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Isolation, characterization and *N*-terminal sequences of the CNBr-cleavage peptides from human complement Factor B

Localization of a free thiol group and a sequence defining the site cleaved by Factor $\bar{D}$

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Nine CNBr-cleavage peptides from Factor B (a component of the alternative pathway of complement) were isolated. Each was characterized by amino acid analysis and automated Edman degradation. One peptide contained a methionyl bond resistant to cleavage by CNBr. The number of CNBr-cleavage peptides is in agreement with the results of amino acid analysis of Factor B and the fragments Ba and Bb. A total of 358 unique residues were identified from the *N*-terminal sequences of the CNBr-cleavage peptides. These represent approx. 50% and 60% of the total residues of Factor B and fragment Bb respectively. Alignment of two CNBr-cleavage peptides (CB-VIc and CB-IV) provided a continuous segment of 140 residues. This sequence contained the site cleaved by Factor $\bar{D}$ to generate the Ba and Bb fragments during the activation of complement. Peptide CB-IV contained a free thiol group at a position corresponding to residue 33 of fragment Bb. Amino sugar analyses of Factor B and of fragments Bb and Ba indicated that all the carbohydrate structures of Factor B are *N*-linked to asparagine through *N*-acetylglucosamine. The two carbohydrate-attachment sites of the Bb fragment were identified.

Factor B is the zymogen of a proteinase, essential for the activation of complement by the alternative pathway. It is a serum glycoprotein, consisting of a single polypeptide chain with a molecular weight of approx. 90000 (Götze, 1975; Curman *et al.*, 1977). Factor B interacts reversibly with complement component C3b in the presence of Mg^{2+} and is activated by the complement proteinase Factor $\bar{D}$. A single Arg–Lys bond is cleaved, forming an *N*-terminal Ba fragment and a *C*-terminal Bb fragment with molecular weights of approx. 30000 and 60000 respectively (Kerr, 1979; Lesavre *et al.*, 1979; Niemann *et al.*, 1980). The Bb fragment in association with component C3b forms the complex proteinases $\overline{C3}$ convertase ($C3b\bar{B}\bar{b}$) and C5 convertase $(C3b)_n\bar{B}b$ (for reviews see Fothergill & Anderson, 1978; Porter, 1979; Müller-Eberhard & Schreiber, 1980; Reid & Porter, 1981).

Abbreviations used: h.p.l.c., high-pressure liquid chromatography; SDS, sodium dodecyl sulphate; Tos-Phe-CH_2Cl ('TPCK'), 1-chloro-4-phenyl-3-L-tosylamidobutan-2-one. The nomenclature of complement components is that recommended by the World Health Organisation (1968).

Medicus *et al.* (1976) showed that C3 convertase and C5 convertase of both pathways of activation are inhibited by di-isopropyl phosphorofluoridate, and that, if radioactive reagent was used, it is located in the Bb fragment, indicating that Factor B is a serine proteinase with its active site in the Bb portion of the molecule. We have recently reported the results of studies of the partial amino acid sequence of Factor B (Christie *et al.*, 1980). An extended *N*-terminal sequence of fragment Bb (40 residues) showed no homology with the conserved *N*-termini of the activated forms of well-characterized serine proteinases (Neurath & Walsh, 1976). However, the sequence of two segments of 110 and 60 residues from the *C*-terminal portion of the molecule showed extensive homology with serine proteinases. The active-site histidine, aspartic acid and serine residues were each found in positions corresponding to those present in typical serine proteinases. Sequence studies demonstrating that Factor B is a serine proteinase were also reported by Mole & Niemann (1980). Both these studies showed that Factor B is a novel type of serine proteinase, with a catalytic chain of approximately twice the molecular weight of

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proteinases previously studied and probably with a different mechanism of activation.

We wish to determine as much of the primary sequence of Factor B as possible in an attempt to relate further its structure with its biological activity. The results of such studies may also be applicable to component C2, a protein analogous to Factor B but from the classical pathway of complement (Goodkofsky & Lepow, 1971; Kerr & Porter, 1978; Kerr, 1979). Interestingly, the genes for both Factor B and component C2 are encoded by closely linked genes in the major histocompatibility locus (Barnstaple *et al.*, 1979).

In the present paper we give details of the isolation, characterization and *N*-terminal sequences of the CNBr-cleavage peptides from Factor B. The data include an extended sequence around the site cleaved by Factor $\bar{D}$ and the localization of a free thiol group present in fragment Bb.

Materials and methods

Materials

Outdated human plasma was obtained from the John Radcliffe Hospital, Oxford, U.K.

Sephadex G-15, Sephadex G-50 (superfine grade), Sephadex G-100, CM-Sephadex C-50, DEAE-Sepharose CL-6B and DEAE-Sephacel were obtained from Pharmacia (G.B.), Hounslow, Middx., U.K.

Trypsin (treated with Tos-Phe-CH_2Cl) was from Worthington Biochemical Corp., Freehold, NJ, U.S.A. V-8 proteinase from *Staphylococcus aureus* was from Miles Laboratories, Stoke Poges, Slough, Berks., U.K. Clostripain from *Clostridium histolyticum* was supplied by Institut Pasteur Productions, Paris, France.

Iodo[2-^{14}C]acetic acid (57mCi/mmol) and iodo-[2-^{3}H]acetic acid (64mCi/mmol) were from The Radiochemical Centre, Amersham, Bucks., U.K. Succinic anhydride, chemicals for polyacrylamide-gel electrophoresis and iodoacetic acid were from BDH Chemicals, Poole, Dorset, U.K. Dithiothreitol (Calbiochem A grade) was supplied by CP Laboratories, Bishops Stortford, Herts., U.K. Guanidinium chloride (Ultrapure) was from Bethesda Research Laboratories, Rockville, MD, U.S.A. Polybrene (1,5-dimethyl-1,5-diaza-undecamethylene polymethobromide) and CNBr were from the Aldrich Chemical Co., Gillingham, Dorset, U.K. Propan-2-ol (h.p.l.c. grade) and Quadrol [*NNN'N'* - tetrakis - (2 - hydroxypropyl)ethylenediamine trifluoroacetate] were from Fluka and supplied by Flurochem Ltd., Glossop, Derbyshire, U.K. Methanol (h.p.l.c. grade), acetonitrile (h.p.l.c. grade S) and all other chemicals for automated sequencing were from Rathburn Chemicals, Walkerburn, Peeblesshire, Scotland, U.K. Fluram (fluorescamine) was from Roche Diagnostics, NJ, U.S.A. Toluene-*p*-sulphonic acid was from the Pierce Chemical Co., Rockford, IL, U.S.A.

