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# The Location of Tyrosine-*O*-sulphate in Fibrinopeptides

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The occurrence and location of tyrosine-*O*-sulphate in fibrinopeptides B from dog, cat, sheep, pig, and horse has been determined by means of quantitative and sequential analysis.

The occurrence, sulphation mechanism, and phylogenetic importance of the tyrosine-*O*-sulphate residues is discussed.

The fibrinogen molecule is built up by three peptide chains A, B and C.[1-3] ** During the conversion of fibrinogen to fibrin by the action of thrombin two main fibrinopeptides designated A and B are split off from the N-terminal end of the corresponding peptide chain of the molecule.[4-7]

Recently the amino acid sequences of fibrinopeptides from several different mammalian species have been established (*cf.* Ref. 8).

Tyrosine-*O*-sulphate has been found to be a constituent of ox and rabbit fibrinopeptide B.[9-11] The ultraviolet spectrum of fibrinopeptide B from several other species also indicates the presence of tyrosine-*O*-sulphate (*cf.* Ref. 8). Jevons [11] demonstrated that in some fibrinogens tyrosine-*O*-sulphate was present in other parts than the fibrinopeptide moiety of the molecule. Recently tyrosine-*O*-sulphate has been identified as an amino acid residue of gastrin II isolated from hog antral mucosa.[12] Free tyrosine-*O*-sulphate is excreted in the urine of many mammalian species.[13,14]

In the present paper the location of the tyrosine-*O*-sulphate residue in fibrinopeptide B from several animal species has been elucidated.

## MATERIAL AND METHODS

*Fibrinopeptides.* Fibrinopeptides B from dog, sheep, horse, pig, and cat were prepared essentially as has been described earlier.[15] In the case of dog fibrinopeptide B two analogues, $B_1$ and $B_2$, are present. $B_1$ and $B_2$ can be separated by chromatography on Dowex 50 × 2 or by electrophoresis. $B_2$ is more acidic than $B_1$.

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** *Note added in proof.* The nomenclature recommended by the International Committee on Haemostasis and Trombosis (Washington 1967) for the three peptide chains in fibrinogen is α(A), β(B), and γ, respectively.

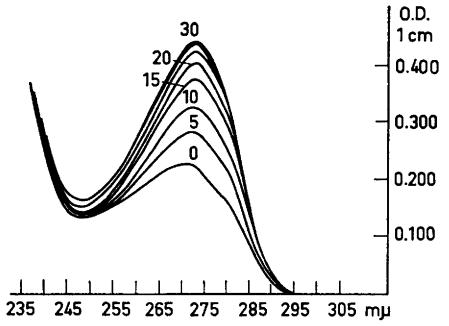


*Fig. 1a.* Increase in optical density during acid hydrolysis of 0.176 mM solution of dog fibrinopeptide $B_1$ (0.2 N HCl, 100°, 2, 5, 10, 15, 20, 25, 30 min). The zero time curve was obtained at room-temperature.

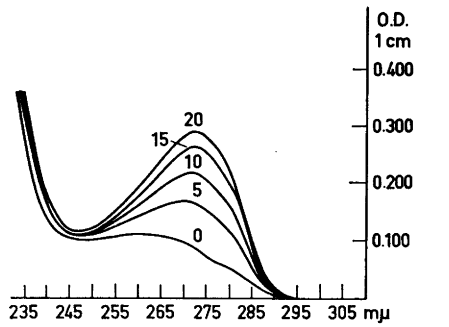


*Fig. 1b.* Increase in optical density during acid hydrolysis of 0.111 mM solution of dog fibrinopeptide $B_2$ (0.25 N HCl, 100°, 5, 10, 15, 20, 30 min). The zero time curve was obtained at room-temperature.

*Synthesis of tyrosine-O-sulphate.* Tyrosine was sulphated with sulphuric acid at low temperature according to the conditions used by Jevons [11] and Tallan *et al.*[13] for the preparation of the crystalline potassium salt. The crystalline product gave 3.5 % of the colour yield of an equimolar amount of tyrosine with the phenol reagent of Lowry *et al.*[16] The ultraviolet spectrum of the compound showed a maximum at 263 mμ. After acid hydrolysis (0.25 N HCl, 30 min, boiling water) the absorption characteristics of tyrosine was obtained. Tyrosine-*O*-sulphate was identified by two-dimensional thin-layer chromatography in solvents A and B (see below) and by column chromatography on Dowex 1 × 8 in acetate form.[13] The elution was accomplished by 2.2 N sodium acetate buffer, pH 5.2. (Elution with 1.1 N sodium acetate buffer, pH 5.0, as used in the original method [13] gave less satisfactory results.) The synthetic compound was found to be homogeneous by these two criteria.

