ENHANCEMENT OF THE SOLUBILITY OF S-ALKYLATED PROTEINS BY CARBOXYL GROUP MODIFICATION

Á.FURKA and F.SEBESTYÉN

Department of Organic Chemistry, Eötvös Loránd University, Budapest, Hungary

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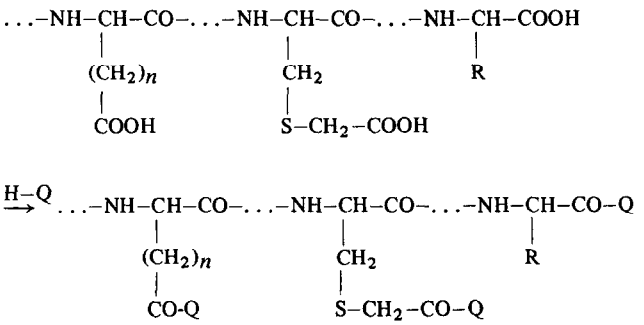
1. Introduction

Reduced and S-carboxymethylated or reduced and S-aminoethylated derivatives of proteins are important starting materials in sequence studies. Their water solubility – like that of other denatured proteins – is generally much lower than that of native proteins. This low solubility causes difficulties when further fractionation or purification is necessary (i.e. the reduction or denaturation leads to formation of individual polypeptide chains). It is often found that the alkylated proteins dissolve at extreme pH values, when the net electric charge (positive or negative) of the molecules is maximal. At low pH values, for example, the dissociation of the carboxyl groups is repressed, the net electric charge being practically equal to the number of protonated basic groups. High net electric charge – and, as a consequence, better solubility – is expected to be maintained over a broader pH range, if the proteins contain only one type of ionizable groups: either acidic or basic.

2. Methods and materials

In order to enhance solubility, two types of modifictation of different S-alkylated or other denatured proteins were applied: (1) Neutralization of acidic carboxyl groups (by coupling with methyl amine). (2) Transformation of acidic carboxyl groups into basic ones (by coupling with either L-arginine methyl ester or dimethylamino ethylamine).



(Q = –NH–CH_3, –ArgOCH_3 or –NH–CH_2–CH_2–N$(CH_3)_2$ in modifications with methyl amine, arginine methyl ester or dimethylamino ethylamine, resp., Asp: n = 1, Glu: n = 2).

Modifications were carried out in 6-molar guanidine hydrochloride at pH 4.8 by means of water-soluble carbodiimide – a method described by Hoare and Koshland [1]. To avoid reaction with free amino groups of the protein, the amino component was applied in large excess. The conversion of the free carboxyl groups to amides, at least in the case of arginine methyl ester modification, was estimated as 80 to 100%. Table 1 shows a very close correspondance between the quantity of the incorporated arginine and the number of free carboxyl groups.

The solubilities of reduced and aminoethylated ovalbumin and that of its three modified derivatives were compared as a function of pH (see fig. 1, and for details experimental part). Fig. 1 shows that, while aminoethyl ovalbumin dissolved only below pH 3 or

Table 1
Arginine methyl ester incorporation (mole/mole protein), calculated from amino acid analysis.

Protein	Arg found	COOH calculated	Arg incorporated
AE-Ovalbumin	15.8	41–46[a]	
Modified AE-Ovalbumin	58.7		42.9
CM-CHT-B, B-chain	0.9	21[b]	
Modified CM–CHT–B, B-chain	23.7		22.8
CM–CHT–A, B-chain	1.2	15[c]	
Modified CM–CHT–A, B-chain	12.6		11.4

Abbreviations: AE = aminoethyl; CM = carboxymethyl; CM–CHT = carboxymethyl-chymotrypsin; a: calculated from found aspartic and glutamic acid content, with the number of amide groups reported in the literature [2,3] taken into account; b and c: number of free carboxyl groups based on known sequences [4] and [5], respectively.

