INFLUENCE OF THE SUBSTITUTION OF TRYPTOPHAN 215 IN BOVINE CHYMOTRYPSINOGEN A ON ITS POTENTIAL ENZYMIC ACTIVITY

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1. Introduction

Large similarities exist in the primary and three-dimensional structures and in the enzymic properties of chymotrypsinogen and trypsinogen which both belong to the serine-proteases family. Recently we have shown that the specific substitution of tryptophan-199 in bovine trypsinogen by Koshland's reagent (2-hydroxy-5-nitrobenzyl bromide) resulted in the complete loss of both its potential enzymic activity and of its ability to bind the basic pancreatic trypsin inhibitor [1]. In the three-dimensional model of trypsin trytophan-199 is within 6 Å of the active centre. These results prompted us to investigate the role of tryptophan residues in the potential function of chymotrypsinogen.

In contrast to trypsinogen which contains four tryptophan residues, there are eight tryptophans in chymotrypsinogen. In an earlier study Delaage et al. [2] have shown that at pH 7 and 10° none of these tryptophan residues reacts significantly with Koshland's reagent. Spande et al. [3] have found that at neutral pH only one tryptophan residue was oxidized with *N*-bromosuccinimide whereas at acidic pH all the tryptophans of chymotrypsinogen A were modified. These authors also investigated the effect of buffer concentration and temperature on the extent of oxidation of tryptophan with *N*-bromosuccinimide. However, in none of these works were the substituted tryptophans identified.

Two chemically closely related reagents have been recommended for the specific substitution of tryptophans in the proteins: an acetone solution of 2-hydroxy-5-nitrobenzyl-bromide* [4] and the water soluble dimethyl (2-hydroxy-5-nitrobenzyl) sulphonium bromide [5]. The advantage of the latter is that it is water soluble and hence the risk of denaturation of the protein during substitution is diminished.

In this work we have compared the specificity of substitution of bovine chymotryosinogen A by these two reagents and the resultant functional changes in this protein.

2. Materials and methods

Chymotrypsinogen (lot CGC) was obtained from Worthington, subtilopeptidase A type VIII was the crystalline lyophilized product of Sigma. 2-Hydroxy-5-nitrobenzyl bromide and dimethylsulphide were purchased from Fluka. Acetyl-tyrosine-ethylester was obtained from Calbiochem. Sephadex derivatives were the products of Pharmacia. Cellulose powder Whatman CC 31 was bought from W and R Balston Ltd. Silica-gel TLC-Ready Plastic Sheets F 1500 LS 254 were obtained from Schleicher and Schüll. Dimethyl-(2-hydroxy-5-nitrobenzyl) sulphonium bromide was synthesized according to Horton and Tucker [5].

2.1. *Substitution*

The reaction of chymotrypsinogen with HNB-bromide was performed at 22° with 225 mg of chymo-

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* *Abbreviations:*
HNB, 2-hydroxy-5-nitrobenzyl.

trypsinogen dissolved in 15 ml of 0.001 N HCl. HNB-bromide (400 mg) dissolved in acetone (0.3 ml) was added in small portions. The pH of the solution was maintained at 3.1 N NaOH. After 10 min the reaction mixture was centrifuged and desalted on a Sephadex G-25 column (2.5 × 85 cm) which had been equilibrated with 0.02 M $(NH_4)_2CO_3$, pH 8.5. The first peak which contained the modified protein was lyophilized.

For the reaction of chymotrypsinogen with dimethyl-(2-hydroxy-5-nitrobenzyl) sulphonium bromide 250 mg of chymotrypsinogen were dissolved in 25 ml of 0.001 N HCl and pH of the solution adjusted with 0.1 N NaOH to pH 4.0. Dimethyl-(2-hydroxy-5-nitrobenzyl) sulphonium bromide powder (400 mg) was added in small portions over a period of 60 min at 20°. The substituted protein was separated from the products of the reaction and lyophilized described above. The modified proteins resulting from the two different methods of substitution were readily soluble in water.

2.2. *Isolation and analysis of substituted peptides from subtilisin digests*

The disulphide bridges of substituted proteins were cleaved by oxidative sulphitolysis according to Pechere et al. [7]. The desalting of the reduced, sulphitolyzed protein was performed on a Sephadex G-25 column (2.5 × 85 cm) equilibrated with 1% NH_4OH.