*Amino acids.* Complete amino acid composition was generally determined in a Technicon Auto Analyzer (Technicon Instruments Corporation, Chauncey, N.Y.). Tyrosine residues in the peptides were determined quantitatively by the Folin phenol reagent using tyrosine as reference.[16]

*Inorganic sulphate* was determined turbidimetrically as barium sulphate.[11] Calibration curves were constructed with known amounts of ammonium sulphate (0.1—0.5 μatom S).

*Alkaline hydrolysis* [11] was performed in 0.2 N $Ba(OH)_2$ at 125° for 24 h in tubes filled with $N_2$.

*Two-dimensional thin-layer chromatography* was performed on silica gel (Silica gel DF5, Camag AG, Muttenz, Switzerland). The thickness of the gel layer was 0.25 mm.

The following solvent systems were found to be convenient.[17] 1) Solvent A: chloroform, methanol, 17 % (v/v) ammonium hydroxide (40:40:20, v/v). 2) Solvent B: phenol-water (75 g phenol + 25 ml water). Ascending chromatography was used. In the first dimension the solvent was run for 3 h, and in the second dimension over night (15 h). The spots were located by spraying with ninhydrin.

*Electrophoresis.* A conventional horizontal apparatus was used. The runs were made in 0.1 M pyridine-acetate buffer (pH 4.1) at a voltage of 8 V/cm for 3 h. The samples were applied to paper strips (Whatman No. 1) with a width of 2—3 cm. In some experiments two-dimensional electrophoresis-chromatography was performed. 1) First dimension: electrophoresis on silica gel plates in 0.1 M pyridine-acetate buffer, pH 4.1 for 3 h, 2) Second dimension: chromatography using solvent B (see above).

*Digestion with leucine aminopeptidase.* A product of Arzneimittelwerk Dresden, DDR, was used (Batch: 070565). 1 mg was dissolved in 0.2 ml 0.1 M tris buffer, pH 8.5 and stored in small test-tubes at −20°. $C_1$ for hydrolysis of L-leucine amide was by us found to be 11.

The digestions were performed in 0.2 M ammonium acetate, pH 8.4 and 40°. Weight ratio of enzyme:substrate was 1:14. Before digestion the enzyme was activated by $Mn^{2+}$.[18] 5 µl of enzyme solution in Tris buffer were added to a mixture of 10 µl 0.025 M manganese chloride and 30 µl 0.5 M ammonium acetate, pH 8.4, and kept at 40° for 30 min. A second addition of 10 µl manganese chloride and 40 µl ammonium acetate buffer was then made. The solution was mixed gently and 0.10—0.15 µmole of peptide B was added. Immediately afterwards a 10 µl sample was taken off ("0" time sample), mixed with 2 µl of 0.2 M sodium citrate buffer, pH 5, (inhibition of enzyme) and subjected to two-dimensional thin-layer chromatography or electrophoresis-chromatography (see above). At different times of incubation similar samples were withdrawn and treated as mentioned above.

## EXPERIMENTAL AND RESULTS

*Determination of tyrosine-*O*-sulphate in dog fibrinopeptide B.* Samples (0.2—0.4 mg) of dried fibrinopeptide B were treated with 1 ml of 0.25 N HCl and heated by immersion in boiling water for 30 min. The change in ultraviolet spectrum in the course of hydrolysis of dog fibrinopeptides $B_1$ and $B_2$ are shown in Figs. 1a and 1b. The hydrolysis curves for the B-peptides of the other species are similar to those shown for the dog peptides. The phenolic tyrosine residues determined before and after hydrolysis of the different fibrinopeptides are presented in Table 1. In dog fibrinopeptide B as well as in those of sheep and cat there are 2 moles of phenolic tyrosine per mole of peptide after complete hydrolysis. In peptide $B_1$ of the dog the ratio of

*Table 1.* Tyrosine and sulphur per µmole of fibrinopeptide B from different species determined before and after acid hydrolysis (0.25 N HCl, 30 min, 100°).

