

Research Paper

Tyrosine Sulfation of Statherin

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Tyrosylprotein sulfotransferase (TPST), responsible for the sulfation of a variety of secretory and membrane proteins, has been identified and characterized in submandibular salivary glands (William et al. Arch Biochem Biophys 1997; 338: 90-96). In the present study we demonstrate the sulfation of a salivary secretory protein, statherin, by the tyrosylprotein sulfotransferase present in human saliva. Optimum statherin sulfation was observed at pH 6.5 and at 20 mM MnCl₂. Increase in the level of total sulfation was observed with increasing statherin concentration. The K_m value of tyrosylprotein sulfotransferase for statherin was 40 μ M. Analysis of the sulfated statherin product on SDS-polyacrylamide gel electrophoresis followed by autoradiography revealed ³⁵S-labelling of a 5 kDa statherin. Further analysis of the sulfated statherin revealed the sulfation on tyrosyl residue. This study is the first report demonstrating tyrosine sulfation of a salivary secretory protein. The implications of this sulfation of statherin in hydroxyapatite binding and Actinomyces viscosus interactions are discussed.

Key words: Saliva, statherin, tyrosine sulfation

1. Introduction

Human saliva, which has several components, plays a crucial role not only in diffusion of acids but also in clearance of bacterial adhesion from the oral cavity. The importance of carbohydrate sulfation in the processing of specific salivary glycoproteins [2] and the involvement of these glycoproteins in bacterial aggregating activity has been reported earlier [3]. Therefore, it has been suggested that tyrosine sulfation may also play a role in the biological activity of tyrosine-containing salivary proteins as it has been demonstrated in other proteins [4-8]. Sulfation of tyrosyl residues in cholecystokinin is required for its optimal biological activity [4] and in the case of hirudin, tyrosine sulfation increases the binding efficiency to thrombin [5]. In M31 hybridoma cell line, tyrosine sulfation has been shown to serve as a signal for the secretion of IgG [6]. In p-selectin glycoprotein ligand -1 (PSGL-1), tyrosine sulfation required for its binding to p-selectin [7] and the tyrosine sulfation of chemokine receptor 5 (CCR5) facilitates HIV-1 entry [8]. Although the significance of tyrosine sulfation in these and several other proteins has been studied in detail, its role in salivary protein is yet to be investigated. In naturally occurring substrates of tyrosylprotein sulfotransferase, the sulfated tyrosyl residues are predominantly surrounded by acidic amino acids [9], and the frequency distribution of basic amino acids in these sites is extremely low [10]. In 1992, Lin et al. [11] had shown increased tyrosine sulfation of substrates containing acidic amino acids by TPST. These studies suggest that acidic

environments are the important determinants for tyrosine sulfation. Salivary statherin is a 43-residue polypeptide, which is tyrosine-rich and contains 7 tyrosine residues. The peptide also contains a sequence (residues 26-30) required for tyrosine sulfation as suggested by Lin et al., [11] and Bundgard et al., [12]. In this paper, we have examined the sulfation of salivary statherin. The results demonstrate, for the first time, the tyrosine sulfation of a salivary protein, statherin, by the tyrosylprotein sulfotransferase from human saliva.

2. Materials and Methods

Materials

³⁵S-3' phosphoadenosine 5'-phosphosulphate (PAPS) (2 Ci/mmol) was purchased from DuPont New England Nuclear. Poly(Glu⁶, Ala³, Tyr¹) (EAY) and 5'-AMP were from Sigma-Aldrich. All other reagents were of analytical grade.

Human Saliva

Unstimulated saliva was obtained from healthy individuals. The saliva was centrifuged at 6,000 rpm for 10 min and the clear supernatant was used as enzyme source. Protein concentration was estimated by the BCA (No. 23225) protein assay reagent (Pierce Chemical Co.). Statherin was purified from human submandibular-sublingual saliva collected from three male donors as described previously [13]. Amino acid composition, N-terminal sequencing and SDS-PAGE were used to check the purity of statherin.

