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Further Characterization of Protein Sulfotransferase(s) of Rat Brain by Alkaline Hydrolysis of Sulfated Proteins

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An *in vitro* protein sulfation in the soluble fraction of rat brain was characterized further by an improved method of alkaline hydrolysis and thin layer cellulose electrophoresis (TLE). The protein sulfation was carried out in a reaction system containing [^{35}S]3′-phosphoadenosine-5′-phosphosulfate (PAPS), Tris-maleate buffer (pH 8), $MgCl_2$, and soluble proteins from rat brain. The sulfated proteins were precipitated by acetone and alkaline hydrolysis was performed to obtain sulfated amino acids. The hydrolysate was separated further by TLE and the separated residues were identified by fluorography. The fluorography of one-dimensional TLE showed at least nine sulfated residues including tyrosine-O-sulfate. The other spots were not identified yet positively. General properties of protein sulfotransferases (PST) using this method were re-examined such as effects of concentrations of PAPS, pH, incubation temperature and Mg^{2+}. These results suggest a possible occurrence of several PST corresponding to each sulfated residue in rat brain and that the sulfation can occur not only in tyrosine but also in other residues as well.

KEY WORDS: ***Protein sulfotransferases, Protein sulfation, Sulfated amino acid, Tyrosine*-O-sulfate, Alkaline hydrolysis of protein.**

Recently, protein sulfation is being studied as a kind of posttranslational modifications. There are two types of sulfation in proteins: One is the sulfation to amino acid residues and the other is the sulfation to carbohydrate moieties of proteins. Nowadays, most of the protein sulfations reported are focused mainly on the sulfation of tyrosine residues since these sulfations are very similar to the tyrosine phosphorylation by various protein kinases. The sulfated tyrosine residue which was identified first was in bovine fibrinopeptide B in 1954 (Bettelheim, 1954). Thereafter, a number of oligopeptides and proteins have been shown to be sulfated in tyrosine. These are the sulfated proteins of a rat pheochromocytoma cell line (Huttner, 1982; Lee and Huttner, 1983), human complement component C-4 (Karp, 1983), fibronectin (Liu and Lipmann, 1985), cholecystokinin derivatives (Vargas *et al.*, 1985), a membrane protein of 66,000 dalton (P61) in A431 cells (Liu and Baenziger, 1986), sheep pituitary prolactin (Kohli *et al.*, 1988), yolk protein 2 of Drosophila melanogaster (Baeuerle *et al.*, 1988; Friederich *et al.*, 1988), and gastrin in rat gastric mucosa (Chen *et al.*, 1989).

In vitro, using exogeneous substrate like acidic polymer (Glu; Ala; $Tyr)_n$, a tyrosylprotein sulfotransferase activity was found in the fraction enriched in Golgi membrane vesicle of bovine adrenal medulla (Lee and Huttner, 1985) and

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similar activity was also detected in the microsomal fraction of rat brain (Vargas and Schwartz, 1987). More recently the presence of tyrosylprotein sulfotransferase in the soluble fraction of rat adrenal gland was also observed (Kim and Choi, 1989). Generally, the function of tyrosine sulfation in proteins are not yet understood. However, some studies of secretory proteins implicate the role of tyrosine sulfation as a necessary modification in processing, sorting, or functioning of several secretory proteins (Hille *et al.*, 1984; Huttner, 1988).

Previously, we have shown an in vitro protein sulfation in the soluble fraction of rat brain using [^{35}S] 3'-phosphoadenosine-5'-phosphosulfate (PAPS) as sulfate donor (Lee and Choi, 1990). In this report a technique for direct identification of sulfated residues by alkaline hydrolysis with Ba $(OH)_2$ was adopted (Huttner, 1984). And the hydrolysate was further separated by thin layer cellulose electrophoresis (TLE). The sulfated amino acids separated were visualized by fluorography. Using this improved method, unidentified but new several sulfated residues beside of tyrosine-O-sulfate were observed in two dimensional TLE and general properties of soluble protein sulfotransferases corresponding to selected indivisual sulfated residues were reinvestigated.

