# Heterogeneity in the Tyrosine Sulfation of Chinese Hamster Ovary Cell Produced Recombinant FVIII

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ABSTRACT: By the use of recombinant technology, several stable Chinese hamster ovary (CHO) cell lines expressing human FVIII were established. Thrombin treatment and SDS–PAGE analysis of the purified recombinant FVIII (rFVIII) revealed a striking difference from plasma-derived FVIII (pFVIII). A 43-kDa fragment of the FVIII heavy chain appears as a double band from rFVIII, while a single band from pFVIII is observed. All other fragments from the two samples appeared similar by SDS–PAGE. The heterogeneity is caused by incomplete tyrosine sulfation of one or more of the three potential tyrosine sulfation sites ($Tyr_{718}$, $Tyr_{719}$, $Tyr_{723}$). To investigate if there is a general limitation and heterogeneity in the tyrosine sulfation of rFVIII, two other potential tyrosine sulfation sites on the FVIII light chain ($Tyr_{1664}$, $Tyr_{1680}$) were analyzed. The results show that both sites on the pFVIII light chain and on the rFVIII light chain are completely sulfated. The limitation of CHO cells to tyrosine sulfate rFVIII is therefore only restricted to a few sites. The two sulfated forms of rFVIII can easily be separated by ion-exchange chromatography, indicating the importance of the sulfate groups on the charge and/or conformation of FVIII. Both forms of rFVIII possess identical in vitro coagulation activity, von Willebrand factor binding, and thrombin activation profile. However, the difference in tyrosine sulfation may change other biological properties of FVIII.

Coagulation factor VIII (FVIII) participates in blood coagulation, where it acts as a cofactor in the activation of factor X to $X_a$ by factor $IX_a$. Factor VIII is absent or defective in the plasma of individuals with hemophilia A.

FVIII is synthesized as a single-chain protein with a molecular mass of approximately 330 kDa (Vehar et al., 1984; Toole et al., 1984; Truett et al., 1985). FVIII is isolated from plasma (Vehar & Davie, 1980; Fass et al,. 1982; Fulcher & Zimmerman, 1982) or recombinant cell cultures as a two-chain complex (Eaton et al., 1987). A few percent full-length FVIII can be obtained if enzyme inhibitors are added during purification (Rotblat et al., 1985). FVIII–light chain (FVIII–LC) with a molecular mass of 77/80 kDa is derived from the C-terminal part of full-length FVIII. FVIII–heavy chain (FVIII–HC) with a molecular mass of 92–210 kDa is derived from the N-terminal part of full-length FVIII.

Thrombin treatment changes FVIII coagulant activity through specific cleavages. By this process, 43- and 50-kDa protein fragments are generated from the heavy chain of FVIII, and a 41 amino acid peptide and a 70/67-kDa fragment are generated from FVIII light chain (Eaton et al., 1986). Using the criteria proposed by Huttner (1987) to predict tyrosine sulfation sites, it can be deduced from the primary sequence that FVIII contains seven potential tyrosine sulfation sites. These sites are distributed as follows: one site on the 50-kDa fragment ($Tyr_{346}$), four sites on the 43-kDa fragment ($Tyr_{395}$, $Tyr_{718}$, $Tyr_{719}$, $Tyr_{723}$), and two sites on the FVIII–LC ($Tyr_{1664}$, $Tyr_{1680}$). The functions of tyrosine sulfate on FVIII are unknown. Experiments have suggested that mutation of some of the tyrosine residues on FVIII to phenylalanine had negligible effect on in vitro coagulant activity; other biological activities were not analyzed (Pittman et al., 1987). For other proteins, it has been shown that tyrosine sulfation influences stability and biological activity (Huttner, 1987; Suiko & Liu, 1988). It is therefore important to verify that recombinant FVIII (rFVIII) intended for treatment of hemophiliacs has the same tyrosine sulfation pattern as plasma-derived FVIII (pFVIII). In this paper, we report that rFVIII produced from transfected Chinese hamster ovary (CHO) cells is secreted in two different tyrosine sulfated forms. One of the two forms of rFVIII is tyrosine sulfated in a different way than pFVIII.

