Tyrosine O-Sulfation

Tyrosine O-sulfation of proteins is a widespread post-translational modification that occurs in all animals. The sulfate transfer to tyrosine residues is catalyzed by one of two tyrosylprotein sulfotransferase (TPST) isoenzymes (EC 2.8.2.20; Fig. 14.7.1), which are integral membrane proteins of the trans-Golgi network (TGN), with their catalytic site facing the lumen. Consequently, all tyrosine-sulfated proteins must transit through the TGN. Moreover, the recognition by TPST of the tyrosine residue to be sulfated in a particular protein requires the presence of certain structural features (see Strategic Planning). Tyrosine sulfate in proteins can be identified by various methods, including metabolic labeling using inorganic [^{35}S]-sulfate (see Basic Protocol 1) followed by tyrosine sulfate analysis (see Basic Protocol 2). The [^{35}S]-labeling may be performed with whole animals, tissue explants, tissue slices, and cells in culture. This unit outlines procedures to determine whether a protein of interest contains a tyrosine-sulfated residue, using mammalian adherent cells.

STRATEGIC PLANNING

Prediction of Tyrosine Sulfation Sites

The first step to investigate whether the protein of interest contains a tyrosine-sulfated residue is to determine the presence of a potential tyrosine sulfation site. In order to be a substrate for TPST, a tyrosine residue in the protein must meet the following three criteria.

1. The tyrosine residue must be situated either on a secretory protein or on the lumenal domain of a transmembrane protein. This is a consequence of the fact that the catalytic site of TPST is oriented toward the TGN lumen. Practically, this means that a tyrosine can only be sulfated if the protein contains an N-terminal signal sequence or at least one transmembrane domain. For prediction of tyrosine sulfation sites in transmembrane proteins, the topology of the membrane insertion must be considered (*UNIT 2.2*).
2. The tyrosine residue must be situated in an acidic environment. Although no strict consensus sequence for tyrosine sulfation sites exists, all sequences exhibit the

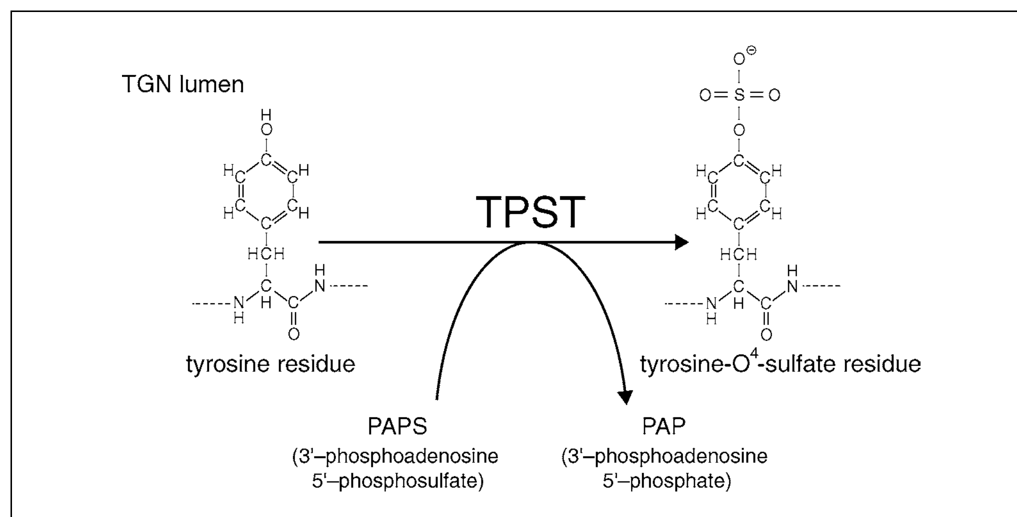

Figure 14.7.1 The formation of a tyrosine-O^4-sulfate residue is catalyzed by tyrosylprotein sulfotransferase (TPST) using 3′-phosphoadenosine 5′-phosphosulfate (PAPS) as co-substrate and sulfate donor. This enzymatic reaction occurs in the lumen of the trans-Golgi network (TGN).

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presence of acidic amino acid residues in the vicinity, i.e., positions −5 (N-terminal) to +5 (C-terminal), of the sulfated tyrosine residue. Turn-inducing amino acids (P, G) are also frequently present. The few examples of tyrosine sulfation sites lacking proline and glycine are located near the N- or C-terminus of the protein and/or contain several of the three other amino acid residues (D, S, N) that have significant turn-conformational potential.