Materials and Methods

Materials

Wister rats were supplied by the Animal Bleeding Laboratory of Seoul National University. ATP, inorganic pyrophosphatase, PPO, and nonradioactive 3'-phosphoadenosine-5'-phosphosulfate (PAPS) were purchased from Sigma Chemical Co. Dimethyl POPOP was obtained from Packard. Thin layer cellulose chromatography plastic sheets were from Merck. Radioactive carrier free $^{35}SO_4^{2-}$ (1,041 Ci/mmol) and hyper film^{TM}-β_{max} were from Amersham. All other chemicals used in this study were analytical grade available commercially.

Preparation of Soluble Proteins

Brain of Wistar rat was obtained by decapitation and the whole rat brain was homogenized in 3 volumes of ice cold isotonic phosphate buffer (pH 7) by using ultra-turrax homogenizer. The homogenate was centrifuged in the Beckman L8-70M ultracentrifuge for 1 hour at 100,000 × g. The supernatant was used not only as enzyme source but also contained endogeneous substrates. The protein concentration of the supernatant was estimated by the Lowry method with bovine serum albumin as a standard protein (Lowry *et al.*, 1951)

Preparation of Radioactive [^{35}S] PAPS

[^{35}S] PAPS was synthesized by re-optimizing the enzymatic method of Lee (1989). The liver of Wistar rat was excised, chilled and homogenized by using ultra-turrax homogenizer in 5 volumes of ice cold 0.25 M sucrose solution. The homogenate was centrifuged in the Beckman L8-70M ultracentrifuge for 1 hour at 100,000 × g. The supernatant was used as an enzyme source for [^{35}S] PAPS synthesis. The updated reaction mixtures which contained 50 mM Tris-HCl buffer (pH 8), 15 mM ATP, 12 mM $MgCl_2$, inorganic pyrophosphatase 10 units, 1 mCi of carrier free $^{35}SO_4^{2-}$ and 0.4 mg/ml of the soluble proteins of rat liver in 100 μl. The resultant mixture was incubated at 30 °C for 2 hours. The reaction was quenched by boiling for 40 seconds and the denatured proteins were pelleted by centrifugation in a clinical centrifuge. The [^{35}S] PAPS was isolated by preparative paper electrophoresis as before. Overall yield of [^{35}S] PAPS in this method is about 30%. Because the amount of nonradioactive PAPS in the enzyme source was not known, the specific activity of [^{35}S] PAPS was assumed to be that of carrier free $^{35}SO_4^{2-}$ (1,041 Ci/mmol).

In Vitro Reaction Condition of Protein Sulfation

The standard reaction mixture (100 μl) contained 50 mM Tris-maleate buffer at pH 8, 20 mM $MgCl_2$, 0.25 mg of soluble protein, 1~2 μci [^{35}S] PAPS with 3.3 μM of nonradioactive PAPS (final specific activity; 6.3 Ci/mmol), which were controled as a final concentration. The resultant reaction mixture was incubated for 2 hours at 30 °C. Controls were mostly taken as zero-time incubation but in some case enzyme-free system was taken as control. After incubation the reaction

mixture was cooled and immediately transferred into an ampoule. The reaction was then stopped by addition of 800 μl ice cold acetone (final 88%) and centrifuged to obtain protein pellet.

Hydrolysis of Sulfated Proteins

In the studies of protein sulfation, alkaline hydrolysis was effectively used by Huttner group to differentiate tyrosine sulfate from carbohydrate sulfates (Huttner, 1984). In this study Huttner's alkaline hydrolysis was modified extensively and developed to simple but reproducible method. For alkaline hydrolysis, 0.2 M Ba $(OH)_2$ solution was added to the ampoule containing protein precipitate, followed by sealing in vacuum. The sealed proteins were then hydrolyzed in an oven keeping 110 °C ~ 120 °C for 40 hours. The sample was cooled at 4 °C and centrifuged for 10 minutes by a clinical centrifuge. The resulting supernatant was transfered to a test tube and the ampoule was washed twice further with 0.5 ml water. The combined supernatant was neutralized with 1 N sulfuric acid using the phenol red as indicator. The sulfuric acid was carefully added into the supernatant until phenol red turned to yellow. In order to adjust pH to 7 ~ 8, the solution was titrated back with solid $BaCO_3$ until phenol red turned to pink color. By centrifugation the precipitate was removed and the collected supernatant was freeze-dried. The dried sample was dissolved in 20 μl water and an aliquot of 5 μl was counted to estimate the total sulfated residues recovered. The radioactivity was measured in a LKB liquid scintillation counter (Model 1219-001) with a cocktail consisted of 667 ml toluene, 333 ml Triton X-100, 5.5 g PPO, and 0.1 g dimethyl POPOP. The counting efficiency was determined to be about 85%. The remaining sample solution was used to separated sulfated residues by TLE.