Substituted chymotrypsinogen was digested with subtilisin at pH 8.8 for 3 hr. The enzyme:substrate ratio was 1:100 (w:w) and the enzyme was added in two aliquots. The digestion was stopped after 3 hr and the products were freeze dried.

For the purification of HNB-peptides the same procedures were used in the cases of both hydrolysates. After a first separation on a Sephadex G-25 column (2.5 × 85 cm) the fractions which contained yellow HNB-peptides were further purified by gel filtration on a Sephadex G-10 column (2 × 65 cm). The successive separation of substituted peptides from the other ninhydrin positive fragments was achieved on a cellulose powder column (1 × 10 cm) which was equilibrated with a solvent composed of 3-methyl-butan-1-ol–pyridine–water (7:7:6). HNB-peptides were further purified by paper electrophoresis at pH 2.4 in 2 M acetic acid at 750 V, 0.3 mA/cm for 165 min on Whatman no. 1 paper. The substituted peptides were revealed as yellow bands after exposure of the dried paper to NH_3 and then were eluted with 0.05 N NH_4OH containing 5% ethanol. When necessary HNB-peptides were additionally purified by thin-layer chromatography on silica-gel in the solvent system 2-methylbutan-2-ol:butan-2-one:acetone:methanol:water:NH_4OH (25:10:5:8:8:8). The yellow bands were scraped from the plates and then the silica-gel powder was eluted in small columns (0.5 × 1 cm) with 0.05 N NH_4OH contained 5% ethanol.

Quantitative determination of HNB-groups in peptides was performed according to the method of Barman and Koshland [6]. All corresponding protein and peptide concentrations were determined by amino acid analysis of acid hydrolysates.

2.3. *Activation assay*

Activation of chymotrypsinogen and substituted chymotrypsinogens with trypsin was carried out simultaneously and under the same conditions. The concentration of chymotrypsinogen was 40 mg/ml and that of trypsin 1.2 mg/ml. The proteins were incubated at 0° in 0.05 M Tris-hydrochloride buffer, pH 7.8 which was 0.02 M in calcium chloride. Samples were taken at intervals over a period of 1 hr and the extent of activation determined by estimation of esterase activity with acetyl-tyrosyl-ethyl ester as substrate [8].

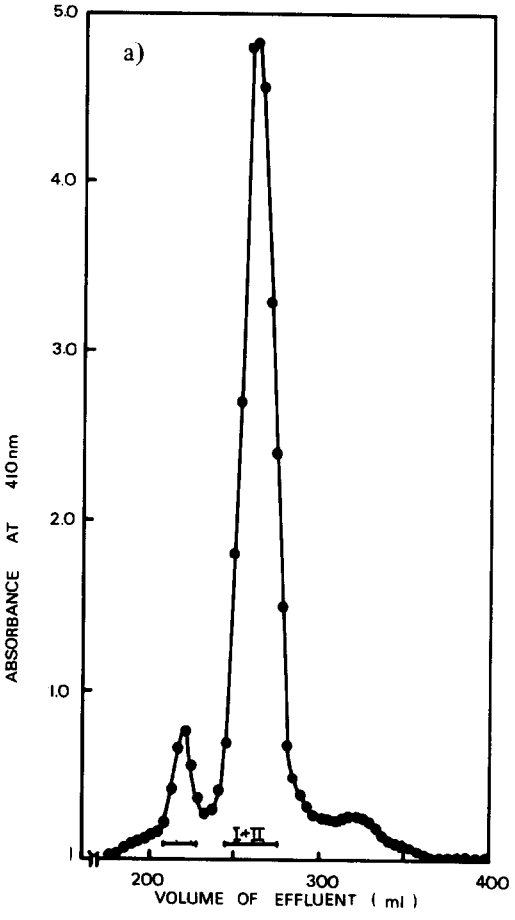
3. Results

3.1. *Attempted activation of substituted chymotrypsinogen*

After incubation with trypsin non-substituted chymotrypsinogen had an esterase activity of 233 μeq/min/mg. In contrast no esterase activity was detected in the case of the HNB-derivatives prepared by two different methods after similar treatment with trypsin.