Assay of Statherin Sulfation

The statherin sulfation assay mixture contained the following components: 100 μ M statherin (acceptor), 5 μ M 35 S-PAPS (4000,000 cpm), 10 mM MnCl_2 ·20 mM MES, pH 6.5, and saliva (100 μ g) in a final volume of 50 μ l. Assays were initiated by the addition of the enzyme and incubated for 30 min at 37°C. Controls without statherin were also incubated for 30 min at 37°C. Statherin sulfation was obtained by subtracting controls from the total sulfation. The reactions were stopped by spotting 35 μ l aliquots of the reaction mixture on 2.4 x 2.4 cm Whatman No. 3 MM filter paper [1]. The papers were washed three times for 15 min in 10% trichloroacetic acid/10 mM Na_2SO_4 and rinsed for 5 min in 95% ethanol. The dried papers were placed in vials containing scintillation solution and counted in a TriCarb-1500 scintillation counter. In experiments involving the analysis 35 S-sulfate labeled statherin, the assay volume was increased to 200 μ l. Reactions were stopped by addition of 100 μ l of a solution containing 6.25% SDS, 10% glycerol and 0.005% bromophenol blue. The samples were processed for SDS-PAGE as described below.

SDS-Polyacrylamide Gel Electrophoresis and Autoradiography

The separating gels contained 15% (w/v) acrylamide, bis(acrylamide) in the ratio of 37.5:1, 0.375 M Tris-HCl, pH 8.8, 0.1% SDS, 0.026% TEMED, and 0.026% ammonium persulfate. The stacking gel contained 3% acrylamide, bis(acrylamide) in the same ratio as before, 0.125 M Tris-HCl, pH 6.8, 0.1% SDS, 0.05% ammonium persulfate, and 0.4% TEMED. Electrophoresis was performed in a Mighty Small gel apparatus (Hoefer Scientific Instruments, San Francisco, CA) at 4°C at a constant current of 20 mA per gel for approximately 1 h. After electrophoresis the gel was stained for 30 min. with Coomassie blue and destained in a solution containing 40% methanol, 10% acetic acid. After destaining, the gels were soaked in water for 30 min and then in 1M sodium salicylate for another 30 min. The gels were then dried and autoradiographed at -70 °C on Hyper Film B-max (Amersham).

Identification of Tyrosine Sulfate in Statherin

To determine the radioactive tyrosine-O-sulfate in statherin, the gel containing 35 S-sulfate labeled statherin was cut, lyophilized, incubated for 18 h at 37°C in 1 ml of a solution containing 75 μ g of trypsin, 50 mM ammonium bicarbonate, pH 8, and a trace of phenol red, and then lyophilized again. The lyophilized material was dissolved in 1 ml of 0.2 M $\text{Ba}(\text{OH})_2$, and the tubes were evacuated, sealed and incubated for 24 h at 110°C. The sample was then centrifuged at 5 000Xg for 20 min. The supernatant was neutralized with 1N H_2SO_4 using phenol red as pH indicator and centrifuged at 5 000Xg. The supernatant thus obtained was lyophilized and the dry residue was dissolved in 10-20 μ l of electrophoresis buffer (5% acetic acid-0.5% pyridine),

pH 3.5. The solution was centrifuged at 5 000Xg for 5 min and applied on plastic-backed cellulose thin-layer sheets. Electrophoresis was performed until the phenol red marker migrated 7-8 cm, i.e., just past the middle of the cellulose sheet. In our electrophoresis system this took about 3 h at 450 V. The cellulose sheet was dried, and the unlabeled amino acid standards were detected by spraying the cellulose sheet with 1% ninhydrin in acetone and developing the color at 110°C for three minutes. Radioactive tyrosine sulfate spot was detected by autoradiography on Hyper Film B-max (Amersham) at -70°C.