Thin Layer Cellulose Electrophoresis

Samples of the hydrolysate were spotted on 20 cm × 20 cm thin layer cellulose plastic sheet up to 10 samples per sheet. Unlabeled authentic residues of tyrosine sulfate, serine sulfate, threonine sulfate, and cystein sulfate were used for positive identification of sulfate amino acids. The authentic samples were synthesized by nonenzymatic organic method (Reitz *et al.*, 1946). The cellulose sheet was masked with pH 5.2 electrophoresis buffer (10% pyridine-5% acetic acid) by using Whatmann 3MM paper. The electrophoresis in this buffer was carried out for 3 hours at 400 volts. When the marker, phenol red, migrated approximately 8 ~ 8.5 cm from the origin, the electrophoresis was stopped. After drying in air, the sheet was used for fluorography. And at later time the sheet was dissected out by comparing to the fluorography and counted for any radioactive sulfated residues. For better separation, two dimensional electrophoresis was carried out. The sample was spotted on the site of 3 cm from both bottom at right edges of cellulose sheet. After the cellulose sheet was wetted by a mask of Whatmann 3MM paper, it was electrophoresed in pH 3.5 buffer (5% acetic acid -0.5% pyridine). Then the cellulose sheet was dried carefully and the pH 5.2 electrophoresis buffer (20% pyridine-10% acetic acid) was sprayed uniformly on the sheet. The second electrophoresis in this buffer was carried out to the vertical direction of the first. After terminating electrophoresis, the fluorography and counting were followed as before.

Fluorography

The cellulose sheet, subjected to electrophoresis, was transiently socked in 20% PPO solution dissolved in ether. The PPO coated cellulose sheet was covered with high performance film (Hyper film^{TM}-β_{max}) and put into X-omatic cassette for amplification. The film was kept in deep freezer of -70 °C for 4 ~ 7 days depending on the radioactivity of the samples.

Results and Discussion

Alkaline Hydrolysis of Sulfated Proteins

The advantage of alkaline hydrolysis is to eliminate directly the carbohydrate sulfate moiety from the sulfated amino acid residues. It is well known that carbohydrate sulfates are labile in alkali while amino acid sulfates like tyrosine sulfate is relatively stable. In this experiment, Ba $(OH)_2$ was used as an alkaline source because it effectively precipitated free SO^{2-}_4 generated from carbohydrate sulfate as form of $BaSO_4$ as well as it hydrolyzed proteins. Previously, acid treatment

which hydrolyzed preferentially the sulfated amino acid residues had been used indirectly to differentiate tyrosine sulfate from carbohydrate sulfate (Lee and Choi, 1990). For the comparison of these two assay methods, the amount of carbohydrate sulfate estimated from the acid treatment was compared to that of sulfated amino acid residues from the alkaline hydrolysis (Table 1). In the acid treatment, the amount of total sulfated proteins was estimated after 7% SDS-PAGE and the amount of carbohydrate sulfate was found to be about 34% of the total sulfated proteins. It left the acid labile sulfated residues which included residues of amino acids was 66%. In the alkaline hydrolysis, the amount of total sulfated protein was estimated after acetone precipitation and the amount of sulfated hydrolysate which contained the amino acid sulfates was estimated to be about 11%. Therefore the sulfated amino acid obtained by alkaline hydrolysis is only one-sixth of the acid labile residues. This indicates that two assay methods are considerably different from each other to estimate the extents of sulfation in amino acid residues. In the acid treatment, it was likely to offer an overestimation of the amino acid sulfation, since it tended to wash out too much when the gel was soaked in the hot acid. On the other hand, the outcome of the alkaline hydrolysis could yield a lower limit of sulfated amino acids, since the hydrolysis was carried out at elevated temperature for a long time. However, the alkaline method has a definite advantage over the acid method because the sulfated amino acids are directly recovered from the hydrolysate and the residues are identified positively by the combination of TLE and fluorography.