3. The tyrosine residue must be sterically accessible to TPST. This usually requires the absence of cysteine residues or potential N-glycosylation sites (NXS or NXT) in the vicinity (positions −7 to +7), which could give rise to disulfide bridges or bulky carbohydrate structures, respectively.

The prediction of potential tyrosine sulfation sites can be supported by bioinformatics. The most recent software is called Sulfinator, developed by the SWISS-PROT group (Monigatti et al., 2002) and available at the ExPASy server (*http://www.expasy.org/tools/sulfinator/*). Sulfinator compares the input sequence with a consensus pattern derived from a multiple sequence alignment of 68 stretches with known tyrosine-sulfated residues.

Note that Sulfinator restricts itself to the above criterion 2, while criteria 1 and 3 are not covered.

BASIC PROTOCOL 1

LONG-TERM [^{35}S]-SULFATE LABELING OF MAMMALIAN CELLS IN CULTURE AND IMMUNOPRECIPITATION

To investigate whether a protein of interest contains a sulfated tyrosine residue, long-term [^{35}S]-sulfate labeling is used. The radiolabeled protein can be recovered by immunoprecipitation either from the cell lysate or, in the case of a secreted protein, the culture medium, and further analyzed by SDS-PAGE and autoradiography or fluorography (see Support Protocol 1). This assay is appropriate for a protein of interest endogenously expressed in a given cell line or upon its heterologous expression in host cells.

If antibodies capable of precipitating the protein of interest are not available, an alternative approach is epitope tagging, whereby the protein of interest is engineered to carry an antigenic determinant to which a commercial antibody is available. It is important that the suitability of the antibody for immunoprecipitation is thoroughly established, and the target epitope does not contain a potential tyrosine sulfation site.

Materials

Mammalian adherent cells expressing protein of interest and appropriate control cells lacking the protein of interest or expressing the protein of interest containing a point mutation in the tyrosine sulfation site
Complete tissue culture medium appropriate for cell line
Labeling medium with and without label (see recipe), prewarmed
PBS (*APPENDIX 2E*), cold
Immunoprecipitation buffer (see recipe) with protease inhibitors, ice cold
RIPA lysis buffer (see recipe) with protease inhibitors, ice cold
Antibody
Protein A-agarose beads
Wash buffers A, B, and C (see recipes)
2× SDS-PAGE sample buffer (see recipe)

Disposable pipets
Centricon concentrator (Amicon)
2.0- and 1.5-ml microcentrifuge tubes
Cell scraper

15-ml centrifuge tubes
End-over-end rotator

Additional reagents and equipment for immunoprecipitation (*UNIT 13.2*) and SDS-PAGE (*UNIT 10.1*)

NOTE: All reagents and equipment coming into contact with live cells must be sterile, and proper sterile technique should be used accordingly.

NOTE: All culture incubations are performed in a 37°C, 5% CO_2 humidified incubator unless specified otherwise. Some media (e.g., DMEM) may require altered levels of CO_2 to maintain the pH at 7.4.

NOTE: The size of the cultures to be labeled depends on the amount of the protein of interest present in the cells. Typically, 100- to 500-mm diameter culture dishes are required.

CAUTION: Radioactive materials require special handling. See *APPENDIX 2B* concerning safe use of radioisotopes.

Prepare cells

1. Culture the mammalian adherent cells expressing the protein of interest and appropriate control cells to an appropriate stage of growth in complete tissue culture medium appropriate for the cell line (see Critical Parameters and Troubleshooting).

   For maximal $^{35}SO_4$ incorporation, cells should be subconfluent (60% to 80%). It is recommended to replace the medium with fresh growth medium 18 hr prior to labeling.

   Protein expression is often achieved by transient transfection with an appropriate expression vector. Several transfection methods are described in Current Protocols in Molecular Biology, UNITS 9.1-9.4.

   Control experiments with cells lacking the protein of interest or expressing the protein of interest containing a point mutation in the tyrosine sulfation site should be conducted in parallel.

2. Remove complete tissue culture medium from the cultured cells by aspiration. Wash away any residual serum-containing medium by adding prewarmed labeling medium without label (10 ml per 100-mm dish), and aspirate.

