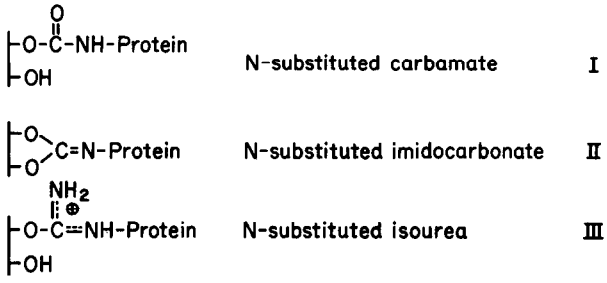
USE OF ISOELECTRIC FOCUSING TO CHARACTERIZE THE BONDS ESTABLISHED DURING COUPLING OF CNBr-ACTIVATED AMYLODEXTRIN TO SUBTILISIN TYPE NOVO

Birte SVENSSON
Carlsberg Laboratory, Chemical Department, DK 2500 Copenhagen, Denmark

Received 21 November 1973

1. Introduction

Proteins are easily bound to water-insoluble polysaccharides treated with cyanogen halides at alkaline conditions [1, 2]. This method of immobilization has been widely applied, but the nature of the covalent bonds between protein and carbohydrate in the coupled product has not been fully determined. Axén et al. [1] suggested that protein amino groups reacted with activated polysaccharide with formation of esters of N-substituted carbamic and N-substituted imidocarbonic acids. Subsequent model experiments have shown that in addition considerable amounts of N-substituted isourea groups can be formed [3, 4]. These three types of derivative are listed below in the forms expected to prevail in neutral aqueous solutions:



Additional evidence supporting that the isourea group is a major reaction product is given in the present report.

The approach taken was to evaluate any change in protein net charge resulting from the modification. Conversion of lysyl residues to N-alkyl carbamates, I, is likely to give a loss of positive charges on the protein surface in the pH region from ~10 to ~0. In order to get an estimate of the base strength of products of type II, the ionization constant of ethyl isobutyl imidocarbonate, which was considered to be an acceptable analog, was determined. The pK_a, found to be 4.7, suggested that imidocarbonates are much weaker bases than the protein amino groups. Substituents of type II would thus cause a decrease in the number of protonated groups in a pH interval from ~10 to ~6. Finally, the formation of isourea groups, III, would essentially be without influence on the protein charge pattern since the dissociation constants of isoureas [5, 6] and protein amino groups are of the same order of magnitude.

The types of derivative that might result from the cyanogen bromide coupling procedure can thus be distinguished by comparison of the pH dependence of the ionization pattern of a protein before and after its attachment to CNBr-activated carbohydrate. With this purpose amylodextrin-subtilisin Novo, a water-soluble enzyme derivative prepared by reaction with CNBr-activated amylodextrin, was subjected to isoelectric focusing. Ottesen and Ralston [7] has previously demonstrated that subtilisin Novo derivatives with different ionization pattern, due to carbamylation of amino groups, could be separated by this procedure.

2. Materials and methods

Amylodextrin consisting mainly of maltotetraose, maltopentaose and maltohexaose (a gift from Dr. B.S. Enevoldsen, the Research Laboratory of Tuborg Brew-

eries Ltd., Copenhagen) was activated with CNBr (Aldrich Chem. Co.), isolated by precipitation with acetone, and finally coupled to subtilisin type Novo (batch 120-2, crystallized and lyophilized, gift from the Novo Industries, Copenhagen) as described previously [8]. Chemical analyses [8] showed that the resulting amylodextrin-subtilisin had on the average been substituted at ~3 lysyl residues and contained 20 glucose equivalents per molecule.

Prior to electrofocusing the enzyme was inhibited with phenyl-methanesulfonyl fluoride, PMSF, (Cyclo Chem. Co.) [7]. However, this treatment does not inactivate the enzyme completely, and products of partial autolysis might, therefore, be responsible for the heterogeneity observed when using a rather shallow pH gradient for electrofocusing.

Isoelectric focusing was conducted with an LKB Ampholine column (110 ml), type 1801, essentially as reported by Vesterberg and Svensson [9]. Solutions (1%) of Ampholines covering the two pH ranges 7–10 and 3–10 were used. Experiments were performed at 4° ± 0.1° for 48 hr and at a maximum of 600 V (pH 7–10) or 300 V (pH 3–10). The column content was pumped out and its transmission registered at 280 nm (LKB 8303A Uvicord absorptiometer).

Ethyl isobutyl imidocarbonate was synthesized, in analogy to diethyl imidocarbonate, from isobutyl cyanate (a gift from Dr. E. Huge-Jensen, the Chemical Laboratory II, University of Copenhagen) [10]. It distilled at 48–50° and 10 mm Hg; IR and NMR spectroscopy showed the expected bands. The dissociation constant of the conjugate acid of ethyl isobutyl imidocarbonate was determined from the inflection point of the potentiometric titration curve obtained with 0.15 M aqueous solution. The compound hydrolyzed at weak acidic conditions (like the reactive groups in CNBr-treated Sephadex [4]) but the decomposition was slow at the low temperature (4°) and did not disturb the measurements.