Separation of Sulfated Residues by TLE

To separate the sulfated residue obtained by alkaline hydrolysis, thin layer cellulose electrophoresis was employed. A typical fluorography pattern of one-dimensional TLE is shown in Fig. 1. The pattern revealed unexpectedly nine spots of sulfated residues. In order to identify each sulfated residues, four amino acid sulfates, tyrosine-O-sulfate, threonine-O-sulfate, serine-O-sulfate, and cystein-S-sulfate were used as authentic samples. By simple comparison, it was able to identify positively the presence of tyrosine-O-sulfate at the spot migrated 10 cm from the origin. However, the other spots were not coincided exactly with the remaining authentic samples, espe-

Table 1. Comparison of assay methods for sulfated amino acids: acid treatment vs. alkaline hydrolysis

Acid treatment			Alkaline hydrolysis		
	DPM	ratio(%)		DPM	ratio(%)
SDS-PAGE (total)	347,590	100	Acetone Precipitate (total)	472,445	100
Acid resistant	117,789	34	Alkaline labile	—	(89)
Acid labile	229,801	66	Alkaline hydrolysate	51,004	11

The result of acid treatment was obtained from reaction system consisted of Tris-maleate buffer (pH 6.7), 5 μCi [^{35}S] PAPS (1,209 Ci/mmol), and soluble proteins. After SDS-PAGE (7%), the sulfated proteins in the gel were acid-treated with hot 1 N HCl solution for 15 min (Lee, 1989). Then, radioactive sulfates of the carbohydrate were determined in the gel. This was called acid-resistant and the amount of it was regarded as that of the carbohydrate sulfates. The result of alkaline hydrolysis was obtained from reaction system consisted of Tris-maleate buffer (pH 8), 3 μCi [^{35}S] PAPS, 20 mM $MgCl_2$, and soluble prtoeins. The procedure of alkaline hydrolysis was described in the Methods part.

cially the two major spots of A and B which accounted almost one half of the recovered radioactivities were not matched. The recovered radioactivities of A, B and tyrosine-O-sulfate were 19%, 24% and 8% of the total radioactivity, respectively (Fig. 1). The spot at the migration distance of 10.6 cm was likely to be matched to threonine-O-sulfate, but the spot was not separated well enough to claim without reservation.

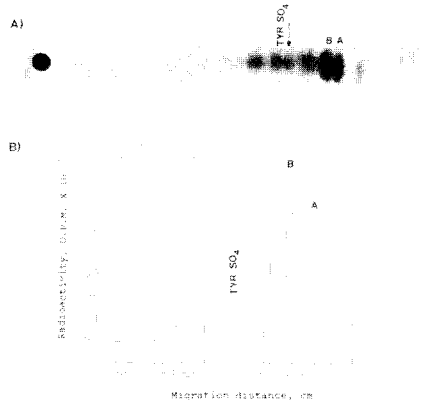

Fig. 1. One dimensional TLE after the alkaline hydrolysis of all sulfated proteins. A) Fluorography; B) Sliced pattern of radioactivity.

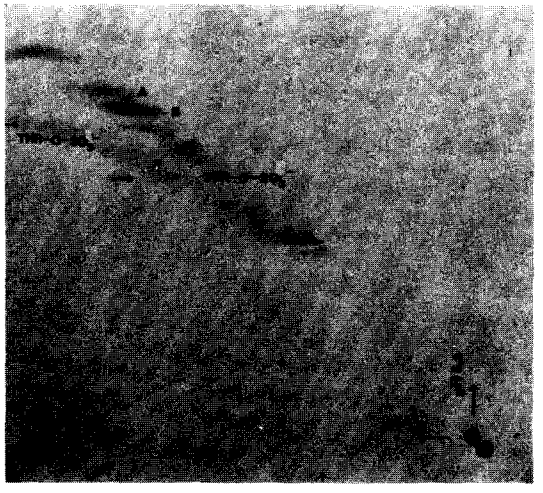

Fig. 2. Two-dimensional TLE of sulfated residues after the alkaline hydrolysis. Tyrosine-O-sulfate and threonine-O-sulfate were positively identified even though their intensity were relatively low.

