Journal of Chromatography, 369 (1986) 357–364
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 18 935

IDENTIFICATION AND ASSAY OF PHOSPHOSERINE AND TYROSINE-O-SULPHATE IN FIBRINOPEPTIDES BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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(First received July 2nd, 1986; revised manuscript received July 15th, 1986)

SUMMARY

A procedure utilizing reversed-phase high-performance liquid chromatography (HPLC) is described for the identification and quantitation of individual phosphorylated and sulphated fibrinopeptides present in fibrin clot supernatants. Fibrinopeptides from human, rabbit and canine fibrinogens, which have different structures and degrees of phosphorylation and sulphation, were used to demonstrate the applicability of these methods. The procedure relies on the increased peptide hydrophobicity following removal of highly charged phosphate or sulphate groups. Dephosphorylated or desulphated peptides are thus more strongly retained on the reversed-phase HPLC column and are eluted later than their corresponding phosphorylated or sulphated peptide counterparts. Dephosphorylation is achieved by treatment of fibrinopeptide-containing clot supernatants with alkaline phosphatase. Phosphorylated peptides are characterized by an increased retention time resulting from loss of phosphate, whereas non-phosphorylated peptides remain unaffected. Similarly, a prolongation of the peptide retention time resulting from desulphation by mild acid hydrolysis serves to verify sulphation of a peptide.

INTRODUCTION

Examples of biological peptides covalently modified by phosphorylation or sulphation include neuropeptides and peptide hormones[1], fibrinopeptides[2,3] and others[4]. The chemical instability of the modified amino acid residues, phosphoserine and tyrosine-O-sulphate, prevents their identification by standard protein-chemical methods such as amino acid composition and sequence analysis. Therefore, specific procedures are required to demonstrate their presence. Enzymatic degradation of the purified peptide has often been used to generate free phosphoserine or tyrosine-O-sulphate which can subsequently be identified by paper electrophoresis or thin-layer

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0021-9673/86/$03.50

chromatography[2,5]. More recent methods have achieved higher sensitivities by radioactive labelling with ^{32}P or ^{35}S, but these are generally applicable only to proteins[6,7].

Fibrinopeptides belong to the earliest peptides found to be modified by phosphorylation or sulphation.[8,9] These peptides are cleaved from fibrinogen by the protease thrombin to form insoluble fibrin, which co-polymerizes to form the major component in the structural network of blood clots. The primary structures and sites of phosphorylation and sulphation have been determined for fibrinopeptides of many different species[2,3]. In order to assign the peptide peaks in high-performance liquid chromatography (HPLC) of fibrin clot supernatants from various species which contain different phosphorylated, sulphated and unmodified fibrinopeptides, a simple, non-radioactive HPLC procedure was developed which enables phosphorylated, sulphated and unmodified peptides to be determined without peptide isolation and individual analysis. A brief description of this work has been given[10].

Due to the small size of a peptide, highly ionic phosphate or sulphate groups contribute considerably to its net charge and polarity. An alteration in these properties leading to a change in peptide retention on a reversed-phase HPLC column would be anticipated upon removal of a phosphate or sulphate group. The individual contributions of tyrosine-O-sulphate as well as other amino acids to the overall retention coefficients of peptides have been investigated and quantitated[11].

Dephosphorylation with alkaline phosphatase followed by HPLC analysis has been applied to the identification of the phosphorylated form of human corticotropin[12] or of human fibrinopeptide A, which in the latter case was cleaved from phosphatase-treated fibrinogen by thrombin[13,14]. In the procedures reported here, fibrinopeptides are first cleaved from fibrinogen and subsequently analysed for phosphorylation or sulphation. HPLC chromatograms of the fibrinopeptide-containing clot supernatants are compared with those of phosphatase-treated clot supernatants in order to identify phosphorylated peptides from human and canine fibrinogen. A similar approach is used for the analysis of sulphated peptides present in rabbit and canine fibrin clot supernatants, whereby sulphate is removed from tyrosine-O-sulphate by hydrolysis with mild acid.

MATERIALS AND METHODS

Clotting of fibrinogen

Approximately 20 mg fibrinogen from human (Deutsche Kabi, Munich, F.R.G.), rabbit (Sigma, St. Louis, MO, U.S.A.) or dog (Sigma) were dissolved in 4 ml of 0.15 *M* ammonium acetate, brought to pH 8.5 with ammonia. Thrombin (Behringerwerke, Marburg/Lahn, F.R.G.) was added to a final concentration of 1 NIH (U.S. National Institute of Health trombin reference standard) unit/ml. After digestion for 2 h at 20°C, the clotted sample was placed in a bath of boiling water for 1 min, then centrifuged (4800 *g*) and part of the supernatant was applied directly to HPLC.