Potentiometric titrations and pH determinations were conducted under N_2 at 4° ± 0.1° using a Radiometer pH meter, type 25, equipped with Radiometer G202 C glass electrode and type K100 calomel electrode.

3. Results and discussion

By isoelectric focusing of PMSF-inhibited subtilisin Novo in the pH range 7–10 a main peak corresponding to an isoelectric point of 8.4 was found (fig. 1A) in accordance with the result of Ottesen and Ralston [7]. The main fraction of amylodextrin-PMS-subtilisin was similarly focused in a zone of isoelectric point 8.4 (fig. 1B). In addition several minor bands were located at lower pH values. To eliminate the possibility that a significant fraction of the amylodextrin-PMS-subtilisin preparation was of even lower isoelectric point, electrofocusing was also carried out in the pH interval 3–10. It confirmed that only negligible quantities had an isoelectric point below pH 6.5. In spite of the heterogeneity, the results suggest that reaction with

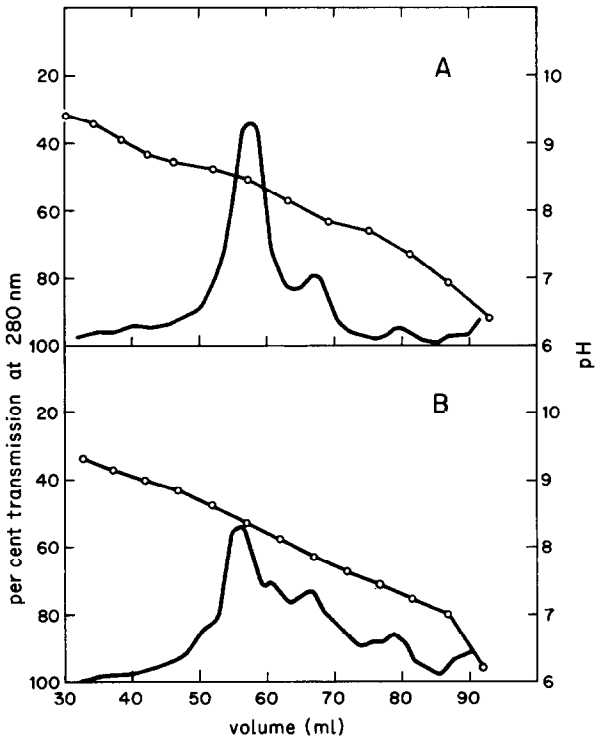


Fig. 1. Isoelectric focusing of PMSF-inhibited subtilisin type Novo (A) and PMSF-inhibited amylodextrin-subtilisin type Novo (B). Percentage transmission of the effluent at 280 nm: ———— (left ordinate); pH: o–o–o (right ordinate).

CNBr-activated amylodextrin does not alter the net charge of the protein markedly.

Subtilisin type Novo in which presumably three ϵ-amino groups plus the N-terminus were carbamylated displayed an isoelectric point of 6.3 [7]. Although the amylodextrin-subtilisin preparation likewise contained an average of ~3 substituted lysines per molecule, it was not concentrated around pH 6.3 by electrofocusing, but remained at the more alkaline value. The data obtained by isoelectric focusing of carbamylated subtilisin Novo [7] allow only a single derivatized amino group to be unprotonated in species that are isoelectric above pH 7.7. From fig. 1B it is thus seen that the majority of the modified residues in amylodextrin-subtilisin cannot be of essentially lower basicity than the original amino groups. Of the suggested coupling products, I–III, only the isourea type, III, can fulfill this requirement. In agreement with this conclusion Kågedal and Åkerström [11] have reported that no significant loss of nitrogen takes place when glycine reacts with CNBr-activated dextran, and Axén et al. [12] have found in Sephadex-glycylleucine a group of pK_a 10.2 which could account for 95% of the coupled dipeptide.

Acknowledgements

I am indebted to Professor M. Ottesen and Dr. G. Ralston for their helpful advice and criticism. This work was supported by a grant from the Danish "Statens naturvidenskabelige Forskningsråd".

References

[1] R. Axén, J. Porath and S. Ernback, Nature 214 (1967) 1302.
[2] J. Porath, R. Axén and S. Ernback, Nature 215 (1967) 1491.
[3] R. Axén and S. Ernback, European J. Biochem. 18 (1971) 351.
[4] R. Axén and P. Vretblad, Acta Chem. Scand. 25 (1971) 2711.
[5] M. Zief and J.T. Edsall, J. Am. Chem. Soc. 59 (1937) 2245.
[6] S. Basterfield and J.W. Tomecko, Can. J. Research 8 (1933) 458.
[7] M. Ottesen and G. Ralston, Compt. Rend. Trav. Lab. Carlsberg 38 (1972) 457.
[8] B. Svensson, Compt. Rend. Trav. Lab. Carlsberg, in press.
[9] O. Vesterberg and H. Svensson, Acta Chem. Scand. 20 (1966) 820.
[10] K.A. Jensen, M. Due, A. Holm and C. Wentrup, Acta Chem. Scand. 20 (1966) 2091.
[11] L. Kågedal and S. Åkerström, Acta Chem. Scand. 25 (1971) 1855.
[12] R. Axén, P.-Å. Myrin and J.-C. Janson, Biopolymers 9 (1970) 401.