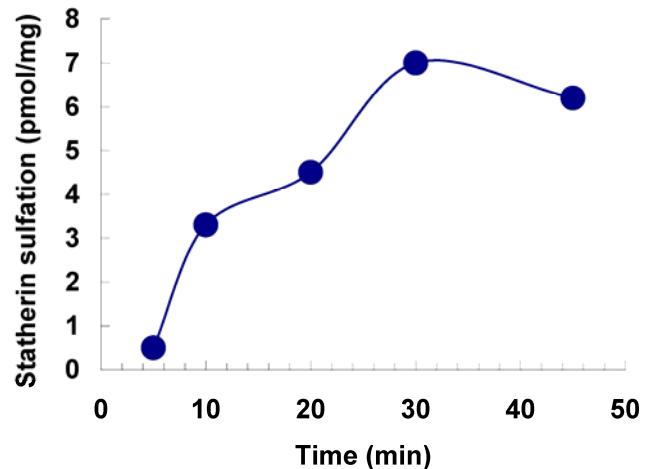


Figure 1. Effect of time on the sulfation of statherin by tyrosylprotein sulfotransferase of human saliva. The statherin sulfation was measured as described under Materials and Methods.

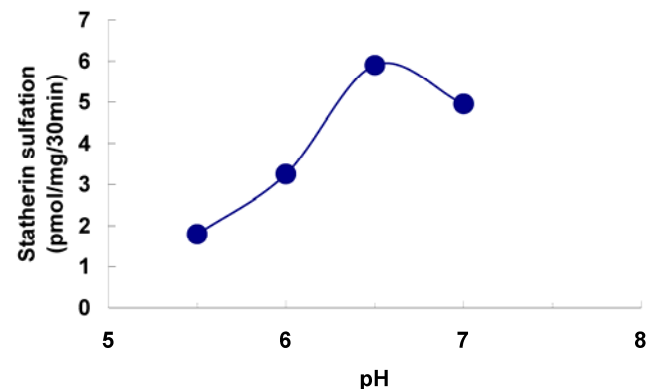


Figure 2. Effect of pH on the sulfation of statherin by tyrosylprotein sulfotransferase of human saliva. The statherin sulfation was measured as described under Materials and Methods except that the pH of the assay buffer was varied.

3. Results and Discussion

The sulfation of statherin by the tyrosylprotein sulfotransferase present in human saliva was demonstrated using statherin as sulfate acceptor and

[^{35}S] PAPS as sulfate donor. Effect of time on sulfation of statherin is shown in Fig. 1. The statherin sulfation activity increased with increased incubation time up to 30 min. A 20% reduction in sulfation level was observed when the reaction was extended for an additional 15 min. The effect of pH on the sulfation of statherin by salivary tyrosylprotein sulfotransferase is shown in Fig. 2. Using the MES buffer, the pH optimum for the sulfation of statherin by tyrosylprotein sulfotransferase was attained at pH 6.5. Dependence of statherin sulfation on MnCl_2 is illustrated in Fig. 3. Statherin sulfation was detected in the absence of exogenous MnCl_2 . However, maximum sulfation required 20 mM MnCl_2 . Effect of statherin concentration on statherin sulfation is shown in Fig. 4. The amount of sulfate incorporation into statherin was dependent upon the concentration of exogenous statherin. The sulfation of statherin increased proportionately with increasing concentration of statherin. The tyrosylprotein sulfotransferase has been identified and characterized in several tissues and cells [14] including submandibular salivary glands and human saliva [15,16]. The substrate used in these studies was poly-Glu₆, Ala₃, Tyr₁. Comparison of the properties of these reactions to the statherin sulfation reveals that both reactions require MnCl_2 and the pH requirement was in the range of 6 to 6.75. A K_m value 40 μM was observed for statherin. The statherin concentration in human saliva is 0.42% of the total salivary protein [17]. The total endogenous statherin concentration in our assay system is less than 1.6% of the exogenous statherin added to the assay. Hence, the endogenous statherin present in the saliva will have minimal effect on the K_m value reported here. The K_m value of 40 μM for statherin was higher than the one reported for the artificial substrate poly-Glu₆,Ala₃,Tyr₁ (Sundaram et al., 1992). Like the natural substrate statherin, a higher K_m value was observed earlier for the cholecystokinin [18].