Enzymatic removal of phosphate

The dephosphorylation was carried out directly on 500 μl of clot supernatant. Alkaline phosphatase (Boehringer Mannheim, Marburg/Lahn, F.R.G.) was added

to a final concentration of 4 U/ml (U = Boehringer units). After digestion for 2 h at 20°C, an aliquot was applied to HPLC.

Removal of sulphate by acid hydrolysis

A 500-μl volume of clot supernatant was lyopholized to remove ammonium acetate and redissolved in 500 μl of 1 *M* hydrochloric acid. After heating for 1 min in a bath of boiling water, the sample was frozen, lyopholized and redissolved in 1 *M* formic acid. Part of the clot supernatant was applied to HPLC.

HPLC conditions

The conditions for the analysis of fibrinopeptides were essentially the same as described previously[15], using an analytical steel column (200 mm × 5.0 mm) packed with LiChrosorb RP-18 (Merck, Darmstadt, F.R.G.). A linear elution gradient was employed, with the following solvents: (A) 0.025 *M* ammonium acetate brought to pH 6.0 with orthophosphoric acid; (B) 0.05 *M* ammonium acetate brought to pH 6.0, plus an equal volume of acetonitrile (Uvasol grade, Merck).

Amino acid analysis

Fractions to be analysed were hydrolyzed under vacuum in 5.7 *M* hydrochloric acid for 24 h at 110°C. The samples were analysed on a Biotronic LC 600 amino acid analyser[16].

RESULTS

Two types of fibrinopeptides, referred to as A and B, are cleaved by the protease thrombin from the Aα- and Bβ-fibrinogen chains. This proteolytic action intitiates the formation of a fibrin blood clot. The resulting fibrinopeptides released into the clot supernatant were dephosphorylated by treating clot supernatants with alkaline phosphatase or desulphated by subjecting clot supernatants to mild acid hydrolysis. The primary structures and sites of phosphorylation or sulphation of the fibrinopeptides investigated here are shown in Fig. 1. In human fibrinopeptides a proportion of the fibrinopeptide A has been shown to be phosphorylated at the single serine residue[2]. Thus two fibrinopeptide A forms exist, a non-phosphorylated and a phosphorylated form, designated as A and AP respectively.

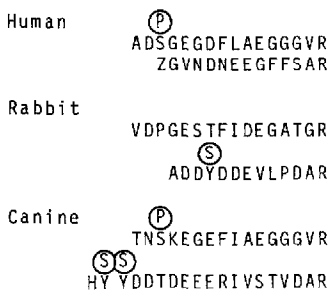

Fig. 1. Primary structures of the fibrinopeptides A and B of human, rabbit and canine. The structures are according to refs. 2–5, using the one-letter code[18]. Encircled P or S denotes phosphate or sulphate groups respectively. Due to incomplete phosphorylation or sulphation, modified fibrinopeptides often exist as mixtures together with unmodified forms.

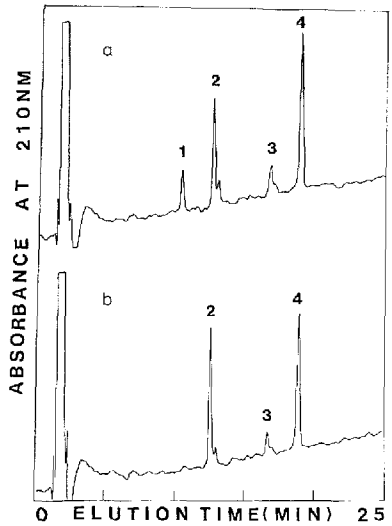

Fig. 2. The HPLC elution pattern of the clot supernatant peptides of human fibrin. The pattern of the untreated clot supernatant (a) is aligned with that of clot supernatant treated with alkaline phosphatase (b) as described under Materials and Methods. Approximately 10 μg were injected in each case onto a column packed with LiChrosorb RP-18 and the peptides separated using a linear gradient from 10 to 30% B over 30 min at a flow-rate of 1.5 ml/min. 0.05 a.u.f.s.