## Experimental Procedures

### *Materials*

The Coatest FVIII kit was from Kabi. L-1-(Tosylamino)-2-phenylethyl chloromethyl ketone treated trypsin was from Sigma. *N*-Glycosidase F (*N*-glycanase) from *Flavobacterium meningosepticum*, neuraminidase from *Clostridium perfringens*, endo-α-*N*-acetylgalactosamidase (*O*-glycanase) from *Diplococcus pheumoniae*, and human thrombin and arylsulfatase from *Helix pomatia* were supplied by Boehringer Mannheim. *Staphylococcus aureus* proteinase was from ICN Biomedicals, IL. Alkaline phosphatase conjugated affinity-purified rabbit anti-mouse IgG was from Dakopatts, Denmark. Nitrocellulose (0.45-μm pore size) was obtained from Schleicher & Schuell.

### *Methods*

*Purification of pFVIII and rFVIII.* Plasma-derived human FVIII was purified from a commercial FVIII concentrate (NORDIOCTO) as described previously (Truett et al., 1985). Recombinant human FVIII was obtained from CHO-conditioned medium using ion-exchange chromatography and monoclonal antibody immunoaffinity chromatography. Purified pFVIII and rFVIII were stored at −80 °C in 20 mM imidazole, pH 7.0, 150 mM NaCl, 10 mM $CaCl_2$, 10% glycerol, and 0.02% NP-40. FVIII coagulation activity (FVIII:C) was measured by Coatest analysis adapted for microplates (Prowsee et al., 1986) and by one-stage coagulation analysis (Proctor & Rapaport, 1961).

*Preparation of pFVIII–LC and rFVIII–LC.* pFVIII–LC was prepared from a FVIII concentrate (NORDIOCTO) by affinity chromatography (Nordfang & Ezban, 1988), and rFVIII–LC was prepared from rFVIII on a Mono-Q column as follows: rFVIII–LC was dissociated from rFVIII–HC by incubating rFVIII in 0.02 M EDTA. To separate the two chains, the solution was applied at 1.0 mL/min to a Mono-Q

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5/5 anion-exchange column previously equilibrated in buffer A: 0.02 M imidazole, pH 6.8, 0.025 M $CaCl_2$, 0.02% NP-40, and 10% glycerol. rFVIII–LC was eluted with a linear gradient of buffer A + 0.3 M NaCl at 1.0 mL/min. rFVIII–LC eluted at approximately 0.15 M NaCl in a single peak. rFVIII–HC was eluted from the column by buffer A + 0.6 M NaCl.

*Separation of the Two Sulfated Forms of rFVIII.* The two sulfated forms of rFVIII were separated on a Mono-Q 5/5 anion-exchange column equilibrated in buffer A: 0.02 M Tris-HCl, pH 7.5, 0.01 M $CaCl_2$, 10% glycerol, and 0.01% Tween-80. rFVIII was eluted with a gradient of buffer B (buffer A + 0.5 M NaCl) at 1.0 mL/min.

The unsulfated form of rFVIII eluted at approximately 0.3 M NaCl, and the sulfated form of rFVIII eluted at 0.5 M NaCl.

*Gel Electrophoresis and Immunoblotting.* Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed on reduced samples by the method of Laemmli (1970) using 7.5% polyacrylamide gels. After electrophoresis, the gels were silver-stained (Morrissey, 1981) or the proteins electrophoretically transferred from the gels to nitrocellulose sheets (Burnette, 1981). Immunoblotting was performed as described by Mikkelsen and Knudsen (1987). FVIII on nitrocellulose blots was detected by treatment with two monoclonal antibodies against the 43-kDa FVIII–HC fragment and the FVIII–LC, followed by an alkaline phosphatase conjugated affinity-purified rabbit anti-mouse IgG. The alkaline phosphatase activity was detected by the method of Blake et al. (1984).

*Thrombin Treatment.* FVIII or FVIII–LC in 20 mM imidazole, pH 7.0, 150 mM NaCl, 5 mM $CaCl_2$, 10% glycerol, and 0.02% NP-40 was incubated with thrombin (0.5 unit/mL) for 1 h at 37 °C. The reaction was stopped by precipitation with 10% trichloroacetic acid or by injecting the sample onto a RP-HPLC column. rFVIII [2.5 units (5 units/mL)] was incubated with 0.04–0.2 unit of thrombin in 20 mM imidazole, 150 mM NaCl, 10 mM $CaCl_2$, and 0.02% Tween 80, pH 7.3 at 22 or 37 °C, over a period of 2 h. At various time points during the incubation, coagulation activity was determined by using the one-stage coagulation assay.