   It is important to hold the dishes at an angle and remove and add medium at one corner so as not to dislodge the cells from the plates.

3. Add 5 ml prewarmed labeling medium per 100-mm culture dish and incubate 7 to 16 hr.

   At the end of the labeling period, the medium can be discarded if the protein of interest is associated with cells. Preliminary experiments should be conducted to determine if the protein of interest is released into the medium.

For proteins released into the medium:

4a. Remove the labeling medium manually from the dish using a disposable pipet, centrifuge 5 min at 300 × *g*, 4°C, and carefully collect the supernatant. Discard the cell pellet and pipet as radioactive waste.

5a. Concentrate the supernatant in a Centricon unit to 500 µl by centrifugation (explicitly follow manufacturer's instructions).

   Carefully choose the cut-off value of the Centricon to ensure retention of the protein of interest.

6a. Dilute to 1.5 ml with cold PBS and repeat the concentration and dilution steps 5a and 6a.

7a. Concentrate to 200 μl as in step 5a and collect the concentrated protein solution.

8a. Dilute to 1.8 ml with ice-cold immunoprecipitation buffer containing protease inhibitors in a 2-ml microcentrifuge tube. Proceed to step 9.

For solutions requiring protease inhibitors, add protease inhibitor cocktail tablets (Roche Molecular Biochemicals) following the manufacturer's instructions.

For proteins associated with cells:

4b. Wash cells two times with 5 ml ice-cold PBS per 100-mm dish, and remove wash fluid by aspiration.

CAUTION: *The wash fluid is radioactive—follow applicable safety regulations for disposal.*

5b. Add 5 ml ice-cold PBS per 100-mm dish and scrape cells carefully with a disposable plastic cell scraper.

6b. Transfer the suspension to a 15-ml centrifuge tube, centrifuge 5 min at 300 × *g*, 4°C, and discard supernatant.

7b. Add ice-cold RIPA lysis buffer containing protease inhibitors to cell pellet, using 0.3 to 0.5 ml per 100-mm dish, resuspend the pellet by gentle agitation, and transfer to a 1.5-ml microcentrifuge tube. Incubate 30 min at 4°C.

8b. Clarify the detergent lysate by centrifuging 10 min at 10,000 × *g*, 4°C, in a refrigerated microcentrifuge. Transfer the supernatant (total lysate) to a 2.0-ml microcentrifuge tube and dilute to 1.8 ml with ice-cold immunoprecipitation buffer containing protease inhibitors. Discard the 1.5-ml microcentrifuge tube and pellet as radioactive waste.

It is worthwhile analyzing an aliquot of the clarified total lysate (step 8b) and/or the concentrated medium (step 7a) by SDS-PAGE (UNIT 10.1). The protein of interest may be highly sulfated and readily detectable (see Support Protocol 1) without prior enrichment, even if it is a relatively minor fraction of the total protein.

Immunoprecipitate

9. Carry out immunoprecipitation (*UNIT 9.8*), using the appropriate amount of antibody and incubate 16 hr at 4°C using an end-over-end rotator.

10. After the 16-hr incubation, add an appropriate amount of protein A-agarose beads to quantitatively precipitate the antibody-antigen complexes, and rotate end-over-end for 2 hr at 4°C.

Substitute with protein G beads when using antibodies that are not bound effectively by protein A.

11. Collect immunoprecipitate by microcentrifuging for 10 sec at maximum speed and wash the beads at 4°C successively with the following:

    a. one time with 1 ml immunoprecipitation buffer;
    b. three times with 1 ml wash buffer A;
    c. two times with 1 ml wash buffer B;
    d. one time with 1 ml wash buffer C.

12. Completely remove wash buffer from the beads with a micropipettor, add 1 vol of 2× SDS-PAGE sample buffer, and incubate 3 min in boiling water.

Analyze protein of interest

13. Analyze the protein of interest, potentially containing radiolabeled tyrosine sulfate, by SDS-PAGE (*UNIT 10.1*).

    After electrophoresis, detection of [^{35}S]-sulfate incorporation into the protein of interest is carried out as described in Support Protocol 1.