3.2. *Isolation of HNB-peptides from chymotrypsinogen modified with dimethyl (2-hydroxy-5-nitrobenzyl) sulphonium bromide*

A subtilisin digest of substituted and S-sulphonated chymotrysinogen was chromatographed on a Sephadex G-25 column. Two peaks with absorbance at 410 nm (fig. 1a) were pooled and lyophilized. The



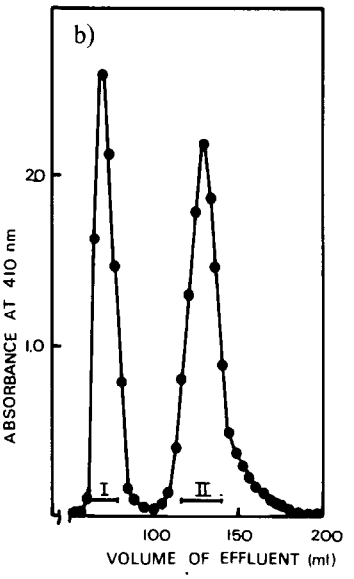
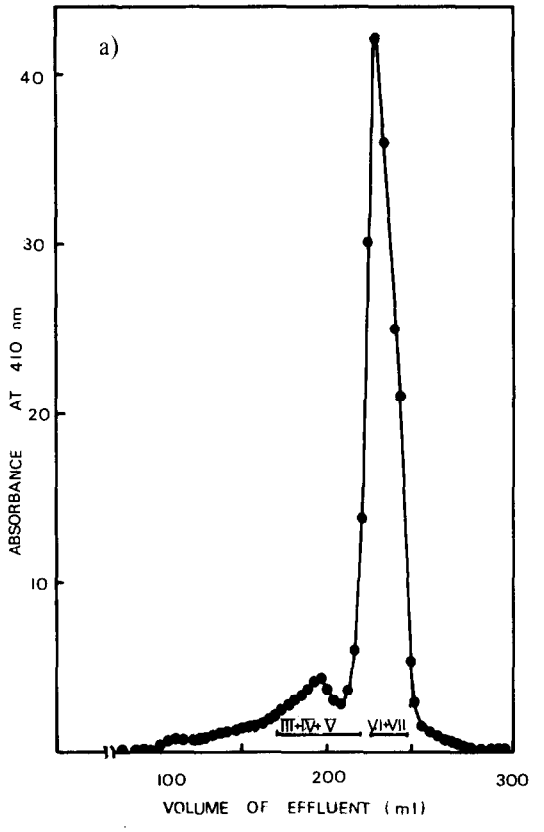
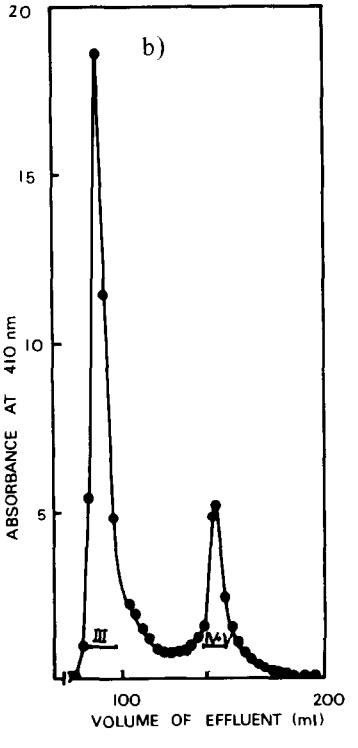


Fig. 1. Isolation of HNB-peptides from the subtilisin digest of chymotrypsinogen modified with dimethyl-(2-hydroxy-5-nitrobenzyl) sulphonium bromide. a) The column of Sephadex G-25 (2.5 × 85 cm) was equilibrated with 0.1 N ammonia + 50 ml/l ethanol at room temp. The flow rate was 16 ml per hr; 4 ml fractions were collected. The bar indicates the fractions containing the HNB-substituted products that were pooled. b) Purification of fraction I + II on Sephadex G-10. The column (2 × 65 cm) was equilibrated with ethanol–ammonia–water (50:10:940 by volume). The flow rate was 12 ml per hr; 4 ml fractions were collected.