When one-dimensional TLE was switched to two-dimensional TLE for better resolution, the threonine-O-sulfate was able to separate among the nine spots, although the intensity of the threonine spot was relatively low (Fig. 2). This finding suggests a strong possibility of occurrence of sulfation in other amino acid residues besides of tyrosine-O-sulfate. And it opens up a research avenue toward various protein sulfotransferases corresponding to individual sulfated amino acid residues.

Characterization of Protein Sulfotransferases Corresponding to Individual Sulfated Residues

In the present study, the assay of protein sulfotransferases (PST) was limited to the two major spots and tyrosine-O-sulfate because A and B were contained almost one half of the recovered total radioactivity of the hydrolysate and tyrosine-O-sulfate was the only residue identified positively in one-dimensional TLE, although its radioactivity was relatively small (8% of the total). The PST assay was routinely performed by one-dimensional TLE. Fig. 3 shows the effects of PAPS concentrations on the PST activities corresponding to these three residues individually. And each of the PST activity seems to reach its maximal activity around $3 \sim 4\,\mu M$ of PAPS. When the effects of pHs on the PST activities of the three residues were examined, the optimal pH for each activities was around 8 (Fig. 4). This pH optimum was shifted to alkaline pH from that obtained by the acid treatment method (Lee and Choi, 1990). Effects of incubation temperatures for each residue differ from each other as shown in Fig. 5. While the PST activity for unknown A showed a broad temperature dependence with an optimal temperature at around 20 °C, the maximal temperature for unknown B was found to be at 30 °C. On the other hand, tyrosine-O-sulfate had an optimal temperature of 37 °C. The effects of Mg^{2+} concentration on the PST were shown in Fig. 6 and it also revealed quite different effects from each other. The PST activity of unknown B showed an increased activity following the addition of Mg^{2+} ions up to 20 mM. However, the others did not show any significant dependence on Mg^{2+} ion. These different properties of PST

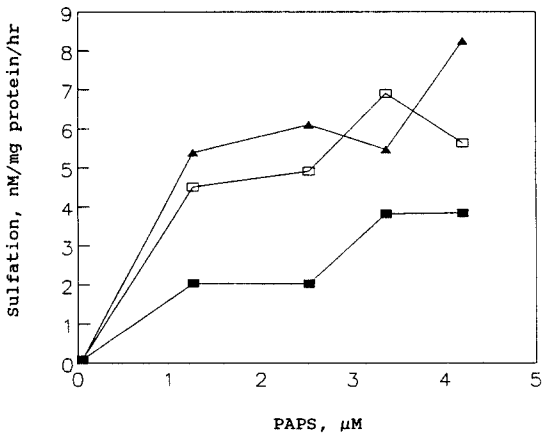

Fig. 3. Effects of PAPS concentration on the PST activities for each residue. Unknown A (—▲—); unknown B (—□—); tyrosine-O-sulfate (—■—).

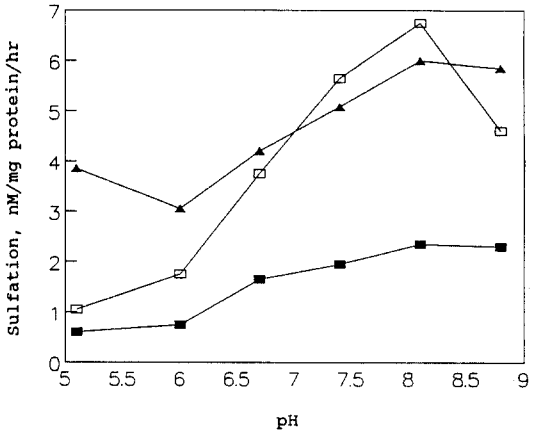

Fig. 4. Effects of pH on the PST activities for each residue. Unknown A (—▲—); Unknown B (—□—); tyrosine-O-sulfate (—■—).