SUPPORT PROTOCOL 1

DETECTION OF [^{35}S]-SULFATED PROTEINS

To detect [^{35}S]-sulfated proteins after SDS-PAGE, several methods are available. The fixed gel, after being dried onto filter paper, can be exposed using a phosphorimager. Alternatively, the fixed, wet gel can be treated with sodium salicylate (*UNIT 14.3*) and fluorographed (*UNIT 10.2*). A rapid procedure to obtain an autoradiogram is to transfer labeled proteins from the gel to a nitrocellulose membrane using the blotting technique (*UNIT 10.7*), and to expose the nitrocellulose membrane to film.

Additional Materials *(also see Basic Protocol 1)*

Gel containing protein of interest (see Basic Protocol 1)
1 M sodium salicylate, pH 5 to 7
20% PPO (2,5-diphenyloxazole) in toluene (optional)

Whatman 3MM filter paper or nitrocellulose membrane
X-ray film (e.g., Kodak XAR-5 or equivalent) or phosphorimager

Additional reagents and equipment for staining and destaining gels (*UNIT 10.5*), fluorography (*UNIT 10.2*), and blotting (*UNIT 10.7*)

CAUTION: Solutions that come into contact with the gel may become radioactive. Gloves should be worn at all times. Sodium salicylate can cause allergic reactions and is readily absorbed through the skin.

To detect [^{35}S]-sulfated proteins by phosphorimaging:

1a. After electrophoresis, fix the gel and (if necessary) stain and destain by standard methods (*UNIT 10.5*).

    Because the tyrosine sulfate ester is acid-labile, the use of acetic acid rather than trichloroacetic acid is recommended.

2a. Dry the fixed gel onto Whatman 3MM filter paper and then detect labeled proteins directly using a phosphorimager.

    Time of exposure will depend on the amount of radioactivity in the sample and, in most cases, will have to be determined empirically by making multiple exposures for different lengths of time.

To detect [^{35}S]-sulfated proteins by fluorography:

1b. After electrophoresis, fix the gel and (if necessary) stain and destain by standard methods (*UNIT 10.5*).

    Because the tyrosine sulfate ester is acid-labile, the use of acetic acid rather than trichloroacetic acid is recommended.

2b. Soak the fixed gel for 1 to 5 hr in 20 vol water to remove residual acid, which could cause precipitation of salicylic acid from sodium salicylate in step 3b.

3b. Soak gel for 30 min in 10 vol of 1 M sodium salicylate, pH 5 to 7. Dry the gel and proceed with fluorography (*UNIT 10.2*) at −70°C.

The sodium salicylate method is recommended for fluorography because salicylate is water-soluble and can, therefore, easily be removed from the gel after fluorography. This renders the proteins in the gel suitable for further biochemical analysis such as tyrosine sulfate analysis (see Basic Protocol 2).

If the labeling of proteins is intense, salicylate treatment of the gel can be omitted and the dried gel can be subjected to autoradiography.

To prevent cracking of gels that are >10% polyacrylamide or >1.5-mm thick, 2% (v/v) glycerol can be added to the sodium salicylate solution.

To detect [^{35}S]-sulfated proteins by membrane autoradiography:

1c. After electrophoresis, transfer labeled proteins from the gel to a nitrocellulose membrane using the blotting technique (*UNIT 10.7*).

2c. Air-dry the nitrocellulose membrane and autoradiograph, or dip the nitrocellulose membrane in 20% PPO (2,5-diphenyloxazole) in toluene, dry, and fluorograph at −70°C.

This is an alternative, rapid procedure to obtain an autoradiogram after SDS-PAGE given that the time needed for autoradiography after transfer to nitrocellulose membrane is shorter than with dried polyacrylamide gels.

BASIC PROTOCOL 2

TYROSINE SULFATE ANALYSIS—ALKALINE HYDROLYSIS METHOD

Since incorporation of $^{35}SO_4$ into proteins may occur not only on tyrosine but also on carbohydrate residues, it is important to obtain positive evidence that the [^{35}S]-sulfated protein of interest indeed contains [^{35}S]-sulfated tyrosine. This protocol presents a simple chemical method based on exhaustive alkaline hydrolysis followed by thin-layer electrophoresis (see Support Protocol 2) to demonstrate the presence of radiolabeled tyrosine residues. The immunoprecipitate containing the protein of interest (see Basic Protocol 1) can be used as starting material.

