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Notes & Tips

A target-specific approach for the identification of tyrosine-sulfated hemostatic proteins

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ABSTRACT

A simple methodology for the identification of hemostatic proteins that are subjected to posttranslational tyrosine sulfation was developed. The procedure involves sequence analysis of members of the three hemostatic pathways using the Sulfinator prediction algorithm, followed by [^{35}S]sulfate labeling of cultured HepG2 human hepatoma cells, immunoprecipitation of targeted [^{35}S]sulfate-labeled hemostatic proteins, and tyrosine *O*-[^{35}S]sulfate analysis of immunoprecipitated proteins. Three new tyrosine-sulfated hemostatic proteins—protein S, prekallikrein, and plasminogen—were identified. Such a target-specific approach will allow investigation of tyrosine-sulfated proteins of other biochemical/physiological pathways/processes and contribute to a better understanding of the functional role of posttranslational tyrosine sulfation.

Posttranslational protein modification by tyrosine sulfation, first discovered in bovine fibrinogen [1], is now known to have widespread occurrence among proteins of multicellular eukaryotic organisms [2,3]. This unique protein modification has been implicated in the alteration of biological activities of proteins [4,5], proteolytic processing of bioactive peptides [6], change in half-life of proteins in circulation [7], intracellular transport of secretory proteins [8], and modulation of extracellular protein–protein interactions [9]. It has been estimated that as much as 1% of an organism's total proteins may be subjected to tyrosine sulfation [10]. Among a limited number of tyrosine-sulfated proteins identified to date are several blood coagulation factors, including fibrinogen and factors V, VIII, and IX [11–15]. In view of the abundance of tyrosine-sulfated proteins that remain unidentified, it is possible that some other members of the blood coagulation pathway, as well as those involved in anticoagulation and fibrinolysis, may also be subjected to tyrosine sulfation.

Amino acid sequences encompassing sulfated tyrosine residues are known for a number of proteins and peptides [16,17]. Comparison of these sequences had revealed that a consensus feature is the presence of acidic amino acid residues (Asp and Glu) within –5 (N terminal) and +5 (C terminal) of the sulfated tyrosine [16,17]. Turn-inducing amino acids (e.g., Pro, Gly) are also found near the sulfated tyrosine (from –7 to +7) [16,17]. Based on these sequence features, a software tool dubbed Sulfinator (available at http://www.expasy.org/tools/sulfinator) has been developed to aid in the prediction of potential tyrosine sulfation sites in proteins [18]. Although Sulfinator may enable users to conveniently identify potential tyrosine sulfation sites in proteins, it is not a guarantor for the identified proteins to be actually sulfated in cells. Therefore, in the current study we attempted to develop a simple methodology for the identification and verification of new tyrosine-sulfated proteins.

Using hemostatic proteins as a model, we first performed a sequence analysis of members of the three hemostatic pathways using the Sulfinator prediction algorithm. Table 1 shows the potential tyrosine sulfation sites identified for three previously reported tyrosine-sulfated hemostatic proteins (fibrinogen, factor V, and heparin cofactor II) and three not-yet-identified but potentially tyrosine-sulfated proteins (protein S, prekallikrein, and plasminogen). To verify whether these three latter proteins are indeed subjected to posttranslational tyrosine sulfation, we used the HepG2 human hepatoma cells that are known to produce many plasma proteins for which there are antibodies commercially available. HepG2 cells were labeled with [^{35}S]sulfate (0.3 mCi/ml, 1 Ci = 37 GBq) in sulfate-free minimum essential medium (prepared by omitting streptomycin sulfate and replacing magnesium sulfate with magnesium chloride) without serum. After an 18-h incubation, the labeling

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Table 1
Potential tyrosine sulfation sites of the plasma proteins identified using Sulfinator.