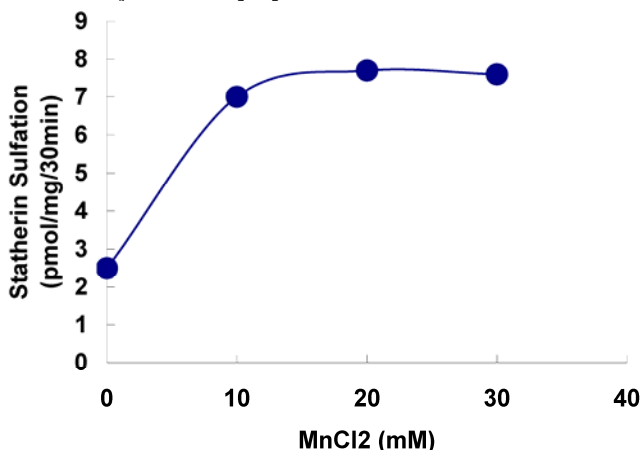


Figure 3. Effect of MnCl_2 on the on the sulfation of statherin by tyrosylprotein sulfotransferase of human saliva. The statherin sulfation was measured as described under Materials and Methods except that the MnCl_2 concentration of the assay buffer was varied.

The sulfation of ^{35}S -labeled statherin on SDS-PAGE followed by autoradiography is shown in Fig. 5. A major ^{35}S -labeled endogenous salivary mucin at the top of the separating gel was observed in all three lanes (unpublished observation). A polypeptide poly-Glu₆, Ala₃, Tyr₁ [19], which is being used as a substrate for tyrosine sulfation in several studies, was used as a positive control. The autoradiogram depicted in Fig. 5, demonstrate the sulfation of a 5 kDa statherin (lane, 3) and 25 kDa polypeptide poly-Glu₆, Ala₃, Tyr₁ (lane 2). This, together with the above kinetic experiments, clearly demonstrates the sulfation of statherin by TPST. Further analysis was carried out to identify the sulfated amino acid in statherin. As shown in Fig. 6, the sulfation of statherin occurred on tyrosine residues.

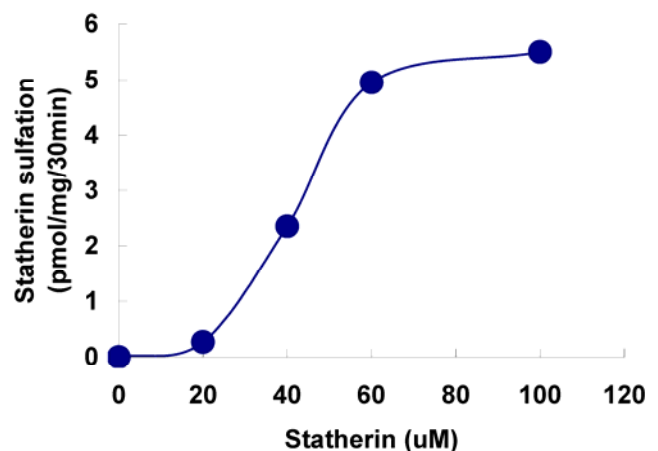


Figure 4. Effect of statherin concentration on the sulfation of statherin by tyrosylprotein sulfotransferase human saliva. The statherin sulfation was measured as described under Materials and Methods.

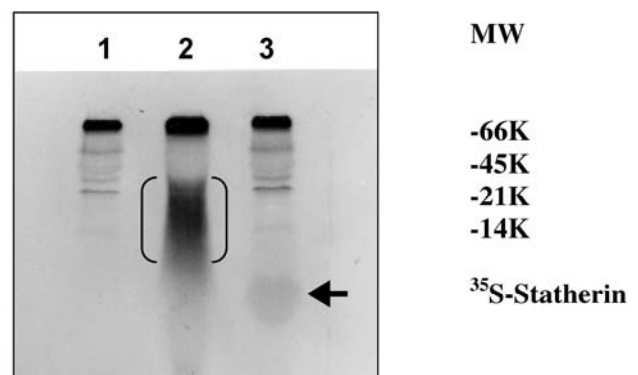


Figure 5. Identification of the ^{35}S labelled statherin in an autoradiogram of an SDS-polyacrylamide electrophoretic gel. Statherin and poly-Glu₆,Ala₃,Tyr₁ was sulfated as described under Experimental Procedures. Lane 1, Human saliva; Lane 2, Human Saliva + EAY; Lane 3, Human Saliva + Statherin. EAY sulfation is marked by double bracket. Statherin sulfation is shown by an arrow.