| Species | Number of expts. | Tyr * before hydrolysis (µmole) | Tyr * after hydrolysis (µmole) | S before hydrolysis (µatom) | S after hydrolysis (µatom) | Ratio Tyr/S |
|---|---|---|---|---|---|---|
| Dog $B_1$ | 8 | 0.95 | 1.84 | 0 | 0.82 | 2.20 |
| Dog $B_2$ | 8 | 0.45 | 1.88 | 0 | 1.51 | 1.2 |
| Sheep | 5 | 0.97 | 1.86 | 0 | 0.92 | 2.1 |
| Cat | 3 | 0.0 | 1.76 | 0 | 1.90 | 0.9 |
| Horse | 4 | 0.05 | 0.95 | 0 | 0.86 | 1.1 |
| Pig | 3 | 0.11 | 0.92 | 0 | 0.71 | 1.3 |

* Determined with the modified phenol reagent.[16]

phenolic tyrosine before and after hydrolysis is 1:2, whereas in peptide $B_2$ this ratio is 1:4. This indicates that in peptide $B_1$ one of the hydroxyl groups is free and the other blocked. On the other hand, in peptide $B_2$ 75 % of the hydroxyl groups seems to be blocked. The small amounts of unblocked tyrosine residues in peptide $B_2$ are most likely due to contamination by peptide $B_1$ in the $B_2$ sample (Fig. 2). It is likely that part of peptide $B_2$ has been converted to peptide $B_1$ during isolation of the peptide. The results of the hydrolysis experiments further indicate that in the sheep B peptide one hydroxyl group

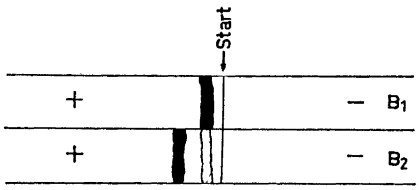


*Fig. 2.* Low-voltage paper electrophoresis of dog fibrinopeptides $B_1$ and $B_2$ (0.1 mg of each peptide in 15 µl of 0.1 N pyridine solution was applied).

of tyrosine is free and one is blocked. In the cat fibrinopeptide B both hydroxyl groups are blocked. The tyrosine residue of horse and pig fibrinopeptides are also blocked.

Inorganic sulphate was consistently found to be liberated in the course of the hydrolysis (Table 1). This suggests that the increase in phenolic tyrosine during acid hydrolysis is due to the hydrolysis of a tyrosine-*O*-sulphuric acid ester in the peptide. It is evident that the relation tyrosine to sulphur in dog fibrinopeptide $B_1$ as well as in sheep peptide is close to 2:1, whereas in dog fibrinopeptide $B_2$ and in cat peptide it is close to 2:2. In the case of horse and pig fibrinopeptides this relation is 1:1 (Table 1).

Further evidence of the occurrence of tyrosine-*O*-sulphate in the fibrinopeptides was obtained by chromatographic identification of this compound in alkaline hydrolysates of the peptides. Dog fibrinopeptide $B_1$ (0.2—0.5 mg) was heated in 0.1 ml of 0.2 N barium hydroxide at 125° for 24 h in a sealed tube. Afterwards the hydrolysate was divided in two aliquots. One aliquot was directly subjected to two-dimensional chromatography on silica gel, the second aliquot was after neutralization (with 0.5 N hydrochloric acid), hydrolysed in 0.25 N hydrochloric acid (boiling water, 30 min). In the former sample both tyrosine-*O*-sulphate and tyrosine could be identified, whereas in the latter the spot corresponding to tyrosine-*O*-sulphate had disappeared. In alkaline hydrolysates of dog peptide $B_2$ a strong spot corresponding to tyrosine-*O*-sulphate was found. Only traces of tyrosine were detected. After acid hydrolysis of the alkaline hydrolysate tyrosine was demonstrated. After alkaline hydrolysis of cat, horse, and pig fibrinopeptide B tyrosine-*O*-sulphate but no tyrosine was demonstrated. As in the previous experiments tyrosine could be demonstrated after acid hydrolysis of the alkaline hydrolysates. In sheep fibrinopeptide tyrosine as well as tyrosine-*O*-sulphate were disclosed in the alkaline hydrolysates. From all the above experiments we conclude that dog peptide $B_1$ and the B-peptides of horse, pig, and sheep contain one tyrosine-*O*-sulphate residue. In the dog peptide $B_2$ and in the cat B peptide two such residues are present.

*Location of tyrosine-*O*-sulphate in the fibrinopeptides.* In dog fibrinopeptide $B_1$ and sheep peptide B where only one of the two tyrosine residues is sulphated, the location of the tyrosine-*O*-sulphate residue in the chain became important. This was done by means of digestions of the fibrinopeptides with leucine aminopeptidase. Both peptide $B_1$ and $B_2$ of dog fibrinogen were digested with leucine aminopeptidase. After 0.5, 1, 3, 6, and 24 h aliquots (10 µl) were withdrawn from the digest and subjected to thin-layer chromatography. In peptide $B_1$ histidine and tyrosine were the first amino acids to appear (Fig. 3).

