from its COOH-terminal is of interest, but apparently still gives trouble in general use (2). The selective tritium labelling by Matsuo has provided the most promising method; however even by this excellent method it is difficult to distinguish between Asn and Asp (3).

Another promising method, but not yet practical, is the reduction with metal hydrides. In many cases, $LiAlH_4$ or $LiBH_4$ have been used in anhydrous tetrahydrofuran, more or less accompanied by the simultanious reduction of peptide bonds. In spite of many attempts, the reaction conditions for the selective reduction of carboxyl or ester groups has still remained unestablished (4).

In previous papers (5), it has been shown that sodium borohydride ($NaBH_4$) in aqueous solution, better than in organic solvents, reduced selectively and almost quantitatively ester groups in some model peptides to the corresponding amino alcoholes, indicating the potential utility in protein chemistry. The present paper shows the first example of the application of the $NaBH_4$ reduction method for the COOH-terminal determination of proteins.

METHODS

Amino alcohol analysis The amino alcohol analyses were performed on a Hitachi KLA-3B Amino Acid Analyzer with the following three buffers: 1 = 0.35N sodium citrate, 0.2 % benzyl alcohol (pH 5.28); 2 = 0.35N sodium citrate, 20 % propanol (pH 5.28); 3 = 0.2N sodium citrate, 10 % propanol (pH 3.50).

Esterification of protein 1) with HCl-MeOH Proteins were esterified with 0.1N hydrogen chloride in anhydrous methanol (6) or 12N hydrochloric acid in methanol (1 : 100) (7). 2) with triethyloxonium fluoroborate $Et_3O^+ BF_4^-$ (Meerwein reagent; MR) A stirred aqueous or 8M urea solution of protein (5 - 50 mg, 0.1 - 1mM) was treated with the dropwise addition (1 hr) of large excess of MR (1 - 2M) in acetonitrile (8). During the reaction, the pH was kept at 4.5 or 7.0 by the continuous addition of 5N NaOH from an automatic titrator.

Reduction with $NaBH_4$ An 8M urea solution of an esterified protein (1 - 3 mg, 0.5mM) containing 1 drop of n-octanol and $NaBH_4$ (0.2M) was kept at 30° for 6 hr. After cooling in an ice-bath, the protein was precipitated from the reaction mixture with a solution of acetone and 1N HCl (39 : 1) and washed with this solvent (9). The reduced protein was hydrolyzed with 6N HCl and subjected to amino alcohol analysis.

RESULTS AND DISCUSSION

Analysis of amino alcohol

In previous papers (5), the reduction rate in model peptides was ana-

lyzed by two methods: trinitrophenylation for the resultant amino alcohol and gas chromatography for the recovered amino acids. However, the amino alcohols have now been analyzed in a manner similar to the method by Gallop et.al. (10) using an amino acid analyzer, which provides a more convenient method. In order to establish analytical conditions a mixture of authentic amino alcohols was first analyzed under the usual conditions for the basic amino acids (buffer 1). As shown in Table 1, a fairly good separation of aliphatic amino alcohols was obtained. For aromatic amino alcohols the second buffer system was applied. At lower pH (3.50), ethanolamine and

Table 1. Analyses of amino alcohols

Amino alcohol	Relative elution time (NH_3 = 1)			Relative color
	Buffer 1	Buffer 2	Buffer 3	yield (Leu = 1)
methioninol			0.50	
serinol	0.79		0.71	0.686
aspartidiol	0.79		0.71	0.218
ethanolamine	1.00		0.79	0.753
alaninol	1.09		0.84	0.100
homoserine-lactone	1.24			
β-amino-γ-butyrolactone	1.39		1.32	0.177
valinol	2.00		1.32	0.205
leucinol	3.42		1.87	0.366
isoleucinol	3.67		1.92	0.290
tyrosinol		2.08		0.608
phenylalaninol		2.37		0.578

alaninol were clearly separated from ammonia. With the above three conditions, most of the usual amino alcohols except those derived from basic amino acids can be analyzed.

COOH-Terminal analysis of lysozyme

As the first example of the application to COOH-terminal determination of protein, the reduction with $NaBH_4$ was applied to lysozyme, whose COOH-terminal amino acid is Leu. According to the procedure described by Donovan (7) lysozyme was esterified with 12N HCl in anhydrous MeOH to give its methyl ester, which was dissolved in 8M urea solution and treated with

excess $NaBH_4$ at 30°. After 6 hr, the reduced protein was precipitated by the addition of 1N HCl in acetone. The precipitate was hydrolyzed with 6N HCl, then analyzed for amino alcohols with the amino acid analyzer. Only leucinol was detected in 99 % yield (internal standard: Arg or His).

The treatment of lysozyme with MR in aqueous solution at pH 4.5 or 7.0 also gave its ethyl ester (8, 11), after the reduction of which leucinol was detected without side-reaction product, though in poor yield (Table 2). Esterification in 8M urea solution*1 gave improved results; especially with 2M of MR at pH 7.0 leucinol was detected in nearly quantitative yield as a single product. Guanidine-HCl can not be used as a denaturant because HCl reacts with the reagent.

Table 2. Yield of leucinol from the COOH-terminal of lysozyme

Solution	MR (mol/l)	pH	leucinol (%)
H_2O	1	4.5	10
	1	7.0	36
	2	4.5	49
8M urea	1	4.5	26
	1	7.0	76
	2	4.5	70
	2	7.0	96

Bovine insulin

The COOH-terminals in esterified insulin (6) were analyzed in a manner similar to that described above. Both alaninol (from B-chain) and β-amino-γ-butyrolactone (from Asn of A-chain) were detected in 88 % yield. This result indicates that Asn as well as Ala was easily analyzed without any detectable side-reaction.

Concanavalin A

The COOH-terminal (Asn) of concanavalin A from jack bean meal has been determined by digestion with carboxypeptidase A (12) and recently X-ray analysis gave unequivocal proof of its presence (13). The presence of Asn has now been confirmed by the $NaBH_4$ reduction method. Esterification with

*1 Urea did not affect the rate of hydrolytic decomposition of MR (at pH 6.1, in H_2O, $k = 0.135\ min^{-1}$; in 8M urea, $k = 0.094\ min^{-1}$).

12N HCl in methanol caused undesirable hydrolysis, i.e., besides quantitative yield of β-amino-γ-butyrolactone, alaninol and aspartidiol were detected in amounts (over 30 %) corresponding to the presence of substantial quantities of chains terminating in these amino acids. However esterification with MR in 8M urea gave much better result. After the reduction β-amino-γ-butyrolactone was analyzed in 89 % yield with only a few % of alaninol and aspartidiol*2.

The results presented here indicate that through the esterification with MR followed by the reduction with $NaBH_4$ in aqueous solution COOH-terminal amino acid of protein can be detected as the corresponding amino alcohol in good yield without any appreciable side-reaction, and this simple method may provide a practical method for COOH-terminal determination of protein, especially with Asn. Studies on the establishment of analytical methods for all of the amino alcohols derived from the usual amino acids and on the further application of this method for COOH-terminal determination of protein are still in progress.

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*2 The yields were corrected for the composition (α : β = 5 : 6) of concanavalin A analyzed by gel filtration (12).

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