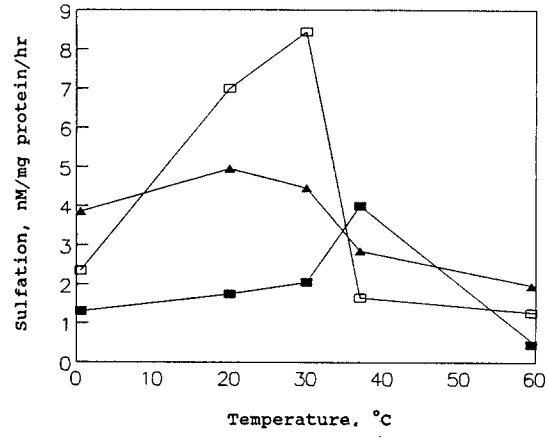

Fig. 5. Effects of incubation temperature on the PST activities for each residue. Unknown A (—▲—); Unknown B (—□—); tyrosine-O-sulfate (—■—).

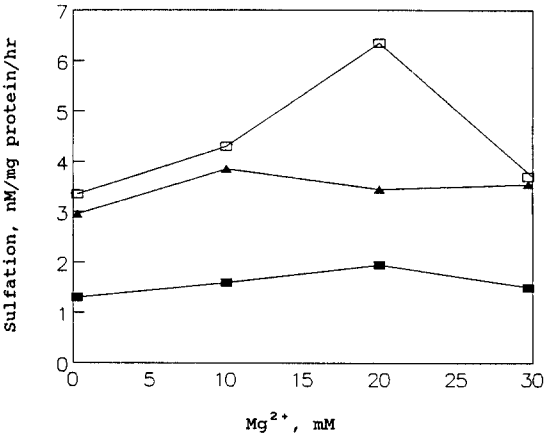

Fig. 6. Effects of Mg^{2+} concentration on the PST activities for each residue. Unknown A (—▲—); Unknown B (—□—); tyrosine-O-sulfate (—■—).

for each sulfated residue could be used as an indicative of multiplicity of the PST activities corresponding to individual sulfated residues derived from the supernatant of rat brain.

On the basis of the improvement made in the current assay technique of PST, a direct identification of sulfated residue and an assay for individual residue, the present data offer more reliable properties of PST than previous one which could not separate the individual sulfated residues. However, the current approach still has a handicap of including all the sulfated protein in the reaction mixture, thereby it offered only an average property of PST corresponding to each sulfated residues that may originated from different proteins. Nonetheless the following conclusion can be drawn safely within the context of present experiment: *In vitro* protein sulfation using the supernatant of rat brain demonstrates that sulfation can occur not only in tyrosine residue but also in other amino acid residues and there is a strong possibility of presence of more than one protein sulfotransferase corresponding to each sulfated residue in the supernatant.

References

Bettelheim, F. R., 1954. Tyrosine-O-sulfate in A peptide from fibrinogen. *J. Am. Chem. Soc.* **76**:2838-2839.

Baeuerle, P. A., F. Lottspeich, and W. B. Huttner, 1988. Purification of yolk protein 2 of drosophila melanogaster and identification of its site of tyrosine sulfation. *J. Biol. Chem.* **263**:14925-14929.

Chen, L. J., G. L. Rosenquist, and I. H. Segel, 1989. Enzymatic sulfation of gastrin in rat gastric mucosa. Abstract presented in 5th FAOB congress, p. 358, Aug 13 ~ 18, Seoul.

Friederich, E., P. A. Baeuerle, H. Garoff, B. Hovemann, and W. B. Huttner, 1988. Expression, tyrosine sulfation, and secretion of yolk protein 2 of drosophila melanogaster in mouse fibroblasts. *J. Biol. Chem.* **263**:14930-14938.

Hille, A., P. Rosa, and W. B. Huttner, 1984. Tyrosine sulfation: a post-translational modification of proteins destined for secretion? *FEBS Letters* **177**:129-134.

Huttner, W. B., 1982. Sulphation of tyrosine residues — a widespread modification of proteins. *Nature* **299**:273-276.

Huttner, W. B., 1984. Determination and Occurrence of tyrosine-O-sulfate in proteins. *Methods in Enzymology* **107**:200-223.

Huttner, W. B., 1988. Throsine sulfation and the secretory pathway. *Ann. Rev. Physiol.* **50**:363-376.

Karp, D. R., 1983. Post-translational modification of the fourth component of complement. *J. Biol. Chem.*, **258**:12745-12748.