Swiss–Prot name	Description	Site(s)[a]	Sequence surrounding sulfatable tyrosine residue
FIBA_HUMAN	Fibrinogen alpha chain [precursor]	ND[b]	None
FIBB_HUMAN	Fibrinogen beta chain [precursor]	255	ETSEM**Y**LIQPDSSVKPY
FIBG_HUMAN	Fibrinogen gamma chain [precursor]	300	PEADK**Y**RLTYAYFAGGD
		306	RLTYA**Y**FAGGDAGDAFD
		444	PAETE**Y**DSLYPEDDL
FA5_HUMAN	Coagulation factor V [precursor]	693	DDEDS**Y**EIFEPPESTVM
		724	ESDAD**Y**DYQNRLAAALG
		726	DADYD**Y**QNRLAAALGIR
		1522	KDGTD**Y**IEIIPKEEVQS
		1538	SSEDD**Y**AEIDYVPYDDP
		1543	YAEID**Y**VPYDDPYKTDV
		1593	EISWD**Y**SEFVQRETDIE
HEP2_HUMAN	Heparin cofactor II [precursor]	79	EEDDD**Y**LDLEKIFSEDD
		92	SEDDD**Y**IDIVDSLSVSP
PLMN_HUMAN	Plasminogen [precursor]	173	DPEKR**Y**DYCDILECEEE
		175	EKRYD**Y**CDILECEEECM
		323	NLDEN**Y**CRNPDGKRAPW
		554	RKLYD**Y**CDVPQCAAPSF
KLKB1_HUMAN	Plasma kallikrein [precursor]	40	DVASM**Y**TPNAQYCQMRC
		46	TPNAQ**Y**CQMRCTFHPRC
PROS_HUMAN	Vitamin-K-dependent protein S	297	NLDTK**Y**ELLYLAEQFAG

Note. Sulfinator was developed by Monigatti and coworkers [18]. Additional details can be found at the Sulfinator website (http://www.expasy.org/tools/sulfinator).
[a] Potential tyrosine sulfation sites refer to the positions of the potential sulfatable tyrosine residues in the amino acid sequences of individual proteins deposited in the Swiss–Prot database.
[b] No potential tyrosine sulfation sites were detected.

medium was collected and a protease inhibitor cocktail was immediately added to prevent protein degradation. For immunoprecipitation, 1-ml aliquots of the labeling medium were incubated individually with 50 μg each of antibodies against fibrinogen, factor V, heparin cofactor II, protein S, prekallikrein, and plasminogen. After an overnight incubation on ice, 50 μl of protein G–Sepharose CL-4B was added to each sample and the mixture was agitated by rotation at 4 °C for 30 min. Protein G–