Materials

Immunoprecipitate containing the protein of interest (see Basic Protocol 1)
Wash buffer D (see recipe)
Pronase solution: 100 μg/ml Pronase (Roche) in 5 mM NH_4HCO_3, pH 8
0.2 and 0.02 M barium hydroxide ($Ba(OH)_2$)
N_2 source
1 and 0.1 M sulfuric acid
Uncapped 3-ml glass centrifuge tube
110°C oven

CAUTION: Barium hydroxide may be fatal if swallowed, harmful if inhaled, and causes irritation to skin, eyes, and respiratory tract. It affects muscles and the central nervous system. Use gloves when handling and perform this experiment in a chemical fume hood.

1. Elute the [^{35}S]-sulfated protein of interest from antibody-protein A–agarose complexes (see Basic Protocol 1, step 11, with an additional final wash with 1 ml wash buffer D) by adding 200 μl Pronase solution. Incubate 12 to 24 hr at 37°C and microcentrifuge 10 sec at maximum speed.

The Pronase solution should be preincubated according to the manufacturer's instructions.

2. Transfer eluate into a disposable 3-ml glass centrifuge tube. Lyophilize eluate.

   The disposable glass tubes must tolerate the subsequent steps, i.e., centrifugation at 10,000 × g, and pH 14 at 110°C; they must also be suitable for gastight sealing. Uncapped 3-ml glass centrifuge tubes work well.

3. Prepare a fresh solution of 0.2 M barium hydroxide. Add 1 ml of 0.2 M $Ba(OH)_2$ to lyophilized eluate and seal tube under N_2 so that it is gas-tight.

   Use degassed water to reduce formation of $BaCO_3$.

   Usually, the barium hydroxide does not dissolve completely. While it is being stirred, take 1 ml of the barium hydroxide solution and add it to the residue of the lyophilized eluate.

   For uncapped glass tubes, freeze sample, evacuate, and seal by melting in flame.

4. Place the sealed tube in a 110°C oven for 20 to 24 hr.

5. Cool the sample to 4°C and break the seal manually by wrapping tube in a paper towel. Centrifuge for 10 min at 10,000 × *g*, 4°C, collect the supernatant and transfer it to another centrifuge tube.

6. (Optional) Add 0.4 ml water to pellet, vortex, centrifuge for 10 min at 10,000 × *g*, 4°C. Pool the second supernatant with first one.

   This step can be omitted if only qualitative results are required.

7. Discard the tube with the pellet as radioactive waste.

8. Neutralize the (pooled) supernatant by adding sulfuric acid, using the phenol red in the sample as indicator. Centrifuge sample for 10 min at 10,000 × *g*, 4°C.

   Carefully add 1 M sulfuric acid with frequent vortexing until the phenol red turns transiently yellow at the site of sulfuric acid addition. Then continue the neutralization with 0.1 M sulfuric acid until the sample is homogenously red-orange (pH ~7).

   If excess sulfuric acid is added accidentally (yellow color), back-titrate with 0.02 M barium hydroxide.

9. Collect the supernatant, and transfer to a 2-ml microcentrifuge tube.

10. (Optional) Add 0.4 ml water to pellet, vortex, and centrifuge as in step 8. Pool second supernatant with first one. Discard the tube with the pellet as radioactive waste.

    The pooled supernatant is referred to as neutralized supernatant.

    This step can be omitted if only qualitative results are required.

11. Lyophilize the neutralized supernatant.

    The residue of the neutralized supernatant is used for the identification of tyrosine [^{35}S]-sulfate by thin-layer electrophoresis (see Support Protocol 2).

SUPPORT PROTOCOL 2

THIN-LAYER ELECTROPHORESIS OF TYROSINE [^{35}S]-SULFATE

Thin-layer electrophoresis of tyrosine sulfate is performed under the same conditions as that of tyrosine phosphate (UNIT 13.3). Given that the alkaline hydrolysis and neutralization conditions used to liberate tyrosine sulfate from proteins (see Basic Protocol 2) render tyrosine sulfate the major, if not the only, [^{35}S]-labeled product in the neutralized supernatant, 1-D thin-layer electrophoresis is sufficient in most instances to demonstrate the presence of tyrosine sulfate. Because electrophoresis equipment differs considerably in design, the details of the assembly and placement of the plate are not discussed here (see UNIT 13.3). It is assumed that a suitable apparatus is available for use by an experienced operator. For fluorography, EN^{3}HANCE (PerkinElmer Life Sciences) can be used.