Table 1
The amino acid composition and content of HNB-groups of substituted peptides.

Amino acid	Dimethyl-HNB-sulphonium bromide-substituted chymotrypsinogen		HNB-substituted chymotrypsinogen				
	I	II	III	IV	V	VI	VII
Aspartic acid			0.97	2.0	1.0		
Threonine			0.91	1.35			
Serine	1.9	2.0		1.0		1.8	1.2
Glutamic acid				1.07	1.75		
Glycine	1.0	1.0	1.10	1.28		1.0	0.98
Alanine				0.86			
Valine		0.75	0.94	2.08	2.0	0.70	0.83
Isoleucine				0.79			
Leucine				0.79			
HNB-groups	2.0	1.0	2.0	2.0	2.0	2.0	2.0
Sequence	214–217	213–217	204–208	44–55	236–240	213–217	213–216





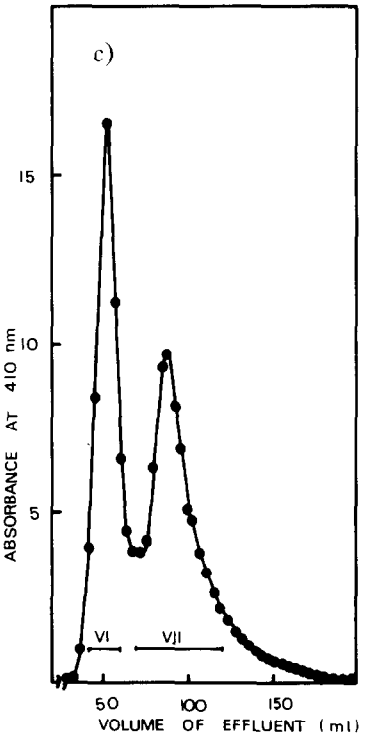


Fig. 2. Isolation of HNB-peptides from the subtilisin digest of chymotrypsinogen modified with 2-hydroxy-5-nitrobenzyl bromide. a) Fractionation of subtilisin digest on Sephadex G-25. The key for the figure is as in fig. 1a. b) Purification of the first minor peak of fig. 2a on Sephadex G-10 column. The conditions are the same as in fig. 1b. c) Fraction of the main peak of fig. 2a on the Sephadex G-10 column (2 × 65 cm). The key for the figure is as in fig. 1b.

Table 2
Yields* of substituted tryptophan residues in chymotrypsinogen A.

Trp no.	HNB-bromide	Dimethyl-HNB sulphonium bromide
26	–	–
28	–	–
51	4.6	–
141	–	–
172	–	–
207	12.5	–
215	33.0	34.0
237	7.5	–

* Based on the amount of substituted S-sulphonated chymotrypsinogen taken for chymotryptic hydrolyses.

first peak was of insufficient quantity for further purification.

The mixture of peptides from the second peak was purified further by gel filtration on Sephadex G-10 (fig. 1b). Two peaks containing the substituted peptides were obtained. Each of these peaks was freed from non-substituted peptides by chromatography on a cellulose-powder column followed by electrophoresis and thin-layer chromatography on silica-gel. Only one HNB-peptide was isolated from fraction 1. Its amino acid composition (table 1) corresponds to the sequence 214–217 contaning the tryptophan residue 215. Two HNB-groups were found per mole of peptide I. A second bisubstituted peptide was isolated from fraction II (fig. 1b). This peptide (213–217) corresponded to the same region of chymotrypsinogen molecule (table 1).

3.3. *Isolation of HNB-peptides from chymotrypsinogen modified with 2-hydroxy-5-nitrobenzyl bromide*

The first fractionation of the subtilisin digest of S-sulphonated HNB-chymotrypsinogen was performed by chromatography on a Sephadex G-25 column (fig. 2a). The first peak was further separated on Sephadex G-10 into two fractions which contained the substituted peptides (III, IV + V) (fig. 2b). Each of these was purified by chromatography on a cellulose powder column, followed by electrophoresis and by thin-layer chromatography on silica-gel. The amino acid composition (table 1) chromatography on silica-gel. The amino acid composition (table 1) shows that the first peak (III) corresponds to the sequence 204–208 in which tryptophan-207 was bisubstituted. Further separation of the mixture in the second peak (fig. 2b) by electrophoresis and chromatography on silica-gel gave two HNB-peptides (IV, V). The amino acid composition of peptide IV corresponds to the residues 44–55 (yield 4.6%); peptide V represents residues 236–240 (yield 7.5%).