Preparation of Factor B

The method of Kerr & Porter (1978) as modified by Kerr (1979), with some additional minor changes, was used to prepare Factor B from human plasma. The column of CM-Sephadex C-50 was equilibrated with 50mM- in place of 0.1M-sodium phosphate buffer, pH6.0. The column of 'aged' CNBr-activated Sepharose 4B was equilibrated with 5mM-sodium 5,5-diethylbarbiturate buffer (pH8.5)/0.5mM-$CaCl_2$/2.0mM-$MgCl_2$/40mM-NaCl and eluted with a linear gradient of NaCl from 0.04 to 0.54M.

Preparation of the fragments Ba and Bb

Factor B (100mg) was incubated with C3 and Factor $\bar{D}$ (B;C3:$\bar{D}$, 100:10:1, by wt.) in 5mM-sodium 5,5-diethylbarbiturate buffer (pH8.5)/0.5mM-$CaCl_2$/2.0mM-$MgCl_2$/40mM-NaCl (30ml) for 4h at 37°C. The digest was applied to a column (2.5cm × 20cm) of DEAE-Sepharose CL-6B equilibrated with the same buffer and developed with a 1-litre gradient of NaCl from 40 to 200mM. The Bb fragment was not retained by the column, and residual Factor B and fragment Ba were eluted after 585 and 765ml of the gradient had been run respectively. Component C3b was eluted immediately after the Ba fragment.

Factor $\bar{D}$ was purified to the stage following gel filtration on Sephadex G-75 as described by Johnson *et al.* (1980). Component C3 was generously provided by Dr. R. B. Sim and Dr. E. Sim and was purified as described by Sim *et al.* (1981).

Reduction and carboxy[^{14}C]methylation

Protein samples were dissolved in 6M-guanidinium chloride/0.5M-Tris/HCl/2mM-EDTA/40mM-dithiothreitol, pH8.1, to a concentration of 5–10mg/ml and incubated under N_2 for 3h at 37°C, with constant stirring. Iodo[2-^{14}C]-acetic acid (50–100μCi; 57mCi/mmol) was added and the mixture incubated for 5min at room temperature. An appropriate volume of unlabelled iodoacetic acid (1.44M in 1M-NaOH) was added to provide a 2-fold molar excess with respect to dithiothreitol, and the mixture was incubated a further 45min at 4°C. Excess reagents were removed by dialysis at 4°C, firstly against distilled water for 3h, then extensively against 5% (v/v) acetic acid.

Alkylation of free thiol group in fragment Bb

The fragment Bb (25mg) in 6M-guanidinium chloride/0.5M-Tris/HCl/2mM-EDTA, pH8.1 (3ml),

was treated with 50μCi of iodo[2-^{3}H]acetic acid (64mCi/mmol) for 5min at room temperature. An appropriate volume of unlabelled iodoacetic acid (1.44M in 1M-NaOH) was added to give a final concentration of 10mM, and the mixture was incubated for 45min at 4°C. Excess reagents were removed by gel filtration on a column (2cm × 20cm) of Sephadex G-15 equilibrated with 10% (v/v) acetic acid.

Succinylation (3-carboxypropionylation) of peptides Bb, CL-3 and CB-II

This was achieved by using the procedure of Koide *et al.* (1978). Samples of the reduced and alkylated peptides were dissolved in 6M-guanidinium chloride to a concentration of 5–10mg/ml and the pH was adjusted to 8.0 with 4M-NaOH. A 10-fold excess of succinic anhydride with respect to protein (by wt.) was added in small amounts over a period of 60min at room temperature, with constant stirring. The pH was maintained within the range 8–8.5 by the addition of 4M-NaOH. Once the pH remained constant the mixture was stirred for a further 30min. Excess reagents were removed by dialysis against 0.1M-NH_4HCO_3.

Separation of peptides by h.p.l.c.

The h.p.l.c. system (Waters Associates) comprised two solvent-delivery pumps (models 6000A and M-45), a sample-injector valve (U6K) fitted with a 2ml loading loop and a solvent programmer (model 660). Peptides were detected from absorbance at 206nm with a LKB Uvicord S instrument (model 2138). All separations were performed at room temperature, with a μBondapak C_{18} column (39mm × 300mm) at a flow rate of 1ml/min. Two solvent systems were employed for peptide separations. In the first system, solvent A consisted of 0.1% NH_4HCO_3 and solvent B was acetonitrile. In the second system, solvent A was 0.1% (v/v) trifluoroacetic acid and solvent B was methanol/acetonitrile/propan-2-ol (1:1:1, by vol.). Linear gradients of solvent B were constructed from an initial to a final ratio of solvents A and B by using curve 6 of the solvent programmer. The specific conditions of each separation are detailed in the text and Figure legends.

Preparation of CNBr-cleavage peptides from Factor B and fragment Bb

Samples of reduced and *S*-carboxy[^{14}C]methylated fragment Bb (812nmol), unreduced fragment Bb (213nmol) that had been treated with iodo-[2-^{3}H]acetic acid and unreduced Factor B (392nmol) were each dissolved in 70% (v/v) formic acid to a concentration of 10mg/ml. A 10-fold excess of CNBr (by wt.) was added, and the mixture was incubated for 20h in the dark at 4°C. The reaction mixture was then diluted 10-fold with water and freeze-dried. The digests were redissolved by the addition of 1.0ml of acetic acid, followed by 5.0ml of water, and applied to a column (3cm × 100cm) of Sephadex G-100 equilibrated with 10% (v/v) acetic acid. The flow rate was 16ml/h and 3.6ml fractions were collected. Peptides were detected from their absorbance at 280nm and by radioactivity. Samples (10–20μl) of column fractions were each added to 3ml of scintillation fluid, composed of 2% (w/v) naphthalene/0.5% (w/v) 2,5-diphenyloxazole in 1,4-dioxan. The radioactivity was determined with a LKB 1210 Ultrobeta counter. Some fractions were further purified by h.p.l.c. as described in the text.