*Endoglycosidase Treatment.* For *N*-glycanase treatment, the thrombin-treated FVIII samples were trichloroacetic acid precipitated and dissolved in 0.1 M imidazole, pH 7.2, 10 mM EDTA, 10 mM DTT, and 1% SDS. The samples were incubated for 10 min at 95 °C and then cooled to 0 °C. Four times the original volume of 0.1 M imidazole, pH 7.2, 10 mM EDTA, and 10 mM DTT was added; the samples were again incubated for 10 min at 95 °C and then immediately cooled to 0 °C; 10% NP-40 was added to the denatured protein solution to a final concentration of 1%. *N*-Glycanase was added at a concentration of 0.6 unit/μg of protein and incubated overnight at 37 °C. The reaction was stopped by heating the samples to 95 °C.

For neuraminidase and *O*-glycanase treatment, FVIII was incubated with neuraminidase (0.005 unit/μg of protein) in 10 mM imidazole buffer, pH 6.0, 100 mM NaCl, and 0.02% Tween-80 for 1 h at 37 °C. After incubation, the samples were thrombin-treated, trichloroacetic acid precipitated, and dissolved as described above. *O*-Glycanase was then added (0.005 unit/μg of protein) and incubated for 1 h at 37 °C.

*Peptide Mapping and Amino Acid Sequence Analysis.* Peptide mapping of the 41.5- and 43-kDa fragments of FVIII was performed as described by Aebersold et al. (1987). Proteins were separated by SDS–PAGE and electroblotted onto nitrocellulose. Protein bands were visualized by Ponceau S, and then in situ digested by trypsin. The extracted peptides were separated on an Applied Biosystem 130A micro-bore HPLC system equipped with a Brownlee Aquapore RP-18 5-μm column (2.1 × 30 mm). The following buffer system was used: buffer A, 0.1% trifluoroacetic acid in $H_2O$; buffer B, 0.08% trifluoroacetic acid/60% 2-propanol in $H_2O$. The peptides were eluted with the following gradient: 0% buffer B in 4 min, 0–50% buffer B in 36 min, 50–70% buffer B in 10 min, 70–0% buffer B in 1 min. Flow was 150 μL/min. Detection was at 214 nm. For amino-terminal sequence analysis, the proteins were first separated on SDS–PAGE and electroblotted to an Immobilon-P transfer membrane. Protein bands were visualized by Coomassie blue and directly sequenced (LeGendre & Matsudaira, 1988). Automated Edman degradation was performed on an Applied Biosystem Model 477A protein sequencer equipped with on-line analysis of PTH-amino acids.

*Purification and Digestion of the N-Terminal Fragment (41 Amino Acid Peptide) of FVIII–LC.* pFVIII–LC and rFVIII–LC were cleaved with thrombin, and the 41 amino acid N-terminal peptide fragment was purified by using the same HPLC system and buffers as described above. The HPLC system was equipped with a Brownlee Aquapore Butyl 7-μm column (2.1 × 30 mm). The peptide was eluted with the following gradient: 0% buffer B in 4 min, 0–100% buffer B in 36 min, 100–0% buffer B in 1 min. Flow was 150 μL/min. The 41 amino acid peptide eluted at approximately 28% buffer B in a single peak.

The 41 amino acid peptide was freeze-dried and dissolved in 100 mM ammonium bicarbonate, pH 7.8. *Staphylococcus aureus* V8 proteinase was added to the peptide in a ratio of 1:100 (E/S), and the digestion was allowed to proceed at 37 °C at 2 h. The digestion was terminated by injecting the sample onto the HPLC or by adding 10 μL of trifluoroacetic acid. The peptides were separated on the same HPLC system, column, and buffers as used for peptide mapping of the 41.5- and 43-kDa fragments. The gradient was changed as follows: 0% buffer B in 4 min, 0–50% buffer B in 26 min, 50–70% buffer B in 10 min, 70–0% buffer B in 1 min. Flow was 150 μL/min.