Kim, H. M. and M. Choi, 1989. Characterization of protein sulfotransferases from rat adrenal gland: Identification of sulfated amino acid residues. 5th FAOB congress (Aug 13 ~ 18, Seoul). Abst. pp. 258.

Kohli, R., N. Chadha, and K. Muralidhar, 1988. Presence of tyrosine-O-sulfate in sheep pituitary prolactin. *FEBS Letters* **242**:139-143.

Lee, J. and M. Choi, 1990. Identification and partial characterization of soluble protein sulfotransferase in rat brain. *Korean Biochem. J.* **23**:62-68.

Lee, R. W. H. and W. B. Huttner, 1983. Tyrosine-O-sulfated proteins of PC12 pheochromocytoma cells and their sulfation by a tyrosilprotein sulfotransferase. *J. Biol. Chem.* **258**:11326-11334.

Lee, R. W. H. and W. B. Huttner, 1985. $(Glu^{32}, Ala^{30}, Tyr^{8})_n$ serves as high-affinity substrate for tyrosylprotein sulfotransferase: a golgi enzyme. *Proc. Natl. Acad. Sci. U.S.A.* **82**:6143-6147.

Liu, M. C. and F. Lipmann, 1985. Isolation of tyrosine-O-sulfate by pronase hydrolysis from fibronectin secreted by fujinami sarcoma virus-infected rat fibroblasts. *Proc. Natl. Acad. Sci. U.S.A.* **82**:34-37.

Liu, N. and J. U. Baenziger, 1986. *In vivo and in vitro* tyrosine sulfation of a membrane glycoprotein. *J. Biol. Chem.* **261**:856-861.

Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randal, 1951. Protein measurement with the folin phenol reagent. *J. Biol. Chem.* **193**:265-275.

Reitz, H. C., R. E. Ferrel, H. Franenkel-Conrat, and H. S. Olcutt 1946. Action of sulfating agents on proteins and model substances. *J. Am. Chem. Soc.* **68**:1024-1035.

Vargas, F., O. Frerot, M. D. Trung-Tung, and J. C. Schwartz, 1985. Characterization of a tyrosylsulfotransferase in rat brain using cholecystokinin derviatives as acceptors. *Biochemistry* **24**:5938-5943.

Vargas, F. and J. C. Schwartz, 1987. Apparent identity of cerebral tyrosylsulfotransferase activities using either a cholecystokinin derivative or an acidic amino acid polymer as substrate. *FEBS Letters* **211**:234-238.

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황산화 단백질의 알칼리 가수분해에 의한 쥐 뇌의 단백질 황산기전달효소의 추가특성 연구

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개선된 방법인 알칼리 가수분해와 얇은 층 cellulose 전기영동 (TLE)으로 *in vitro* 조건에서 쥐 뇌의 용해부분에서의 단백질 황산화의 특성을 추가로 조사하였다. 단백질 황산화는 방사능을 띤 [^{35}S] 3'-phosphoadensoine-5'-phosphosulfate (PAPS), Trismaleate buffer (pH 8), $MgCl_2$, 그리고 쥐 뇌의 용해 단백질이 포함된 반응체 내에서 실행되었다. 황산화된 단백질들은 acetone에 의해 침전된 후 황산화된 아미노산을 얻기위해 알칼리 가수분해를 하였다. 더나아가 그 가수분해물을 TLE로 분리하고 황산화된 잔기들을 fluorography를 통해 확인하였다. 일차원 TLE의 fluorography에서는 tyrosine-O-sulfate를 포함한 적어도 9개의 황산화된 잔기들이 나타났다. Tyrosine-O-sulfate를 제외한 다른 잔기들은 아직 분명하게 밝혀지지 않고 있다. 이런 방법으로 단백질 황산기전달효소 (PST)의 일반적 성질들 즉, PAPS 농도, pH, 반응 온도, 그리고 Mg^{2+} 등의 효과를 재조사하였다. 이런 결과들은 쥐 뇌에서 각 황산화된 잔기들에 해당하는 여러 개의 PST의 존재가 가능하며 단백질 황산화가 tyrosine 뿐만 아니라 다른 잔기들에서도 일어날 수 있음을 암시한다.