Preparation of peptide CB-II,T-1

Reduced and alkylated succinylated peptide CB-II (15mg) in 0.1M-NH_4HCO_3 (20ml) was incubated with Tos-Phe-CH_2Cl-treated trypsin (0.2mg) for 2h at 37°C. The incubation was repeated after the addition of another 0.2mg of enzyme. Then the mixture was freeze-dried. The digest was redissolved in 2.5ml of 0.1M-NH_4HCO_3, and the soluble material was applied to a column (2cm × 90cm) of Sephadex G-50 (superfine grade) equilibrated with 0.1M-NH_4HCO_3. Peptide CB-II,T-1 was eluted as a discrete peak with an elution volume of 143–152ml.

V-8 proteinase digestion of peptide CB-IV

Peptide CB-IV (2.5mg) was dissolved in 50mM-NH_4HCO_3/2mM-EDTA, pH7.8 (0.5ml), and digested with V-8 proteinase (5lμg) for 6h at 37°C and freeze-dried. The digest was redissolved in 0.1% (v/v) trifluoroacetic acid (0.5ml) and injected on to a μBondapak C_{18} column equilibrated with 90% (v/v) 0.1% trifluoroacetic acid and 10% (v/v) solvent B (methanol/acetonitrile/propan-2-ol, 1:1:1, by vol.). After 5min a linear gradient of 60min was applied to the column to give a final solvent composition of 0.1% (v/v) trifluoroacetic acid/solvent B (45:55, v/v).

Preparation of peptide Bb,CL-3,T-2,DE-A

Fragment Bb (43mg) in 20mM-NH_4HCO_3 (13ml) was subjected to limited proteolysis by incubation with clostripain (0.43mg) for 2h at 37°C and the digest was freeze-dried. Clostripain was activated immediately before use by incubation of the enzyme (0.5mg/ml) in 20mM-NH_4HCO_3 (pH8.0)/10mM-dithiothreitol for 1h at 37°C. The digest was redissolved in 0.1M-NH_4HCO_3 (6ml) containing 5mM-iodoacetic acid to inactivate the clostripain and applied to a column (2cm × 90cm) of Sephadex G-75 equilibrated with 0.1M-NH_4HCO_3. A fragment Bb,CL-3, with a molecular weight of approx. 30000, corresponding to the *N*-terminal portion of fragment Bb, was eluted between 148 and 157ml. Reduced and *S*-carboxy-

methylated succinylated peptide Bb,CL-3 (7mg) in 0.1M-NH_4HCO_3 (1ml) was digested with Tos-Phe-CH_2Cl-treated trypsin (70μg) for 2h at 37°C. A further 70μg of enzyme was added and the incubation repeated. The digest was applied to a column (2cm × 90cm) of Sephadex G-50 (superfine grade) equilibrated with 0.1M-NH_4HCO_3. The material (Bb,CL-3,T-2) that was eluted between 153 and 174ml was freeze-dried. This fraction was redissolved in 0.1M-NH_4HCO_3 and applied to a column of DEAE-Sephacel, equilibrated with the same buffer, which was developed with a linear gradient of NH_4HCO_3 (0.1–1.0M) in a total volume of 100ml. The column effluent was monitored at 280nm. Peptide Bb,CL-3,T-2,DE-A was eluted after 70ml of the gradient had been run.

SDS/polyacrylamide-gel electrophoresis

Electrophoresis in 10% and 15% (w/v) polyacrylamide gels in the presence of 0.1% (w/v) SDS was performed as described by Laemmli (1970). When reduction of samples was necessary, these were incubated with 40mM-dithiothreitol/4M-urea/1% (w/v) SDS/50mM-Tris/HCl, pH8.0 at 100°C, for 4min. Apparent molecular weights were calculated by reference to the mobilities of unreduced bovine serum albumin, ovalbumin, chymotrypsinogen and myoglobin.

Amino acid and hexosamine analysis

Amino acid analysis. Samples were hydrolysed in twice-distilled 5.7M-HCl in sealed evacuated glass tubes at 110°C. The amino acid compositions of Factor B and fragments Bb and Ba are based on the average of duplicate 24h, 48h and 72h hydrolysis values, except for threonine and serine, which were extrapolated to zero time, and valine and isoleucine, for which the 72h values were taken. Peptides were hydrolysed for 24h and no correction for the destruction of threonine and serine was made. Half-cystine was determined either as cysteic acid after performic acid oxidation as described by Hirs (1956) or as *S*-carboxymethylcysteine after hydrolysis in 5.7M-HCl containing 0.05% (v/v) 2-mercaptoethanol. Tryptophan was not determined.

Hexosamine analysis. Samples were hydrolysed in 3M-toluene-*p*-sulphonic acid at 110°C for 24h by using the procedure described by Allen & Neuberger (1975).

Samples were analysed on a Durrum D-500 or a LKB model 4400 amino acid analyser.

Automated sequence analysis

Automated Edman degradation was performed in a Beckman 890C sequencer with the 0.3M-Quadrol program of Hunkapiller & Hood (1978). Polybrene was added to the sequencer cup and one cycle of the program was performed before the addition of each sample. Amino acid phenylthiohydantoin derivatives, with the exception of those of histidine and arginine, were identified by h.p.l.c. on a μBondapak C_{18} column with a linear gradient of methanol from 14 to 56% (v/v) in an aqueous buffer (Bridgen *et al.*, 1976) pumped at 2ml/min by a Waters Associates system equipped with a dual-wavelength detector (254 and 313nm) and a Data Module (model 730). Identification of the histidine and arginine derivatives was also performed with the h.p.l.c. system described above, with a linear gradient of methanol from 20 to 35% (v/v) in an aqueous buffer. Assignment of *S*-carboxymethylated cysteine residues was confirmed by determining the radioactivity present in a 200μl sample of the butyl chloride extract by scintillation counting. To minimize the destruction of serine and threonine derivatives, the thiazolinones were extracted with butyl chloride containing dithiothreitol (10mg/l) and tri-*N*-butylphosphine (100μl/l) (Frank, 1979). The extracts were collected under N_2 into tubes containing 1.0M-HCl/0.1% (v/v) ethanethiol (200μl), prepared freshly each day.

Occasionally, to eliminate background during sequence determination, the sequencer was stopped at the end of a cycle when proline was the new *N*-terminal residue in the sequence of interest. Fluorescamine was then added directly to the sequencer cup to block any primary amines, leaving only the proline imino group available for subsequent reaction with phenyl isothiocyanate. The procedure was that of Bhown *et al.* (1981). Excess reagent was extracted with benzene followed by ethyl acetate/0.1% (v/v) acetic acid in accordance with the normal sequence program.