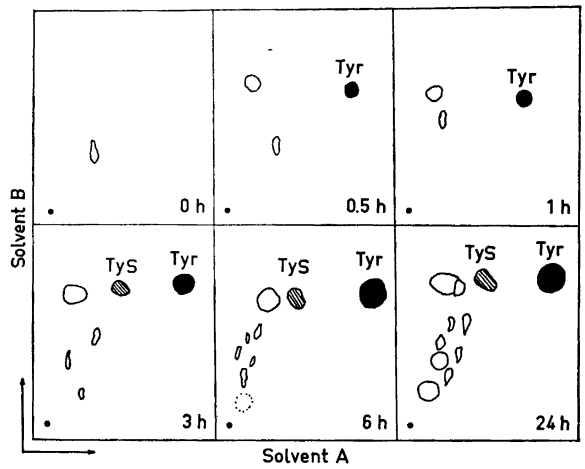


*Fig. 3.* Liberation of amino acid from dog fibrinopeptide $B_1$ by leucine aminopeptidase after different incubation times. Two-dimensional thin-layer chromatography. 10 µl applied.

After 3 h digestion a spot corresponding to tyrosine-*O*-sulphate was visible. This order of release was also confirmed by quantitative amino acid analysis of the samples (Table 2). In peptide $B_2$ (Fig. 4) only tyrosine-*O*-sulphate appeared during the first 3 h of digestion. After longer digestion times small amounts of tyrosine could also be detected. This tyrosine is apparently derived from the $B_1$-peptide contaminating the $B_2$ preparation (Fig. 2).

In the subsequent experiments the location of the tyrosine-*O*-sulphate residue in sheep fibrinopeptide was explored by means of digestion with leucine amino peptidase. Sheep peptide B was digested with the enzyme for 0.5, 1, 3, 6, and 24 h. In this case satisfactory separation of the particular

*Table 2.* Quantitative determination of amino acid split off from dog fibrinopeptides $B_1$ by leucine aminopeptidase. 0.9 µmole of fibrinopeptide in 420 µl of incubation mixture was incubated at an enzyme to substrate ratio of 1:14 (for further details see text). 70 µl of sample were withdrawn at 0, 1/2, 1, 3, 6, and 24 h and applied to the column.

| Time h | Amino acid, µmoles | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | His | Tyr | TyS | Asp | Thr | Glu | Arg | Val | Ala |
| 0 | — | — | — | — | — | — | — | — | — |
| 1/2 | 0.0175 | 0.0219 | 0.0192 | — | — | — | — | — | — |
| 1 | 0.0402 | 0.0391 | 0.0255 | — | trace | — | 0.0020 | — | — |
| 3 | 0.0454 | 0.0458 | 0.0271 | 0.0053 | » | — | 0.0055 | — | — |
| 6 | 0.0849 | 0.0804 | 0.0670 | 0.0094 | » | 0.0067 | 0.0102 | — | — |
| 24 | 0.1141 | 0.1305 | 0.1729 | 0.0417 | » | 0.0454 | 0.0192 | 0.0108 | 0.0095 |

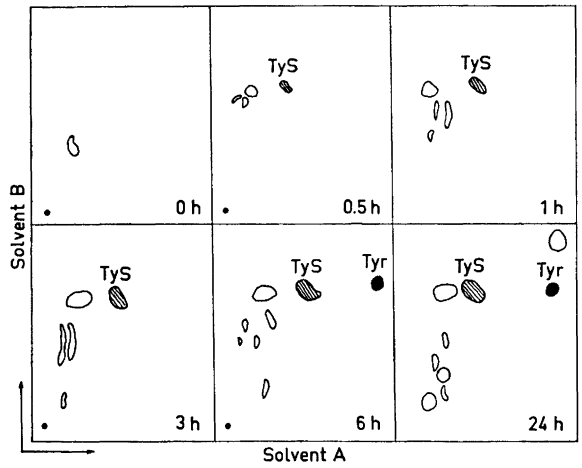


*Fig. 4.* Liberation of amino acid from dog fibrinopeptide $B_2$ by leucine aminopeptidase after different incubation times. Two-dimensional thin-layer chromatography. 10 $\mu$l applied.

amino acids released could only be achieved by two-dimensional electrophoresis-chromatography on thin-layer plates. The first amino acids to appear were glycine, tyrosine, and leucine. After 24 h of incubation the first trace of tyrosine-*O*-sulphate appeared (Fig. 5).