*Desulfation of Tyrosine Sulfate.* Removal of sulfate from tyrosine sulfate by weak acid hydrolysis was performed by a modification of the procedure described by Lucas and Henschen (1986). Samples for desulfation were heated in 1.0 M HCl for 3 min at 100 °C. After being heated, the samples were either lyophilized or injected directly onto the HPLC. For enzymatic desulfation, the peptides (10–50 ng) were lyophilized and incubated with 100 μg arylsulfatase for 3 h in 50 μL of 100 mM ammonium acetate, pH 5.2. After incubation, the samples were analyzed by HPLC.

## Results and Discussion

Transient expression of biologically active recombinant factor VIII from a mammalian cell line (COS-7) has been described (Truett et al., 1985: Burke et al., 1986). We have obtained expression of human factor VIII from several transformed stable CHO cell lines. Thrombin treatment and SDS–PAGE analysis of purified factor VIII show a striking difference between pFVIII and rFVIII. The 43-kDa heavy-chain fragment appears as a double band at 41.5 and 43 kDa in rFVIII, while only a single band of 43 kDa is observed in pFVIII (Figure 1). All other FVIII-related fragments appeared similar. The appearance of a double band in rFVIII could be caused by difference in the amino acid sequence and/or different posttranslational modifications.

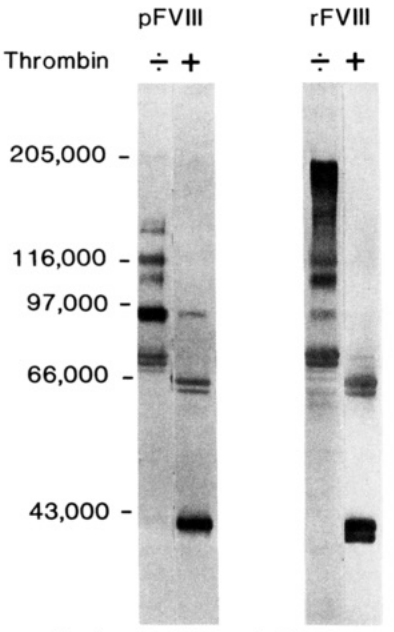


FIGURE 1: Western blotting of pFVIII and rFVIII. 0.4 μg of FVIII or thrombin-activated FVIII was resolved on a 7.5% SDS–polyacrylamide gel. The proteins were transferred to nitrocellulose. To detect FVIII, a mixture of two monoclonal antibodies against the 43-kDa FVIII–HC fragment and FVIII–LC, respectively, was used. Lane 1, pFVIII; lane 2, thrombin-activated pFVIII; lane 3, rFVIII; lane 4, thrombin-activated rFVIII.

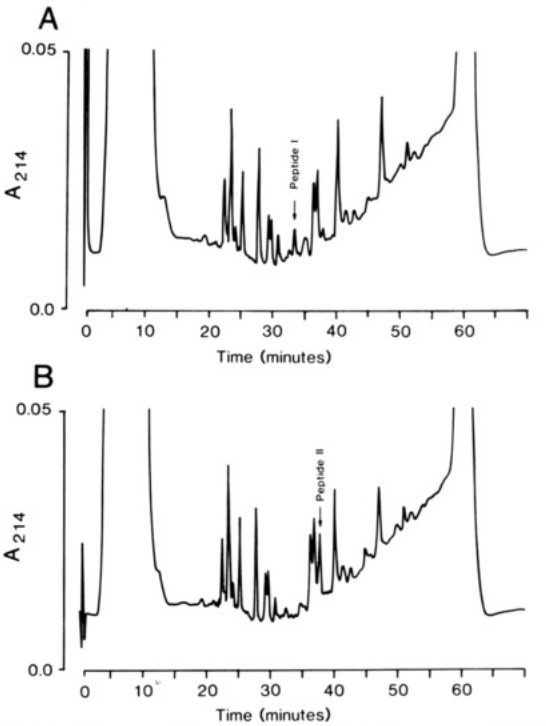


FIGURE 2: Peptide map of the 41.5- and 43-kDa fragments of rFVIII. Thrombin-activated rFVIII (130 μg) was applied on a 7.5% SDS–polyacrylamide gel and after electrophoresis transferred to NT. The peptide maps were performed as described under Experimental Procedures. (A) Proteolytic map of the 43-kDa fragment; (B) proteolytic map of the 41.5-kDa fragment; (—) absorbance at 214 nm.