Result

General strategy

Limited proteolysis of Factor B was employed to simplify the overall task of sequence determination. The complement proteinase Factor D was used to cleave Factor B in the presence of C3b. Separation of the larger more-basic Bb fragment from the more-acidic Ba fragment and the other components of the reaction mixture was achieved by ion-exchange chromatography on DEAE-Sepharose. The fragments Ba and Bb were obtained in yields of 70–80% and each ran as a single band on SDS/polyacrylamide-gel electrophoresis, corresponding to molecular weights of 30000 and 60000 respectively. This approach enabled each polypeptide to be treated separately, facilitating the isolation of fragments generated by specific chemical and enzymic cleavage procedures.

The amino acid and hexosamine compositions of Factor B and fragments Ba and Bb are presented in Table 1. The sum of the total residues of the Ba and

Table 1. *Amino acid and amino sugar compositions of Factor B and fragments Bb and Ba*

Amino acid compositions of Factor B and fragments Bb and Ba were calculated from the average of duplicate 24h-, 48h- and 72h-hydrolysis values. Corrections were made for the destruction of threonine and serine. Valine and isoleucine were estimated from the 72h-hydrolysis values. Half-cystine was determined as cysteic acid after performic acid oxidation (Hirs, 1956). Tryptophan was not determined. The composition of Factor B was calculated assuming a molecular weight of 60000 and a carbohydrate composition of 8.9%, an average of published values (Boenisch & Alper, 1970; Curman *et al.*, 1977). Amino sugar analyses were performed after hydrolysis in 3 M-toluene-*p*-sulphonic acid at 110°C for 24h.

	Composition (mol of residue/mol)			
	Factor B	Fragment Bb	Fragment Ba	Fragments Bb + Ba
Asx	74.5	60.6	14.5	75.1
Thr	39.9	23.7	14.3	38.0
Ser	51.4	29.4	18.6	48.0
Glx	83.4	56.6	27.3	83.9
Pro	45.1	26.8	15.2	42.0
Gly	61.2	38.5	21.3	59.8
Ala	34.0	26.2	8.1	34.3
CyS	20.1	11.2	8.1	19.3
Val	62.5	50.1	11.5	61.6
Met	10.1	8.0	1.0	9.0
Ile	34.9	30.3	6.2	36.5
Leu	55.4	42.5	11.2	53.7
Tyr	32.4	20.6	10.2	30.8
Phe	20.9	14.4	5.1	19.5
His	20.0	13.9	5.0	18.9
Lys	56.2	48.7	5.9	54.6
Arg	42.8	21.5	16.5	38.0
Total	745	523	200	723
Glucosamine	9.9	5.3	3.7	9.0
Galactosamine	—	—	—	—

Bb fragments is in close agreement with the composition of Factor B. Factor B contains 9–10 methionine residues and all but one of these are located within the Bb portion of the molecule. Thus treatment of Factor B or fragment Bb with CNBr should yield relatively large peptides, which would be suited to automated liquid-phase Edman degradation.

Isolation of the CNBr peptides from Factor B and Bb

A total of ten peptides were isolated from CNBr digests of samples of reduced and *S*-carboxymethylated fragment Bb, fragment Bb treated with iodo[^{3}H]acetic acid without prior reduction and intact Factor B. The amino acid compositions and yields of these peptides are presented in Table 2, as well as the results of amino sugar analyses.

In each case, initial separation of CNBr-cleavage peptides was achieved by gel filtration on a column (3cm × 100cm) of Sephadex G-100 equilibrated with 10% (v/v) acetic acid (Fig. 1). Seven pools of material, designated CB-I to CB-VII, were made from the CNBr digest of reduced and *S*-carboxy-[^{14}C]methylated fragment Bb as shown in Fig. 1(*a*). Pools CB-I and CB-III represent products of incomplete cleavage. Pools CB-II, CB-IV and CB-V each contained a single peptide with molecular weights of approx. 30000, 10000 and 6000 respectively. Peptides CB-II and CB-V were re-run on the same column before sequence analysis. Peptide CB-II contained a high proportion of the total half-cystine residues of fragment Bb and was the only peptide not containing homoserine. On this basis and from the results of *C*-terminal analysis with carboxypeptidase Y, peptide CB-II was assigned to the *C*-terminal portion of fragment Bb, as discussed previously (Christie *et al.*, 1980). Pool CB-VI contained two peptides (CB-VIa and CB-VIb), and attempts to separate these by ion-exchange chromatography resulted in poor yields. It was more convenient to isolate these peptides from CNBr digests of non-reduced Factor B or fragment Bb, as described below. Three peptides were isolated from the CB-VII pool by reversed-phase h.p.l.c. on a μBondapak C_{18} column with a 0.1% NH_4HCO_3/acetonitrile-based solvent system (Fig. 2). Peptides

Table 2. *Amino acid and amino sugar compositions of CNBr-cleavage peptides from Factor B and fragment Bb*
Compositions are given as mol of residue/mol of peptide and were calculated from 24h-hydrolysis values. Values of less than 0.2 have been omitted. Values for any particular peptide are normalized with respect to the residue marked with an asterisk (*). Abbreviation: N.D., not determined. Methionine was determined as homoserine or homoserine lactone. Half-cystine was determined either as *S*-carboxymethylcysteine or as cysteic acid after performic acid oxidation. Tryptophan was not determined.