The main fraction obtained after separation of the digest on Sephadex G-25 (fig. 2a) was further separated into two subfractions by gel filtration on Sephadex G-10 (fig. 2c). After their subsequent purification by methods described previously two further homogeneous peptides (VI and VII) were obtained. Both of them were bisubstituted and their amino acid composition (table 1) corresponds to the fragments 213–217 and 213–216 which contain tryptophan-125. The total recovery of the peptides containing tryptophan-125 was 33%.

4. Discussion

The reaction of 2-hydroxy-5-nitrobenzyl bromide and its water-soluble dimethyl sulphonium derivative with proteins is very selective under acidic conditions [9]. The substitution occurs only on certain tryptophan residues providing that the protein is in the native form [1, 10]. On the other hand the substitution of all tryptophan residues occurs when the same proteins are denatured [6].

The study of the substitution of native bovine chymotrypsinogen A with the two reagents gave us an opportunity to compare their relative specificities. Out of the eight tryptopnan residues in the chymotrypsinogen molecule only one tryptophan moiety, residue 215, was found to be substituted with the water-soluble dimethyl-HNB sulphonium bromide. When HNB-bromide in an acetone solution was used for the substitution the results were less unambiguous; in addition to the modified tryptophan-215 which was obtained in the highest yield (33%), some other tryptophan residues were also modified: tryptophan-51 (4.6%), 207 (12.5%) and 237 (7.5%). It seems likely that the presence of acetone in this case caused a partial unfolding of the chymotrypsinogen molecule.

All modified tryptophans in both hydrolysates

were found to be bisubstituted. In our study a twenty fold molar excess of the reagents to protein tryptophan was used and under these conditions bisubstituted tryptophan residues could be expected.

The substitution of tryptophan residue 215 in chymotrypsinogen is accompanied by complete loss of its potential enzymic activity. When the zymogen was modified with 2-hydroxy-5-nitrobenzyl bromide in acetone we could attribute this functional change to the partial unfolding of chymotrypsinogen. This explanation can hardly be forwarded in the other case when the zymogen was substituted with the water-soluble reagent. The fact that only one tryptophan residue 215, was found to be modified is a strong indication that the tertiary structure of the zymogen remained intact during the substitution reaction. The analogy between the primary and tertiary structures of trypsinogen and chymotrypsinogen is well known. These analogies are especially pronounced in the three-dimensional arrangement of the residues essential for the potential enzymatic activity.

Recently we have shown that in trypsinogen the substitution of tryptophan-199 with HNB-bromide resulted in the complete loss of the potential enzymic activity [1]. A similar effect is now observed for chymotrypsinogen as a result of the selective substitution of tryptophan-215.

Both tryptophan residues, 119 in trypsinogen and 215 in chymotrypsinogen are found in analogous positions in the primary structures of the enzymes.

Gln–Gly–Ile–Val–Ser–Trp–Gly–Ser–Gly
(trypsinogen)

Val –Gly–Ile–Val–Ser–Trp–Gly–Ser–Ser
(chymotrypsinogen)

Also the three-dimensional arrangements of the loops containing these sequences are similar and are close to the potential hydrolytic sites of the two zymogens [11, 12]. The importance of this region in active α-chymotrypsin for the formation of a specific β-structure with the substrate has been stressed by Wilcox et al. [13]. Our results report the view that the function of this loop in both zymogens may be similar.

The question remains open as to why the tryptophan-215 reacts so readily with the Koshland's reagent in view of the fact that it is not the only one of the eight tryptophan residues which are situated on the surface of the molecule. The preferential reactivity of a specific residue with the Koshland's reagent, an aromatic substance, may reflect the overall favourable disposition of this region of the molecule to function as the binding site since chymotrypsin has a strong preference for aromatic substrates. It is not possible at present to decide whether the ultimate effect of the substitution on the indole ring of tryptophan-215 is to block directly part of the functional binding site or if it only sterically hinders the approach of the substrate to the main binding site.

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