Also by pulse chase analysis of immunoprecipitated thrombin-cleaved rFVIII produced by CHO cells, the 43-kDa FVIII–HC fragment appears as a double band, indicating that the double band is not an artifact of the purification procedure. A similar double band originating from the FVIII–HC is seen by Pittman and Kaufman (1988), who also obtained rFVIII from CHO cells. The 43-kDa fragment of FVIII contains one potential N-linked glycosylation site. In order to explore the potential role of glycosylation in the observed heterogeneity, thrombin-cleaved rFVIII was treated with *N*-glycanase and a mixture of neuraminidase and *O*-glycanase. The effect of carbohydrate removal was monitored by SDS–PAGE. Neither *N*-glycanase nor neuraminidase/*O*-glycanase treatment had any apparent effect on the electrophoretic migration (data not shown). This indicates that the observed heterogeneity is not caused by a difference in glycosylation.

To analyze if the difference between the 41.5- and 43-kDa fragments was caused by a different thrombin cleavage site, the N-terminal sequences of both fragments were determined. The amino acid sequence of the first eight amino acid residues determined for both fragments was found to be identical with the amino acid sequence of the 43-kDa fragment of pFVIII. As no difference was found in the N-terminal sequence, HPLC peptide mapping was performed on the two fragments after in situ protease digestion of the electroblotted fragments on nitrocellulose (Figure 2). The peptide map originating from the 41.5-kDa fragment differs from the peptide map of the 43-kDa fragment by the presence of an extra peptide (peptide II, Figure 2B) and the absence of one peptide (peptide I, Figure 2A).

The amino acid sequence of peptide II was determined [Asn-Thr-Gly-Asp-Tyr-Tyr-Glu-Asp-Ser-Tyr-Glu-Asp-Ile-Ser-Ala-Tyr-Leu-Leu-(Ser)-(Lys)], and from the reported sequence of FVIII (Truett et al., 1985), it can be concluded that this sequence is identical with the tryptic cleavage peptide (residues 714–733). Therefore, the difference in the peptide maps of the 41.5- and 43-kDa fragments cannot be caused by an alteration in the amino acid sequence. However, the amino acid sequence of peptide II revealed the presence of three potential tyrosine sulfation sites ($Tyr_{718}$, $Tyr_{719}$, $Tyr_{723}$). Tyrosine sulfate cannot be detected during amino acid sequence analysis, as the sulfate group is removed during sequential Edman degradation (Hortin et al, 1986). Sulfation of tyrosine on a peptide will decrease its hydrophobicity; thus, desulfated peptides will be more strongly retained on an RP-HPLC column and elute later than their corresponding sulfated peptide counterpart. Since the tyrosine sulfate ester is remarkably acid-labile, a short acid treatment will lead to loss of sulfate from tyrosine, with minimal hydrolysis of peptide bonds. To confirm whether peptide I corresponds to the sulfated form of peptide II, peptide I was submitted to desulfation by weak acid hydrolysis and rechromatographed in the same gradient system as used for peptide mapping (Figure 3). After subtraction of the peaks from the blank run (Figure 3A), it can be seen (Figure 3B) that peptide I now elutes at the same position as peptide II. This peptide was sequenced and shown to have the same amino acid sequence as previously found for peptide II. These results indicate that the difference between the peptide map of the rFVIII 41.5-kDa fragment and the 43-kDa fragment is due to lack of tyrosine sulfation of the 41.5-kDa fragment. However, by this method, it is not possible to determine the actual number of tyrosine sulfate groups on the peptide.

To examine if the difference in tyrosine sulfation caused the observed change in mobility between the 41.5- and 43-kDa fragments, thrombin-cleaved rFVIII was desulfated by acid hydrolysis before SDS–PAGE (Figure 4). The weak acid hydrolysis increased the relative amount of 41.5-kDa fragment. However, it was not possible to make a complete conversion of the 43-kDa fragment, because the conditions used for complete desulfation would cause extensive cleavage of the polypeptide chain.