	Amino acid composition (mol of residue/mol)									
Peptide ...	CB-N	CB-II	CB-IV	CB-V	CB-VIa	CB-VIb	CB-VIc	CB-VII-1	CB-VII-2	CB-VII-3
Asx	14.3	23.1	13.2	12.0	1.2	4.9	4.2	1.2	2.0	1.2
Thr	11.2	11.0	5.6	1.3	1.8	1.5	2.7	—	—	—
Ser	14.2	9.4	8.5	1.4	2.6	1.1	3.1	2.2	—	1.5
Glx	19.6	30.0	6.9	5.5	3.8	1.3	9.3	2.0	2.9	—
Pro	9.2	14.0	2.9	3.0	1.8	3.3	2.8	—	—	1.0
Gly	18.3	19.7	8.3	5.4	2.2	2.1	5.3	1.6	—	1.3
Ala	6.8	13.6	8.3	2.5	1.0	—	3.3	—	—	—
CyS	9.8	8.0*	1.2	—	1.0	—	—	0.7	—	—
Val	10.3	21.3	10.2	7.9	1.9	2.3	3.0	—	1.0	1.0
Met	1.0*	—	1.3	0.9	1.0*	2.0*	0.8	1.0*	1.0*	1.0*
Ile	6.1	13.0	5.4	4.6	1.8	2.1	1.9	1.0	—	1.0
Leu	10.3	20.1	7.9	5.3	—	2.0	3.1	2.0	1.1	1.1
Tyr	10.5	8.7	6.5	2.6	0.8	—	—	—	0.4	—
Phe	3.9	9.4	1.3	2.2	—	—	0.9	—	1.0	—
His	3.9	5.4	1.6	1.2	3.1	1.8	1.0	—	—	—
Lys	7.3	21.8	11.0*	5.8	4.2	—	2.0*	—	—	1.0
Arg	15.8	10.8	1.9	3.0*	2.3	2.3	1.0	—	—	—
Total residues	173	239	102	65	30	27	44	12	10	10
Yield of peptide (%)	84	75	71	82	60	48	31	41	29	37
Glucosamine (mol/mol)	4.9	—	3.3	—	—	1.6	N.D.	N.D.	N.D.	N.D.
Galactosamine (mol/mol)	—	—	—	—	—	—	N.D.	N.D.	N.D.	N.D.

CB-VII-1, CB-VII-2 and CB-VII-3 were eluted 8, 16 and 21 min from the time of injection respectively.

Pool CB-VI (elution volume 450–500ml) from the separation of the CNBr digest of non-reduced fragment Bb (Fig. 1*b*) contained only a single peptide (CB-VIb). When the CB-II pool from this preparation was run under both non-reducing and reducing conditions on SDS/polyacrylamide-gel electrophoresis, bands corresponding to molecular weights of approx. 33000 and 30000 were found. When non-reduced peptide CB-II was reduced and *S*-carboxymethylated and subjected to gel filtration under the conditions described above, peptide CB-VIa was isolated in good yield. The cysteine-containing peptide CB-VII-1 was also isolated by this procedure, in a yield of 80%. Thus both peptide CB-VIa and peptide CB-VII-1 are linked by disulphide bonds to peptide CB-II.

Alkylation of non-reduced fragment Bb with iodo[2-^{3}H]acetic acid before treatment with CNBr resulted in a peak of radioactivity corresponding to the elution position of peptide CB-IV (Fig. 1*b*), indicating that this peptide contains a free thiol group. Other radioactivity that was eluted from the column was associated from partial cleavage products also containing the CB-IV portion of the molecule.

Two additional peptides were isolated from the CNBr digest of Factor B (Fig. 1*c*). Peptide CB-N, eluted between 300 and 350ml, had a molecular weight of approx. 25000 and an amino acid composition similar to that of fragment Ba (Tables 1 and 2). The glucosamine content of 4.9mol/mol of peptide is consistent with it containing all the carbohydrate-attachment sites present in fragment Ba. The second peptide, CB-VIc, was isolated from the CB-VI pool (elution volume 450–550ml) by reversed-phase h.p.l.c. with a solvent system formed from 0.1% trifluoroacetic acid and methanol/acetonitrile/propan-2-ol (1:1:1, by vol.). Peptide CB-VIc was eluted 54min from the time of injection (Fig. 3). The peptide eluted at 30min corresponds to peptide CB-VIb, and the asymmetry of this peak is attributed to the presence of carbohydrate (Table 2).

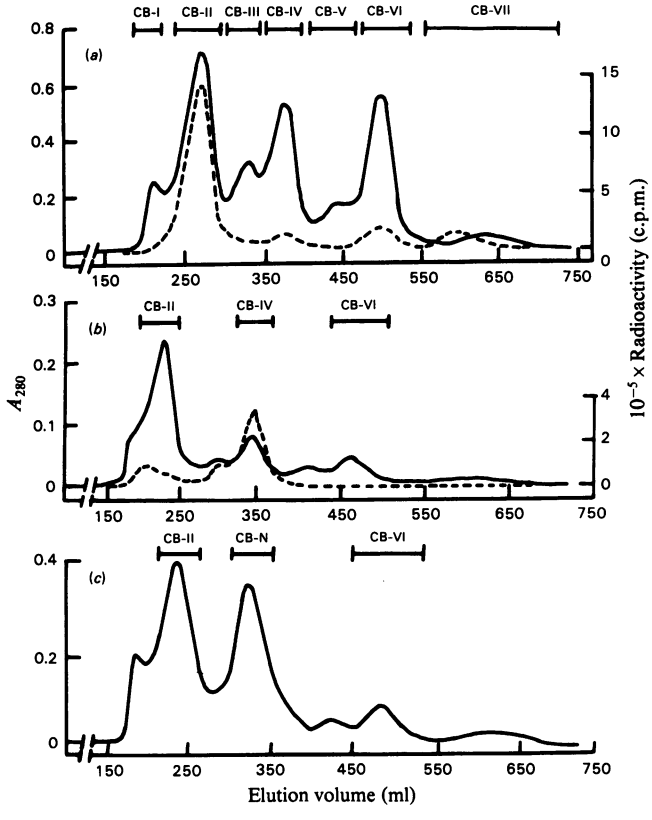

Fig. 1. *Separation of CNBr-cleavage peptides from fragment Bb and Factor B by gel filtration on Sephadex G-100*
CNBr digests of (*a*) reduced and *S*-carboxy[^{14}C]methylated fragment Bb (812nmol), (*b*) iodo[2-^{3}H]acetic acid-treated unreduced fragment Bb (213nmol) and (*c*) unreduced Factor B (392nmol) were dissolved in acetic acid (1ml) and water (5ml), and each was applied to a column (3cm × 100cm) of Sephadex G-100 equilibrated with 10% (v/v) acetic acid. The flow rate was 16ml/h and 3.6ml fractions were collected. Fractions were pooled as indicated in the Figure. Samples (10–20μl) were taken for the determination of radioactivity. ——, A_{280}; – – – –, radioactivity.

A small amount of peptide CB-V present in the sample applied to the h.p.l.c. was eluted between peptides CB-VIb and CB-VIc.