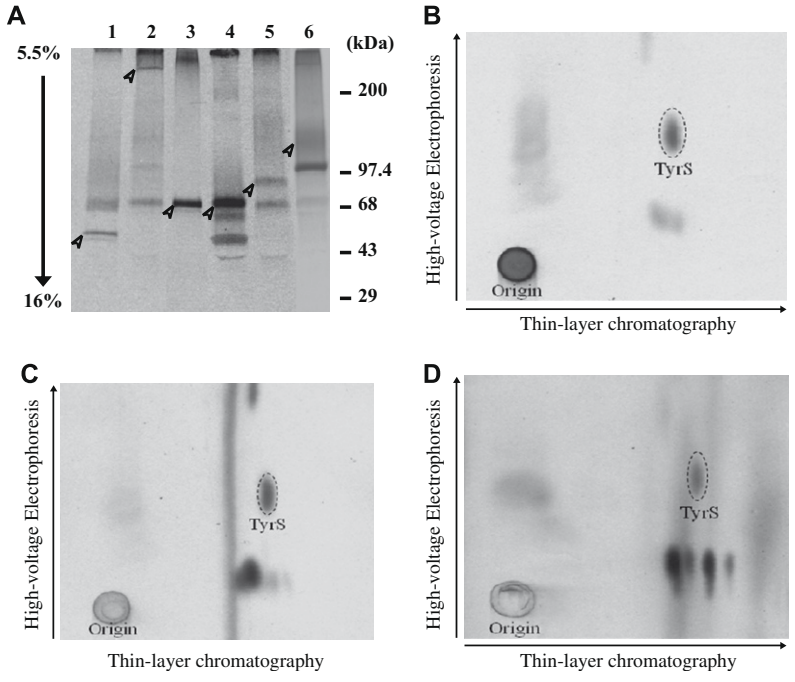

Fig. 1. Immunoprecipitation of known and potentially tyrosine-sulfated hemostatic proteins (A) and two-dimensional thin-layer analysis of the pronase hydrolysates of [^{35}S]sulfate-labeled protein S (B), prekallikrein (C), and plasminogen (D). Panel A shows the autoradiograph taken from the dried SDS–PAGE gel used for the electrophoretic separation of proteins immunoprecipitated from spent medium of [^{35}S]sulfate-labeled HepG2 cells. Samples analyzed in lanes 1 through 6 were fibrinogen, factor V, heparin cofactor II, protein S, prekallikrein, and plasminogen, respectively. Arrowheads indicate radioactive bands corresponding to the immunoprecipitated proteins. Panels B, C, and D correspond to the autoradiographs taken from the thin-layer chromatography plates used for the two-dimensional thin-layer separation of each of the three pronase hydrolysate samples. The dashed-line ovals correspond to the position of synthetic tyrosine *O*-sulfate as revealed by ninhydrin staining.

Sepharose bound with the immune complex was subsequently brought down by centrifugation, washed three times with phosphate-buffered saline, and placed in the sodium dodecyl sulfate (SDS)[1] sample buffer for the subsequent SDS–polyacrylamide gel electrophoresis (PAGE). After the electrophoresis, the gel was stained with Coomassie blue, destained, dried, and subjected to autoradiography. Fig. 1A shows the autoradiograph taken from the dried gel. All six proteins, as indicated by arrowheads on their respective electrophoretic lanes, were found to be [^{35}S]sulfated. The radioactive bands corresponding to these proteins were assigned based on their molecular weights: fibrinogen (M_r of Bβ subunit = 55,000), factor V (M_r = 330,000), heparin cofactor II (M_r = 66,000), protein S (M_r = 70,000), prekallikrein (M_r = 88,000), and plasminogen (M_r = 106,900). It should be noted that nonspecific radioactive bands were also observed on different electrophoretic lanes (Fig. 1A). These could be due to proteins that interacted with specific proteins being immunoprecipitated or protein G–Sepharose gel beads.

To further examine the chemical nature of the bound [^{35}S]sulfate, the radioactive bands corresponding to the three potentially tyrosine-sulfated proteins—protein S, prekallikrein, and plasminogen—were located by autoradiograph and excised from the dried gel and were subjected to pronase hydrolysis, followed by a two-dimensional thin-layer separation combining high-voltage electrophoresis and thin-layer chromatography (TLC), based on a procedure established previously [19]. As shown in Fig. 1B–D, the autoradiographs taken from the TLC plates used for the two-dimensional separation of the pronase hydrolysates of [^{35}S]sulfate-labeled protein S, prekallikrein, and plasminogen clearly revealed the presence of tyrosine *O*-[^{35}S]sulfate. The additional radioactive spots detected on the three autoradiographs are likely due to the carbohydrate-bound [^{35}S]sulfate also present in these three proteins.

Plasminogen, which is a central component in the fibrinolytic system, had previously been shown to undergo posttranslational modifications by O- and N-glycosylation [20]. Prekallikrein, a precursor of kallikrein responsible for cleaving kininogen to generate bradykinin as well as activating several coagulation factors such as factors XII and VII, had also been reported to be subjected to N-glycosylation [21]. For protein S, the occurrence of γ-carboxylation of glutamic acid residue and β-hydroxylation of asparagine residue had been demonstrated previously [22]. Our current results showing the tyrosine sulfation of protein S, prekallikrein, and plasminogen imply that these different posttranslational modifications may collectively contribute to the functioning of these three hemostatic proteins.

In conclusion, the abundance and distribution of tyrosine-sulfated proteins have remained poorly understood due to the lack of suitable analytical methods and tools. In this article, we have reported a simple methodology for the identification of new tyrosine-sulfated hemostatic proteins. The same approach may be employed to identify new tyrosine-sulfated proteins involved in other biochemical/physiological pathways/processes.

[1] *Abbreviations used:* SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; TLC, thin-layer chromatography.

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