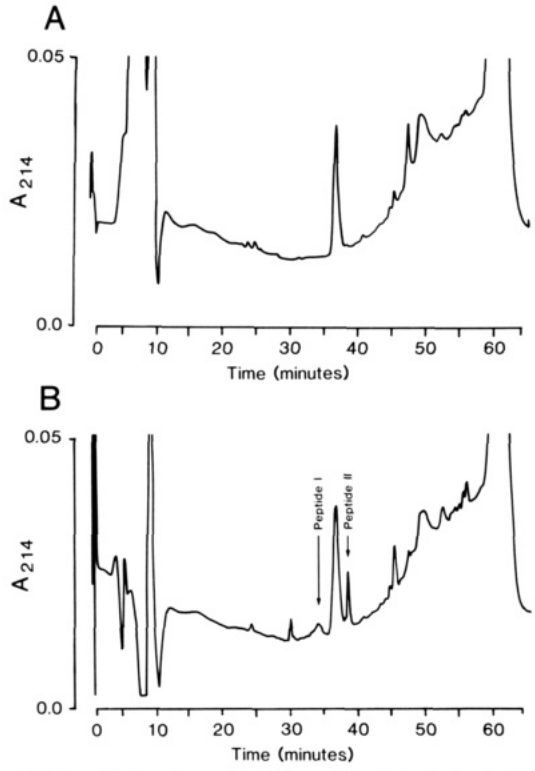


FIGURE 3: Desulfation of peptide I by weak acid hydrolysis. Peptide I from Figure 2A was desulfated as described under Experimental Procedures and rechromatographed under the same condition used for peptide mapping. (A) Blank for the hydrolysis condition; (B) desulfated peptide I. The elution position of peptides I and II from Figure 2 is marked by arrows. (—) Absorbance at 214 nm.

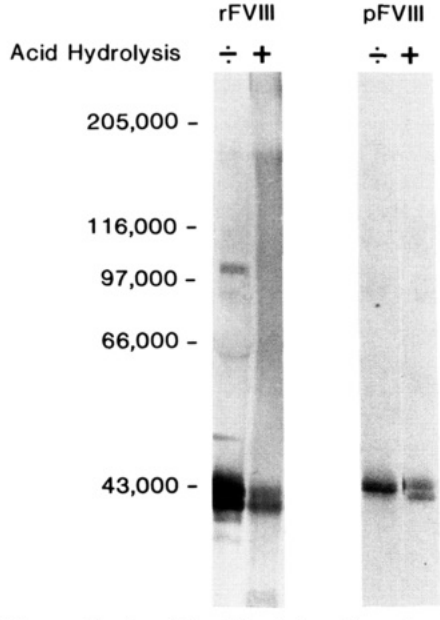


FIGURE 4: Western blotting of desulfated thrombin-activated pFVIII and rFVIII. Thrombin-activted FVIII was precipitated with TCA and desulfated by weak acid hydrolysis as described under Experimental Procedures. Thrombin-activated FVIII and desulfated thrombin-activated FVIII were resolved on a 7.5% SDS–polyacrylamide gel and transferred to nitrocellulose. To detect FVIII bound to NT, a monoclonal antibody against the 43-kDa fragment was used. Lane 1, thrombin-activated pFVIII (2.0 μg of protein); lane 2, desulfated thrombin-activated pFVIII (2.0 μg of protein); lane 3, thrombin-activated rFVIII (2.0 μg of protein); lane 4, desulfated thrombin-activated rFVIII (2.0 μg of protein).

Similarly, it was shown that the 43-kDa fragment of pFVIII appeared as a doublet on SDS–PAGE after acid treatment with the same molecular weight as the 41.5/43-kDa fragment from rFVIII (Figure 4). These results further support that the difference between the 41.5-kDa fragment and the 43-kDa fragment of rFVIII is caused by a limitation of the CHO cells to tyrosine sulfate rFVIII. The sulfated peptide (residues 714–733) from the 43-kDa fragment of pFVIII eluted at the same position as peptide I from the 43-kDa fragment of rFVIII on the HPLC column. The identical elution position of the two peptides indicates that both peptides have the same degree of tyrosine sulfation.