N-Terminal sequence analysis of the CNBr-cleavage peptides of Factor B and fragment Bb

Peptide CB-N. Two sequences were obtained when peptide CB-N was subjected to automated Edman degradation. The main sequence corresponded to that of the *N*-terminal of Factor B and fragment Ba, commencing Thr-Pro-Trp-. The other sequence, CB-N′, commenced Gly-Ser-Cys-, which we recognized as being identical with the sequence of peptide CB-N starting at position 10. The sequencer was stopped after seven cycles of Edman degradation, when proline was the *N*-terminal residue of the CB-N sequence. The minor sequence was blocked by the addition of fluorescamine and automated Edman degradation was resumed. This permitted the identification of residues 1–22 of peptide CB-N (Fig. 4 and Table 3).

Peptide CB-II. Automated Edman degradation permitted the identification of residues 1–42 (Fig. 4 and Table 3). Sequence analysis of peptide CB-II,T-1, derived from a tryptic digest of succinylated peptide CB-II, showed it to result from the cleavage of an Arg–Asp bond at position 34 of peptide CB-II and enabled the sequence of peptide CB-II to be extended to 78 residues (Fig. 4 and Table 4).

Peptide CB-IV. Automated Edman degradation of peptide CB-IV enabled the identification of 42 of the first 43 residues (Fig. 4 and Table 3). As peptide CB-IV contains 3.3mol of glucosamine/mol (Table 2), it is likely that the gap in the sequence at position

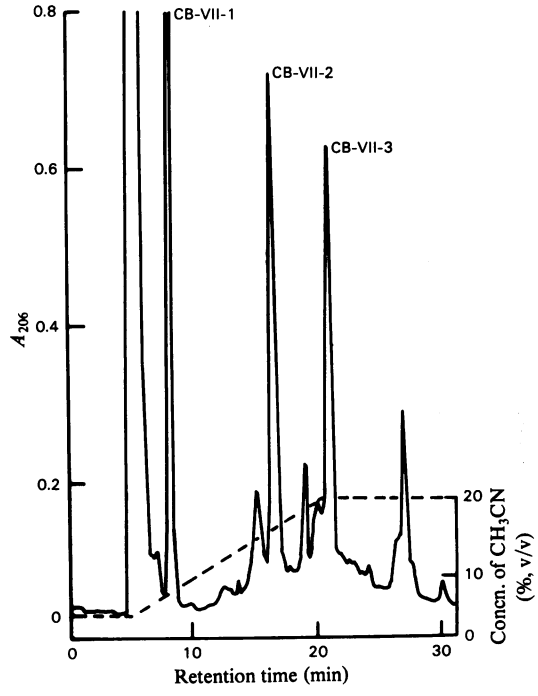

Fig. 2. *Separation of peptides from the CB-VII pool from the CNBr digest of reduced and S-carboxy[^{14}C]methylated fragment Bb (Fig. 1a) by h.p.l.c.*

The freeze-dried material was dissolved in 0.1% NH_4HCO_3 (0.85 ml), and 0.2 ml was injected on to a μBondapak C_{18} column (39 mm × 300 mm) equilibrated with a mixture of 0.1% NH_4HCO_3/acetonitrile (95:5, v/v). After 5 min, a 20 min linear gradient of acetonitrile was pumped to the column at 1 ml/min to give a final mixture of 0.1% NH_4HCO_3/acetonitrile of 80:20 (v/v). The sample was injected at zero time. ——, A_{206}; – – – –, concn. of acetonitrile.

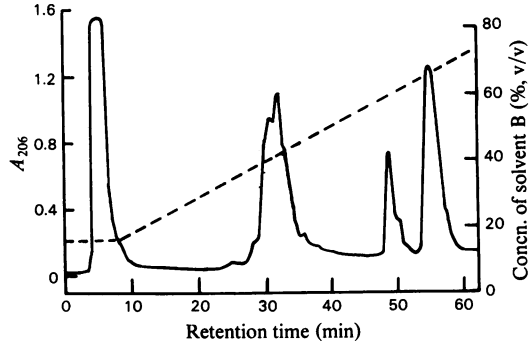

Fig. 3. *Separation of peptides from the CB-VI pool from the CNBr digest of non-reduced Factor B (Fig. 1c) by h.p.l.c.*

The freeze-dried material was dissolved in 10% (v/v) acetic acid (2 ml), and 1 ml was injected on to a μBondapak C_{18} column (39 mm × 300 mm) equilibrated with a mixture of 0.1% trifluoroacetic acid/solvent B (85:15, v/v). Solvent B was methanol/acetonitrile/propan-2-ol (1:1:1, by vol.) After 8 min, a 60 min linear gradient of solvent B was pumped to the column at 1 ml/min to give a final mixture of 0.1% trifluoroacetic acid/solvent B of 20:80 (v/v). The sample was injected at zero time. ——, A_{206}; – – – –, concn. of solvent B.

16 results from the presence of asparagine-linked carbohydrate. The sequence -Asn-Phe-Thr- is consistent with the requirement for the attachment of carbohydrate via glucosamine. To extend the *N*-terminal sequence of peptide CB-IV, the peptide was digested with V-8 proteinase, and the peptides were isolated by h.p.l.c. with the 0.1%-trifluoroacetic acid-based solvent system. Peptides CB-IV, SP-17 and SP-8 were subjected to automated sequence analysis. The *N*-terminal 16 residues of peptide CB-IV,SP-17 were identified (Fig. 4 and Table 4), and the sequence showed it to result from the cleavage of a Gly–Val bond at position 35 (CB-IV numbering). This extended the sequence of peptide CB-IV to 51 residues. Sequence analysis of peptide CB-IV,SP-17 also showed a contaminating peptide present in a yield of approx. 20%. The sequence corresponded to a peptide resulting from cleavage at Glu-57 and Glu-72 of peptide CB-IV. This contaminant accounted for the discrepancy between the sequence and amino acid composition of peptide CB-IV,SP-17 (Fig. 4 and Table 4). Peptide CB-IV,SP-8 was found to contain homoserine (Table 4), indicating that it was the *C*-terminal peptide of peptide CB-IV. Automated sequence analysis permitted the identification of 18 residues (Fig. 4 and Table 4). Sequence analysis of a peptide Bb,CL-3,T-2,DE-A provided an overlap with the sequence of peptides CB-IV,SP-17 and CB-IV,SP-8, completing the identification of residues 1–94 of peptide CB-IV (Fig. 4 and Table 4). The peptide Bb, CL-3,T-2, DE-A was isolated from a tryptic digest of succinylated peptide Bb,CL-3, a fragment generated by the limited proteolysis of fragment Bb by clostripain. Fragment Bb,CL-3 had the same *N*-terminal sequence as fragment Bb and a molecular weight of approx. 30000. A full description of the isolation of peptide Bb,CL-3,T-2,DE-A is presented in the Materials and methods section.