Materials

Lyophilized sample (see Basic Protocol 2)
Electrophoresis buffer, pH 3.5 (see recipe)
Acetone
Unlabeled L-tyrosine-*O*-sulfate (Bachem)
Phenol red
Unlabeled L-serine-*O*-sulfate (Bachem)
Unlabeled L-tyrosine (Bachem)
0.2% (w/v) ninhydrin in acetone (as spray)

Cellulose-coated plastic-backed TL chromatography sheet (20 cm × 20 cm × 100 μm)
Whatman 3 MM filter paper
100°C oven

Additional reagents and equipment for thin-layer electrophoresis (*UNIT 13.3*)

1. Dissolve the sample (residue after lyophilization of the neutralized supernatant, see Basic Protocol 2, step 11) in 20 μl electrophoresis buffer, pH 3.5. If the residue is small and dissolves readily, proceed to step 4 as the sample will run well in the thin-layer electrophoresis. However, if the residue appears to be too big to be loaded on a thin-layer sheet or does not dissolve readily, perform an acetone precipitation (steps 2 to 3).

2. Add 100 μl acetone to a 20-μl sample.

3. Sediment the precipitate by microcentrifuging 5 min. Collect the supernatant and evaporate the acetone. Repeat step 1.

4. Add 3 μg of unlabeled L-tyrosine-*O*-sulfate, and a trace of phenol red (100 to 300 ng) to the sample.

   Usually, the phenol red is already present from the alkaline hydrolysis.

5. Spot 25% to 100% of the sample, in 0.50-μl aliquots, on a 20-cm × 20-cm × 100-μm cellulose thin-layer chromatography sheet, 3 cm from the cathodic edge of the plate and adjacent to unlabeled L-tyrosine-*O*-sulfate, L-tyrosine, and L-serine-*O*-sulfate standards (3 μg each, in electrophoresis buffer, pH 3.5; see Fig. 14.7.2 for arrangement of samples). Between each application, dry the sample spot with compressed air delivered through a Pasteur pipet plugged with cotton.

   Use long, thin plastic micropipet loading tips for sample application, and do not let the tip touch the plate.

   The entire hydrolysate can be spotted onto a single origin; however, streaking may be observed upon overloading.

6. Wet the cellulose sheet with electrophoresis buffer, pH 3.5.

   This can be done either by spraying (if the spot is small) or by using a "mask' of Whatman 3 MM filter paper (if the spot is to be concentrated). Briefly, take a piece of the filter paper that is slightly larger than the cellulose sheet, cut circular holes slightly larger than the sample spots at the position corresponding to that of the sample and standards on the cellulose sheet. Wet the filter paper with electrophoresis buffer, pH 3.5 and place it on the cellulose sheet such that sample spots are not touching the filter paper. Allow the electrophoresis buffer to concentrically migrate toward the center of the spots, thereby concentrating the samples.

   The cellulose sheet should be homogeneously wet.