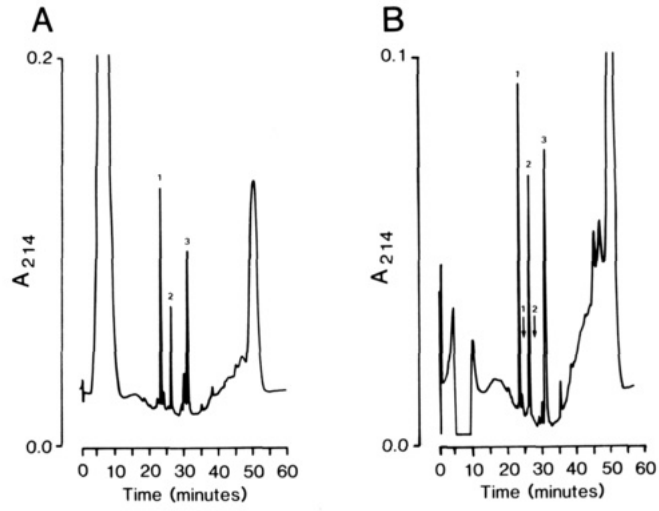


FIGURE 5: *Staphylococcus aureus* proteinase cleavage of the 41 amino acid peptide. The 41 amino acid peptides from pFVIII (A) and rFVIII (B) were cleaved with *Staphylococcus aureus* proteinase as described under Experimental Procedures. By amino acid sequence determination, peptide 1 was determined to be residues 1672–1689; peptide 2, residues 1649–1671; and peptide 3, the uncleaved 41 amino acid peptide. The elution position of arylsulfatase treated peptides 1 and 2 is indicated in (B) by arrows. (—) Absorbance at 214 nm.

FVIII contains four other potential tyrosine sulfation sites besides the three on peptide residues 714–733. To investigate if there is a general limitation and heterogeneity in the tyrosine sulfation of rFVIII, two other potential tyrosine sulfation sites were compared. FVIII–LC contains 2 potential tyrosine sulfation sites ($Tyr_{1664}$, $Tyr_{1680}$) on the 41 amino acid peptide (residues 1649–1689), which is released from FVIII–LC by thrombin activation. This 41 amino acid peptide has an important function as it is the binding region to the von Willebrand factor (Foster et al., 1988).

FVIII–LC from both rFVIII and pFVIII was cleaved with thrombin, and the 41 amino acid peptides were isolated by RP-HPLC. The elution positions of the two 41 amino acid peptides were identical. *Staphylococcus aureus* protease cleaved the 41 amino acid peptide into 2 peptides, peptide 1 (residues 1672–1689) and peptide 2 (residues 1649–1671). The elution positions on an HPLC column of peptide 1 and peptide 2 are the same for the peptides resulting from either pFVIII or rFVIII (Figure 5). The elution positions of peptide 1 and peptide 2 after enzymatic desulfation with arylsulfatase are also shown (Figure 5B). These results show that both potential tyrosine sulfation sites on pFVIII–LC and rFVIII–LC are used and that there is no heterogeneity in the tyrosine sulfation pattern of rFVIII–LC. The biological function of protein tyrosine sulfation has only been established in a few cases (Huttner, 1987; Suiko & Liu, 1988; Ryuichiro et al., 1988; Hortin et al., 1989) even though tyrosine sulfation is a widespread posttranslational modification. Tyrosine residues that are acceptors for sulfation are located at the surface on the protein, as sulfation occurs in Golgi after folding of the protein (Huttner, 1987). Therefore, it is possible that tyrosine sulfation may have a function in the interaction of FVIII with

other coagulation factors or membrane proteins in the hemostatic process.

To examine the biological function of tyrosine sulfation on the peptide (residues 714–733), the two tyrosine sulfated forms of rFVIII were separated from each other on a Mono-Q column. The thrombin activation profile, the activity in Coatest and the one-stage coagulation assay, and the binding to the von Willebrand factor (Leyte et al., 1989) were compared for the two forms of rFVIII. No difference was found, indicating that tyrosine sulfation of the peptide (residues 714–733) does not influence these activities of FVIII (data not shown).

This paper describes that recombinant FVIII produced from CHO cells is secreted in two different tyrosine sulfated forms. The heterogeneity of rFVIII is caused by a limitation of CHO cells to tyrosine sulfate one peptide (residues 714–733). Two other potential tyrosine sulfation sites, $Tyr_{1664}$ and $Tyr_{1680}$, were found to be completely sulfated on both pFVIII–LC and rFVIII–LC. The limitation of CHO cells to tyrosine sulfate rFVIII is therefore not a general limitation, as only a few sites of FVIII are affected. A possible explanation for this can be that CHO cells are not secretory cells, and therefore do not have the capacity to completely sulfate sites which have restricted accessibility. Alternatively, the folding of rFVIII produced in CHO cells is such that some potential tyrosine sulfation sites are not available for sulfation.