Fig. 4. *N-Terminal sequence of the CNBr-cleavage peptides from Factor B and fragment Bb*

Residues identified after automated Edman degradation are shown by —→. The following peptide nomenclature is used: CB, CNBr-cleavage peptides; T, tryptic-digest peptides; SP, V-8-proteinase-digest peptides; CL, clostripain-digest peptides; DE, peptide purified on DEAE-Sephacel. Amino acids are in the single-letter notation: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His, I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; Y, Tyr. It is probable that position 16 of peptide CB-IV and position 12 of peptide CB-Ib are occupied by asparagine residues with carbohydrate residues attached. The ↓ indicates the bond that is cleaved by Factor D̄. A free thiol group is present at position 23 of peptide CB-IV.

Table 3. *Details of the automated Edman degradation of the CNBr-cleavage peptides of Factor B and fragment Bb*

Peptide ...	CB-N	CB-II	CB-IV	CB-V	CB-VIa	CB-VIb	CB-VIc	CB-VII-1	CB-VII-2	CB-VII-3
Amount used in automated sequence analysis (nmol)	90	110	130	120	22	40	120	70	55	78
Initial yield (%)	18	41	26	38	64	60	60	57	73	77
Repetitive yield (%)	95	93	91	92	89	90	92	87	88	87
Amino acids used to calculate repetitive yields	Gly	Leu	Leu	Leu	Val	Val	Leu	Val	Leu	Ile
		Val	Val	Val	Pro	Pro	Val	Asp	Ala	Gln
		Ala	Ala	Ile	Lys		Ala			
		Phe	Tyr							

Two other peptides, CB-IV,SP-6 and CB-IV, SP-21 were isolated from V-8 proteinase digests of peptide CB-IV by h.p.l.c. Sequence analysis of peptides CB-IV,SP-6 and CB-IV,SP-21 confirmed the identification of residues 58–72 and 73–76 of peptide CB-IV respectively.

Peptide CB-V. Automated Edman degradation permitted the identification of residues 1–49 (Fig. 4 and Table 3).

Peptide CB-VIa. Residues 1–27 were identified by automated Edman degradation (Fig. 4 and Table 3).

Peptide CB-VIb. Automated Edman degradation enabled the identification of 19 of the first 20 residues (Fig. 4 and Table 3). It is likely that the gap at position 12 is due to the presence of carbohydrate. This peptide contained 1.6 mol of glucosamine/mol, and the sequence -Asn-Arg-Thr- is consistent with it being a carbohydrate-attachment site. From sequence analysis peptide CB-VIb is longer than would be expected from an amino acid composition based on 1 mol of homoserine/mol of peptide. For this reason, the amino acid composition of peptide CB-VIb has been calculated on the assumption that this peptide contains one methionyl bond resistant to treatment with CNBr (Table 2).

Peptide CB-VIc. The *N*-terminal 42 residues were determined by automated sequence analysis (Fig. 4 and Table 3). The sequence overlapped by six residues with the *N*-terminal sequence of peptide CB-VII-3. Peptide CB-VII-3 was only obtained from CNBr digests of fragment Bb and is the *N*-terminal CNBr-cleavage peptide of fragment Bb (Christie *et al.*, 1980). Thus peptide CB-VIc provided an overlap between the Ba and Bb fragments. The sequence of peptide CB-VII-3 was used to complete the sequence of peptide CB-VIc. The methionine at position 46 was assigned on the basis of the amino acid composition of peptide CB-VII-3.

Peptide CB-VII-1. Residues 1–11 were determined from automated sequence analysis (Fig. 4 and Table 3). The methionine residue at position 12 was assigned on the basis of amino acid analysis.

Peptide CB-VII-2. The entire sequence of ten residues was determined by automated sequence analysis (Fig. 4 and Table 3).

Localization of a free thiol group in fragment Bb

A sample of peptide CB-IV isolated from a CNBr digest of fragment Bb that had been previously treated with iodo[2-^{3}H]acetic acid was subjected to automated sequence analysis (Table 5). The sequence was identical with that previously obtained for peptide CB-IV (Fig. 4 and Table 3), and 81.7% of the expected radioactivity, calculated from the initial coupling yield and the repetitive yield, was recovered at cycle 23. A cysteine residue had previously been assigned to this position (Fig. 4).

Table 4. *Amino acid composition and details of automated Edman degradation of peptides used to extend the N-terminal sequence of CNBr-cleavage peptides CB-II and CB-IV*

Compositions are given as mol of residue/mol of peptide and were calculated from 24h-hydrolysis values. Values of less than 0.2 have been omitted. Values for any particular peptide are normalized with respect to the residue marked with an asterisk (*). For peptide CB-IV,SP-8 methionine was determined as homoserine or homoserine lactone.

	Amino acid composition (mol of residue/mol)			
Peptide ...	CB-II,T-1	CB-IV,SP-17	CB-IV,SP-8	Bb,CL-3,T-2,DE-A
Asx	6.2	1.8	3.9	8.4
Thr	3.4	1.7	2.4	2.6
Ser	0.8	1.2	2.7	3.5
Glx	6.4	1.7	2.0*	7.1
Pro	3.8	1.3	—	0.9
Gly	4.3	1.7	1.4	2.2
Ala	2.3	1.5	3.2	4.3
CyS	2.2	—	—	0.5
Val	3.2	3.6	1.7	5.6
Met	—	—	0.9	1.0*
Ile	5.1	1.2	—	2.2
Leu	5.2	1.5	2.6	4.5
Tyr	3.3	2.2	1.1	2.7
Phe	1.8	—	—	0.9
His	1.2	—	1.1	1.3
Lys	5.7	2.6	4.6	6.6
Arg	2.0*	1.0*	—	—
Total residues	57	23	28	54
Yield of peptide (%)	34	18	23	32
Amount used in automated sequence analysis (nmol)	94	42	54	78
Initial yield (%)	31	36	83	46
Repetitive yield (%)	93	91	90	93
Amino acids used to calculate repetitive yield	Leu	Val	Leu	Leu Val Lys