HPLC analysis of the clot supernatant of human fibrinogen gave the elution pattern shown in Fig. 2a. The amino acid compositions were determined (data not shown) from fractions corresponding to each peptide peak and compared to the known human fibrinopeptide structures shown in Fig. 1. Peaks 1 and 2 had identical amino acid compositions corresponding to the known sequence of human A, whereas peaks 3 and 4 corresponded to the structure of B. In order to to identify the peptide peaks in Fig. 2a corresponding to fibrinopeptides A and AP, clot supernatant was treated with alkaline phosphatase and subsequently analysed by HPLC as described under Materials and Methods, giving the chromatogram shown in Fig. 2b. Upon inspection of Fig. 2b, a peak eluting at the same position as peak 1 of the untreated sample (Fig. 2a) is absent and a concomitant increase in peak 2 is observed. This can be interpreted as the result of the removal of phosphate from AP, thereby converting it into A. Thus peak 1 in Fig. 2a represents AP and peak 2, peptide A. On the basis of peak heights, the relative concentrations are approximately 30% AP and 70% A. Since fibrinopeptide B is not phosphorylated, the retention times of peaks 3 and 4 are not affected by the phosphatase treatment.

As previously reported [13,14], a slow degradation of fibrinopeptide B occurs after its release by thrombin from fibrinogen, resulting in minor amounts of desarginine B, which was found in Fig. 2 to be eluted as peak 3 on the basis of its amino acid composition, since it lacked arginine but corresponded otherwise to the human fibrinopeptide B structure (Fig. 1). Peak 4 was identified as the intact peptide B, since it contained arginine and corresponded fully to the structure of B. It has been suggested that trace amounts of the enzyme responsible for this degradation copurify with Kabi fibrinogen and that the enzyme is specific towards fibrinopeptide B but

not fibrinopeptide A, which also contains a carboxy-terminal arginine[13,14]. This example serves to demonstrate the need to examine peptide retention time shifts which might result from cleavage. For example, the formation of desarginine B from intact B was established after amino acid analyses of both peptides. This degradation is apparently caused by carboxypeptidease-like enzyme contaminants present in Kabi fibrinogen and is not the result of the dephosphorylation procedure, since similar amounts of desarginine B occur in the untreated sample (Fig. 2a, peak 3) as well. Here, and in the following experiments, amino acid analyses were consistently carried out on all peptides before and after dephosphorylation or desulphation to confirm the integrity of their primary structures and to exclude the possibility of proteolytic or hydrolytic cleavage.

Sulphated peptides were investigated using a similar approach to that for phosphorylation, whereby sulphate removal was achieved chemically by mild acid hydrolysis. The rabbit fibrinopeptide B, whose structure is shown in Fig. 1, is sulphated at tyrosine[3], the sulphated form being designated here as BS. The chromatogram of the rabbit fibrin clot supernatant, shown in Fig. 3a, contains two major peaks. Peak 1 was identified by amino acid analysis as B and peak 3 as A. Rabbit clot supernatant was subjected to mild acid hydrolysis to remove sulphate as described in Materials and Methods. The hydrolysis products were analysed by HPLC, giving the chromatogram shown in Fig. 3b. Comparison with Fig. 3a shows that peptide peak 2 in Fig. 3b has a similar relative height but is eluted later than peak 1 of the untreated sample (Fig. 3a). Furthermore, peaks 1 and 2 (in Fig. 3a and b, respectively) had an identical amino acid composition which corresponded to the rabbit fibrinopeptide B structure. These results indicate that peak 1 in Fig. 3a corresponds to BS, which upon sulphate removal is converted into the less polar B, eluted later as peak 2. Peak 3, however, is not altered by mild acid since it represents fibrinopeptide A which does not contain any acid-hydrolyzable sulphate groups.

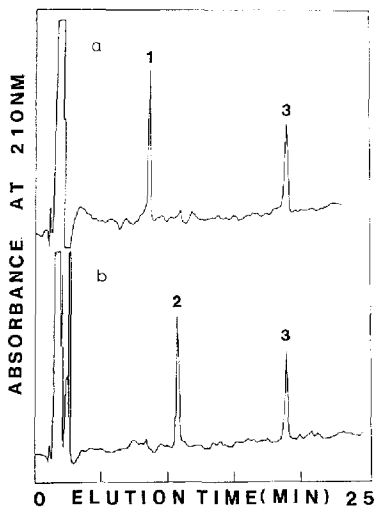

Fig. 3. The HPLC elution pattern of peptides in the clot supernatant of rabbit fibrin. The elution pattern of the untreated control material (a) is aligned with that of clot supernatant subjected to mild acid hydrolysis (b) as described under Materials and Methods. Approximately 10 μg were injected onto the column. Linear gradient: from 5 to 25% B over 30 min at a flow-rate of 1.5 ml/min. 0.05 a.u.f.s.