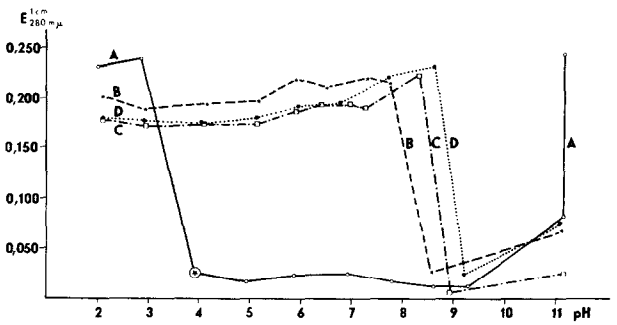


Fig. 1. Solubility of aminoethyl ovalbumin (A) and its derivatives (modified with: methyl amine (B), arginine methyl ester (C) and dimethylamino ethylamine (D), resp.) indicated by optical density of the solution at different pH values. Measurement of o.d. at point ⊙ is uncertain because of the cloudiness of the solution.

Table 2
List of protein modifications.

	Reagents		
	MeA	AME	DMAEA
AE–Ovalbumin	+	+	+
AE–CHT–A	+	+	+
CM–CHT–A		+	
CM–CHT–A, B-chain		+	
CM–CHT–A, C-chain	+		
Denatured CHT–B			+
AE-CHT-B			+
CM–CHT–B, B-chain		+	
CM-CHT-B, C-chain		+	

MeA = methyl amine; AME = arginine methyl ester; DMAEA = dimethylamino-ethylamine. Also see abbreviations of table 1.

above pH 11, each modified derivative became soluble at any pH value below 8. Even the elimination of the acidic groups, i.e. their coupling with methyl amine, improved the solubility, although the properties of the other two derivatives were found to be better.

Eight additional protein derivatives (see table 2) were modified with good results in improving their solubility in neutral and acidic regions. Most of the products were submitted to gel filtration on Sephadex columns using 0.2 N acetic acid as eluant. Good fractionations and almost quantitative recoveries were obtained.

Thus, it is concluded that modifications described above extend the solubility of the alkylated proteins to a much broader pH range and make separations possible under mild conditions.

3. Experimental part

3.1. *Materials*

Ovalbumin was prepared [6] from chicken eggs and six times crystallized. Chymotrypsin-A and chymotrypsin-B were commercial products (Fluka AG and Wilson Laboratories, Inc., Chicago, resp.). Proteins were reduced by β-mercapto-ethanol, aminoethylated [7] or carboxymethylated [8]. B- and C-chains of CM-chymotrypsin-A and -B were separated

according to Hartley [5] and Parkes and Smillie [9], respectively. Chymotrypsin-B was denatured in 6-molar guanidine-HCl. L-arginine methyl ester dihydrochloride was synthetized according to Boissonnas et al. [10]. Methyl amine hydrochloride, 2-dimethylamino ethylamine, guanidine hydrochloride and N-cyclohexyl-N'-(2-(4-β-morpholinyl)-ethyl)-carbodiimide methyl-*p*-toluene sulphonate (CMCI) were Fluka AG reagents.

3.2. *Coupling reaction*

In a typical experiment 100 mM amine component (6.75 g methyl amine hydrochloride, or 26.12 g L-arginine methyl ester dihydrochloride, or 11.9 ml 2-dimethylamino ethylamine) was dissolved in 80 ml 7.5 molar guanidine hydrochloride and the pH of the solution was adjusted to 4.5. Then 0.022 mM (1 g) aminoethyl ovalbumin, and – with stirring – 10 mM (4.23 g) CMCI were dissolved. The pH was adjusted to 4.8 and left at 27° for 5 hr. The pH was then adjusted to 3 and the excess of the reagents was removed by either dialysis at 4° against 5 changes of 4 l 10^{-3} N HCl, or gel filtration on Sephadex G 25.

3.3. *Amino acid analysis*

Samples were hydrolyzed in 6 N HCl at 105° for 20 hr, then analysed on an Evans type analyzer.

3.4. *Comparison of solubilities*

A 100 mg sample was dissolved in 30 ml 0.01 N hydrochloric acid, then the pH of the solution was gradually raised to 11 by the addition of a concentrated solution of sodium hydroxide. After every pH adjustment the solution was left overnight in the refrigerator, centrifuged, then the pH of the clear supernatant and the optical density (with Beckman DU spectrophotometer) were measured. Before each pH adjustment the supernatant and the precipitate were combined. Optical densities were plotted against the pH values of the solution (fig. 1).

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