Table 5. *Details of automated Edman degradation of peptide CB-IV to determine the position of the free thiol group*

Amount used in automated sequence analysis (nmol)	59
Initial yield (%)	24
Stepwise yield based on the yield of leucine at positions 4, 6 and 24 and valine at positions 5 and 25 (%)	94
Radioactivity present in sample used for sequence analysis (c.p.m.)	724700
Radioactivity recovered at cycle 23 (c.p.m.)	38400
Expected yield of radioactivity assuming all the counts are located at position 23, calculated from initial and stepwise yields (c.p.m.)	47000

Discussion

Ten peptides were isolated from CNBr digests of Factor B and fragment Bb in yields of 29–84%, and each was characterized by amino acid analysis and automated *N*-terminal sequence analysis. Peptide CB-VIII-3 was specific to digests of fragment Bb and was a direct result of the initial cleavage of Factor B by Factor D. Of the nine CNBr-cleavage peptides from Factor B, one (CB-VIb) must contain 2mol of homoserine/mol of peptide; the sequence obtained for this peptide is much longer than would be expected if the peptide contained a single residue of homoserine. Only homoserine and no free methionine was detected for this peptide by amino acid analysis (Table 2), which is consistent with the treatment of resistant Met–Thr bonds with CNBr resulting in the formation of an internal homoserine residue without peptide-bond cleavage (Schroeder *et al.*, 1969). Moreover, sequence analysis of a tryptic peptide from fragment Bb has shown the presence of a Met–Thr bond at a position equivalent to residue 21 of peptide CB-VIb (results not shown). Thus a total of nine methionine residues of Factor B can be accounted for from the CNBr-cleavage peptides. This is in agreement with the number based on the amino acid compositions of fragments Ba and Bb.

An additional peptide would be expected, however, from the amino acid composition of Factor B, and the possibility of a small peptide or even free methionine being missed cannot be fully discounted until the complete primary sequence is known.

The *N*-terminal sequences of the CNBr-cleavage peptides account for 358 residues, which represent approx. 50% and 60% of the total residues of Factor B and fragment Bb respectively. During automated sequence analysis of the CNBr-cleavage peptides initial coupling yields ranged from 18 to 77% and repetitive yields from 87 to 95%. As would be expected, there was a correlation between the size of the peptide and these parameters: in general, larger peptides had lower initial coupling and better repetitive yields than had smaller ones (Table 3).

The results of amino sugar analyses of Factor B and fragments Ba and Bb (Table 1) and of the CNBr-cleavage peptides (Table 2) indicate that the carbohydrate structures of Factor B are *N*-linked to asparagine through *N*-acetylglucosamine. The absence of galactosamine rules out the possibility of *O*-glycosidic linkage of carbohydrate to serine or threonine (Kornfeld & Kornfeld, 1976). It appears very likely that the gaps in the sequence of peptide CB-IV at position 16 and of peptide CB-VIb at position 12 result from the presence of asparagine-linked carbohydrate. These peptides contain glucosamine (Table 3), and in both cases the sequence is consistent with the -Asn-Xaa-Thr- requirement for the *N*-linkage of carbohydrate (Neuberger *et al.*, 1972). The glucosamine contents of peptides CB-IV and CB-VIb account for all the glucosamine present in fragment Bb, indicating that there are no additional attachment sites in this fragment. From the glucosamine composition fragment Ba would be expected to contain two carbohydrate-attachment sites.

In a previous paper we showed that peptide CB-II was derived from the *C*-terminus of Factor B and aligned this peptide with peptide CB-VIa (Christie *et al.*, 1980). The portion of the molecule defined by the contiguous peptides CB-VIa and CB-II was demonstrated to contain the essential active-site residues of this unusual type of serine proteinase. It was also shown that peptide CB-IV resulted from cleavage of the methionyl bond at position 10 of fragment Bb. The present work has enabled further alignment of the CNBr-cleavage peptides. The large peptide CB-N has the same *N*-terminal sequence as Factor B and fragment Ba (Kerr, 1979; Niemann *et al.*, 1980), and is clearly from the *N*-terminal portion of the molecule. The reason for the heterogeneity of peptide CB-N on *N*-terminal sequence analysis is unclear. A single sequence, identical with the main peptide CB-N sequence, was obtained when fragment Ba was subjected to automated Edman degradation. The only residue found at position 9 was glutamine, ruling out the presence of some methionine due to polymorphism accounting for the additional site of cleavage by CNBr. Assuming that fragment Ba contains a single methionine residue (Table 1), peptide CB-N can be aligned with peptide CB-VIc. As the sequence of peptide CB-VIc overlaps with that of peptide CB-VII-3, which corresponds to residues 1–10 of fragment Bb, peptides CB-VIc and CB-IV can be aligned. This provides a continuous segment of 140 residues giving extended sequence about the site cleaved by Factor $\bar{D}$, and confirms previous reports that Factor B is cleaved at an Arg–Lys bond at a site containing three basic residues -Lys-Arg-Lys- (Kerr, 1979; Lesavre *et al.*, 1979). In other respects peptide CB-VIc is an acidic peptide containing a relatively high number of glutamic acid residues.

Lesavre *et al.* (1979) showed that Factor B contains two free thiol groups/molecule, one in each of the Ba and Bb fragments. Our results clearly demonstrate the presence of one free thiol group at position 23 of peptide CB-IV, equivalent to position 33 of fragment Bb. Although the thiol group is situated close to the site cleaved by Factor $\bar{D}$ in the linear sequence, Lesavre *et al.* (1979) found that Factor B treated with *p*-chloromercuribenzoate was still cleaved by Factor $\bar{D}$, retaining 47% of its haemolytic activity. The functional importance of this thiol group therefore is uncertain. It may be noted that the thiol group is in close proximity to a carbohydrate-attachment site.

An unusual cleavage was observed when peptide CB-IV was digested with V-8 proteinase. Under conditions specific for cleavage at glutamic acid residues the Gly–Val bond at position 35 was cleaved to generate peptide CB-IV,SP-17 in a yield similar to that obtained for other peptides. Similar 'non-specific' cleavage has been observed previously (Dognin & Wittmann-Liebold, 1977).

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