Both phosphorylation and sulphation have been described for the canine fibrinopeptides[5,17]. Canine fibrinopeptide A is found as a non-phosphorylated and phosphorylated form (A and AP) and the fibrinopeptide B as a mono and disulphated form (BS and BS_2 respectively) resulting from the possible sulphation of one or two of the adjacent tyrosine residues, as seen by their structures shown in Fig. 1. The HPLC elution of the dog fibrinopeptides, presented in Fig. 4a, consists of four major peaks. Peaks 1 and 2 in Fig. 4a were identified as canine A and peaks 3 and 4 as B by their amino acid compositions. Phosphorylation of A was investigated by treatment of the clot supernatant with alkaline phosphatase, as described in Materials and Methods. Subsequent HPLC analysis showed one peak to increase beyond the full scale of detection. Therefore, a smaller amount of phosphatase-treated material was analysed which corresponded to one-half of the untreated material analysed previously in Fig. 4a. The resulting elution pattern is shown in Fig. 4b. From the observed disappearance of peak 1 and the concomitant increase in peak 2, resulting from dephosphorylation of AP to A, it can be concluded that peak 1 represents AP and peak 2, A. Based on peak heights, the relative concentrations are approximately 30% A and 70% AP. Interestingly, these proportions are reversed for the human fibrinopeptides, *i.e.*, 70% A and 30% AP. The mono- and disulphated B peptides were identified as follows; clot supernatants were subjected to mild acid hydrolysis resulting in removal of most of the sulphate groups. The hydrolysis products were then analysed by HPLC and the resulting elution profile (Fig. 4c) aligned with that of the untreated sample (Fig. 4a). It is seen that the disappearance of peak 3 and the decrease in peak 4 is accompanied by the appearance of a more hydrophobic peak 5 in Fig. 4c, which had an amino acid composition identical to that of peaks 3 and 4. Peaks 3 and 4 were thus identified as BS_2 and BS respectively, since upon mild acid hydrolysis apparently all of the BS_2 is degraded to BS and B and most of the

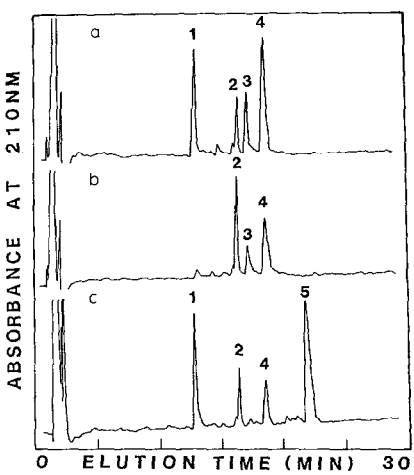

Fig. 4. The HPLC elution pattern of peptides in the clot supernatant of canine fibrin. The elution pattern of the untreated material (a) is aligned with that of phosphatase-treated (b) and of acid-treated (c) clot supernatants as described under Materials and Methods. In (b) half the amount of peptide material was analysed as in (a) and (c) where the amount was approximately 20 μg. Peptides were eluted by a linear gradient from 10 to 30% solvent B over 30 min at a flow-rate of 1.5 ml/min. 0.05 a.u.f.s.

BS is degraded to B. The relative concentrations of the sulphated peptides were 30% BS_2 and 70% BS. Furthermore, all of the canine B peptides were found to be sulphated, since no peak corresponding to desulphated B (peak 5 in Fig. 4c) is observed with the untreated sample (Fig. 4a).

DISCUSSION

The methodology described here enables the identification and assay of individual phosphorylated and sulphated peptides in peptide mixtures by comparison of the peptide elution pattern before and after dephosphorylation or desulphation. This treatment leads to changes in charge and polarity which are sufficient to alter their retention on reversed-phase HPLC columns. This is a property characteristic of peptides containing phosphate or sulphate which enables them to be distinguished from peptides lacking such modifications.

This approach offers an alternative to labelling with ^{32}P or ^{35}S, particularly in cases where radioactive labelling may not be feasible. The analysis is carried out with standard HPLC equipment and the peptides can readily be recovered in a lyophilizable solvent with their primary structures intact. Therefore, these procedures can also be adapted for preparative dephosphorylation or desulphation. Furthermore, the results obtained from the canine fibrinopeptides demonstrate that phosphorylated and sulphated peptides can be individually analysed in sample containing both phosphorylated and sulphated peptides, since the dephosphorylation or desulphation procedures appear to be specific. No side reactions were detected, particularly during the mild acid hydrolysis procedure used for desulphation of rabbit and canine fibrinopeptides. In these cases, retention shifts of sulphated peptides occurred as expected, whereas no alterations of other peptides present were found. The finding that these procedures can be used successfully on fibrinopeptides of differing structures suggests that they may also be useful for other types of peptides.

ACKNOWLEDGEMENTS

The helpful advice and encouragement of Dr. Chris Southan, Dr. David Lane and Mrs. Irene Oetken are gratefully acknowledged.

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