From all the above results and the known amino acid sequences of the fibrinopeptides (*cf.* Ref. 8) the position of tyrosine-*O*-sulphate (TyS) in the investigated fibrinopeptides B could be deduced as follows:

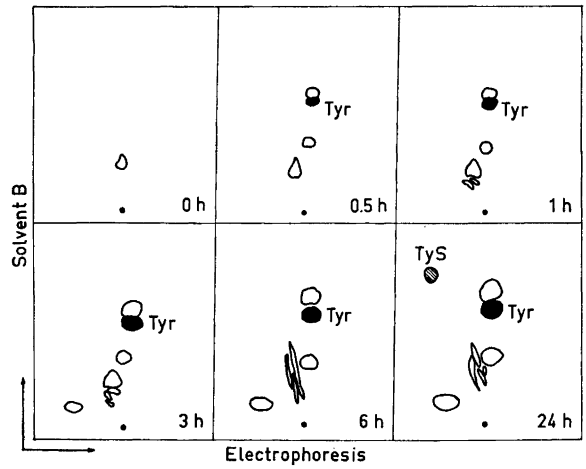


*Fig. 5.* Liberation of amino acid from sheep fibrinopeptide B by leucine aminopeptidase after different incubation times. Separation by electrophoresis-thin layer chromatography.

| | | |
|---|---|---|
| Dog $B_1$ | H-His-Tyr-TyS-Asp- | Arg-OH |
| » $B_2$ | H-His-TyS-TyS-Asp- | Arg-OH |
| Sheep | H-Gly-Tyr-Leu-Asp-TyS-Asp- | Arg-OH |
| Cat | H-Ile-Ile-Asp-TyS-TyS-Asp- | Arg-OH |
| Horse | H-Leu-Asp-TyS-Asp- | Arg-OH |
| Pig | H-Ala-Ile-Asp-TyS-Asp- | Arg-OH |

## DISCUSSION

Tyrosine-*O*-sulphate was first identified by Bettelheim in bovine fibrinopeptide B.[9] The species in which tyrosine-*O*-sulphate has been identified with certainty are so far ox, sheep, pig, horse, cat, and dog. The ultraviolet spectra of fibrinopeptide B from another 20 mammalian species suggest that its occurrence is rather common among fibrinopeptides of the B-type. However, tyrosine-*O*-sulphate is not present in fibrinopeptide B from several primates and not in rodents such as rat and guinea-pig. The presence of tyrosine-*O*-sulphate in proteins is not confined only to the fibrinopeptides. Jevons[11] has found it to be present in other parts of the fibrinogen molecule than the fibrinopeptide part. It has also been identified in gastrin.[12]

It is likely that the sulphation of the tyrosine residues in the protein occurs enzymatically during or after the biosynthesis. Structural features, such as aspartic acid residues, around the tyrosine residue in a peptide chain may be of particular importance for the enzymatic sulphation and/or the stability of the sulphate ester. The fact that in gastrin II[12] the sequence around the sulphated tyrosine is Ala-TyS-Gly indicates that at least in the sulphation reaction a variety of amino acid sequences around the susceptible tyrosine is permissible. It is interesting in this connexion to note that the two tyrosine residues found in fibrinopeptides from cat and dog are both sulphated. In cat the amino acid residue on both sides of the tyrosines is aspartic acid. In dog, however, histidine is on one side and aspartic acid on the other. In the latter case mainly the mono-substituted peptide variant is isolated from the fibrinogen and furthermore the disulphated peptide seems to be degraded into the mono-substituted variety during the isolation procedure. In the cat, on the other hand, only the disulphate peptide is found and no sign of instability has been observed. Like the dog fibrinopeptide B, gastrin has also been isolated in a desulphated (gastrin I) variety. It may be that a $\beta$-carboxyl group of aspartic acid close to the tyrosine-*O*-sulphate residue stabilizes the sulphate ester.

The tyrosine-*O*-sulphate residue in fibrinopeptides from different species provides a good phylogenetic tracer as its position in the chain is the same in the closely related species so far investigated. Thus it is at position 16 in the artiodactyls, at 17 in perissodactyls and 15—16 in several carnivores. During evolution tyrosine-*O*-sulphate seems frequently to have been exchanged with aspartic acid. If we assume that hydrophilic residues usually are on the outside of a molecule this would indicate that the tyrosine-*O*-sulphate residue is on the surface of the fibrinogen molecule.

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