The two sulfated forms of rFVIII can easily be separated by ion-exchange chromatography, indicating that the sulfate groups on the FVIII–HC 43-kDa fragment have a significant impact on the overall charge density of FVIII.

It has been shown (Ryuichiro et al., 1988) for complement component $C_4$ that sulfation of tyrosine residues has a major effect on the activity of proteins participating in protein interactions. Although we have not been able to show any significance of the tyrosine sulfation on the heavy-chain fragment, it is not precluded that this posttranslational modification plays a role in vivo, where FVIII interacts with membrane-bound proteins during the hemostatic process.

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**Registry No.** FVIII, 9001-27-8; L-Tyr, 60-18-4.

## References

Aebersold, R. H., Leavitt, J., & Saavedra, R. A. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 6970–6974.

Blake, M. S., Johnston, K. H., & Russell-Jones, G. J. (1984) *Anal. Biochem. 136*, 175–179.

Burke, R. L., Pachl, C., & Quiroga, M. (1986) *J. Biol. Chem. 261*, 12574–12578.

Burnette, W. N. (1981) *Anal. Biochem. 112*, 195–203.

Eaton, D., Rodriguez, H., & Vehar, G. A. (1986) *Biochemistry 25*, 505–512.

Eaton, D. L., Hass, P. E., & Riddle, L. (1987) *J. Biol. Chem. 7*, 3285–3290.

Fass, D. N., Knutson, G. J., & Katzmann, J. A. (1982) *Blood 59*, 594–600.

Foster, P. A., Fulcher, C. A., & Houghten, R. A. (1988) *J. Biol Chem. 263*, 5230–5234.

Fulcher, C. A., & Zimmerman, T. S. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 1648–1652.

Hortin, G., Sims, H., & Strauss, A. W. (1986) *J. Biol. Chem. 261*, 1786–1793.

Hortin, G. L., Farries, T. C., & Graham, J. P. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 1338–1342.

Huttner, W. B. (1987) *Trends Biochem. Sci. (Pers. Ed.) 12*, 361–363.

Laemmli, U. K. (1970) *Nature 227*, 680–685.

LeGendre, N., & Matsudaira, P. (1988) *BioTechniques 6*, 154–159.

Leyte, A., Verbeet, M., & Brodniewicz-Proba, T. (1989) *Biochem. J. 257*, 679–683.

Lucas, J., & Henschen, A. (1986) *J. Chromatogr. 369*, 357–364.

Mikkelsen, J., & Knudsen, J. (1987) *Biochem. J. 248*, 709–714.

Morrissey, J. H. (1981) *Anal. Biochem. 117*, 307–310.

Nordfang, O., & Ezban, M. (1988) *J. Biol. Chem. 263*, 1115–1118.

Pittman, D. D., & Kaufman, R. J. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 2429–2433.

Pittman, D. D., Wasley, L. C., & Murray, B. L. (1987) *Thromb. Hemostasis 58*, 344 (Abstr.).

Proctor, R. D., & Rapaport, S. I. (1961) *Am. J. Clin. Pathol. 36*, 212–219.

Prowsee, C., Hornsey, V., & McKay, G. (1986) *Vox Sang 50*, 21–25.

Rotblat, F., O'Brian, D. P., & O'Brian, F. J. (1985) *Biochemistry 24*, 4294–4300.

Ryuichiro, D., Kazutomo, I., & Masafumi, K. (1988) *Biochem. Biophys. Res. Commun. 153*, 1209–1213.

Suiko, M., & Liu, M.-C. (1988) *Biochem. Biophys. Res. Commun. 154*, 1094–1098.

Toole, J. J., Knopf, J. L., & Wozney, J. M. (1984) *Nature 312*, 342–347.

Truett, M. A., Blacher, R., & Burke, R. L. (1985) *DNA 4*, 333–349.

Vehar, G. A., & Davie, E. W. (1980) *Biochemistry 19*, 401–410.

Vehar, G. A., Keyt, B., & Eaton, D. (1984) *Nature 312*, 337–342.