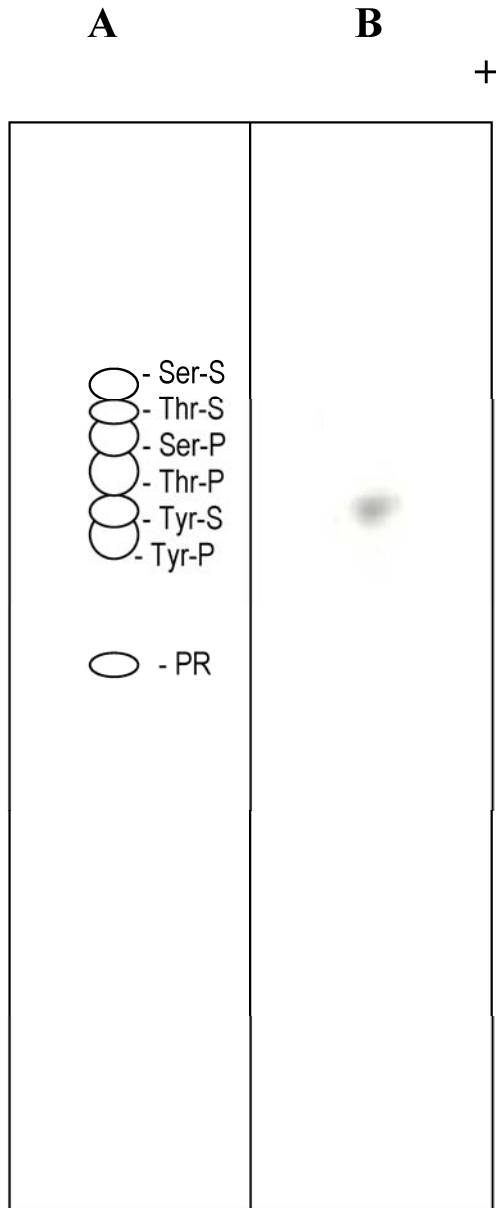


Figure 6. Thin layer electrophoresis of tyrosine sulfate. (A) Separation of non radioactive standards. The dotted lines in A and B indicate the position of tyrosine sulfate. (B). Autoradiogram of separation of an alkaline hydrolysate of sulfated statherin.

4. Summary

In the present study, we have demonstrated the tyrosine sulfation of statherin by the TPST present in human saliva. At present the significance of the tyrosine sulfation of statherin is not known. A possible function of tyrosine sulfation in statherin could be that it may interfere in the mineralization and demineralization process due to its binding to hydroxyapatite [20] and also in *Actinomyces viscosus* interactions [21]. Statherin, a 43-residue polypeptide contains 7 tyrosine residues and at least one sequence

E-Q-P-L-Y (residues 26-30) that satisfies the sequence preference for tyrosine sulfation as demonstrated by Lin et al., [11] and Bundgaard et al., [12] (Fig.7). However, the *Actinomyces viscosus* binding site in statherin is at the C-terminus that also contains a tyrosine residue (Q-Y-T-F). Although this segment does not contain the motif for tyrosine sulfation, the potential sulfation of Tyr 30 residue may affect the binding of statherin to *A. viscosus*.

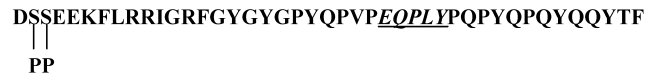


Figure 7. Primary structure of statherin. The potential sulfation site is underlined. Note additional tyrosines in the sequence especially at the C-terminus where *A. viscosus* binding may occur.

Acknowledgement

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Conflict of interest

The authors have declared that no conflict of interest exists.

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