7. Place wet cellulose sheet in the electrophoresis apparatus (follow manufacturer's instructions).

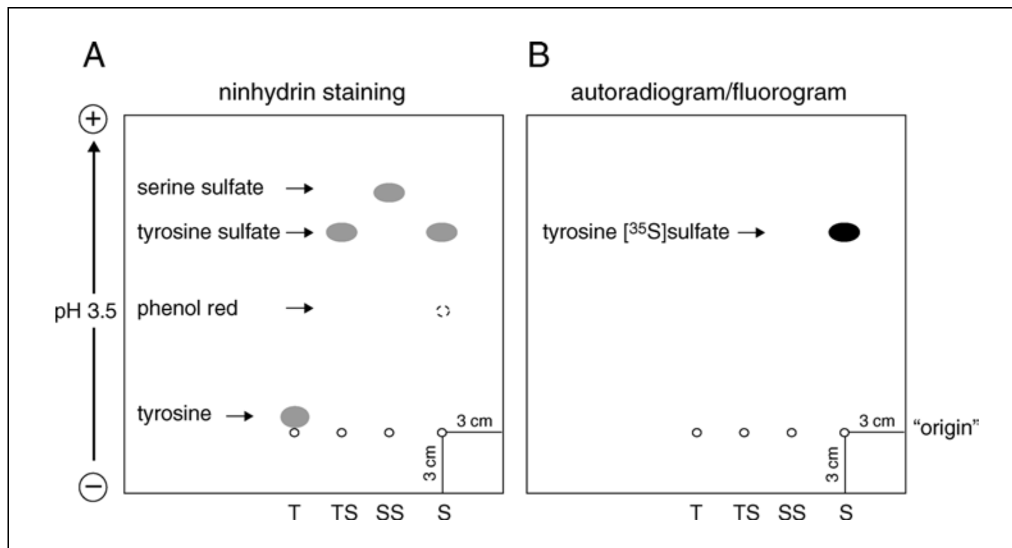

Figure 14.7.2 Thin-layer electrophoresis of tyrosine sulfate. Schematic diagram of a 1-D separation, showing the positions of sulfated and nonsulfated hydroxyamino acids. The sample (S) containing [^{35}S]-labeled tyrosine sulfate, and standards (tyrosine, T; tyrosine sulfate, TS; serine sulfate, SS) are analyzed by ninhydrin staining (**A**) and autoradiography/fluorography (**B**). The position of phenol red is indicated.

8. Electrophorese the samples in electrophoresis buffer, pH 3.5, until the phenol red marker has migrated 7 to 8 cm, i.e., just past the middle of the cellulose plate.

   Electrophoresis conditions will depend on the thin-layer electrophoresis apparatus used. Consequently, the appropriate duration and voltage of the electrophoresis need to be determined empirically by examining the rate of migration of unlabeled standards.

9. Remove the plate and quickly air-dry with a fan without heating.

   It takes ~20 min to dry the plate.

10. Spray the dry plate with 0.2% ninhydrin in acetone. Develop the color in a 100°C oven for a few minutes to visualize the sulfated amino acid standards.

11. Perform autoradiography or fluorography of the plate to detect radioactive tyrosine sulfate.

REAGENTS AND SOLUTIONS

Use Milli-Q-purified water or equivalent for the preparation of all buffers. For common stock solutions, see **APPENDIX 2E**; *for suppliers, see* **SUPPLIERS APPENDIX**.

Dialyzed serum

Dialyze serum overnight against PBS (*APPENDIX 2E*) at 4°C. Use 100 times the serum volume for the dialysis, and change the solution once during dialysis. Sterile-filter after dialysis and store in 1- or 2-ml aliquots in sterile tubes for up to 1 year at −20°C. After thawing, store dialyzed serum for up to 1 month at 4°C.

Electrophoresis buffer, pH 3.5

5% (v/v) acetic acid
0.5% (v/v) pyridine
Store up to 1 year in a sealed bottle at room temperature

Immunoprecipitation buffer

1% Triton X-100
0.1% SDS
150 mM NaCl
10 mM EDTA
50 mM Tris · Cl, pH 7.8 (*APPENDIX 2E*)
Store up to 1 year at 4°C

Labeling medium

Sulfate-free tissue culture medium (see recipe) containing:
$MgCl_2$ instead of $MgSO_4$
10% of the normal concentration of methionine and cysteine
1% dialyzed serum (see recipe)
Carrier-free $Na_2{}^{35}SO_4$ (1.0 mCi/ml; >900 Ci/mmol) (Amersham Biosciences)
Prepare fresh

See Critical Parameters for more information.

Low-Met/Cys amino acid mixture, 10x

For 1 liter:
0.84 g L-arginine · HCl
0.063 g L-cystine
0.30 g glycine
0.42 g L-histidine · HCl
1.05 g L-isoleucine
1.05 g L-leucine
1.46 g L-lysine · HCl
0.03 g L-methionine
0.66 g L-phenylalanine
0.42 g L-serine
0.95 g L-threonine
0.16 g L-tryptophan
0.94 g L-valine
Dissolve in sterile Milli-Q-purified water and adjust volume to 1 liter
Store up to 6 months at −20°C

Having low methionine and cysteine improves the [^{35}S]-sulfate incorporation by reducing the amount of unlabeled sulfate that may arise from the metabolism of sulfur-containing amino acids and that would reduce the specific activity of the radioactive sulfate. Methionine and cysteine at 10% the normal concentration is usually sufficient to maintain protein synthesis and keep the cells healthy.

Tyrosine is omitted from the above 10× amino acid mixture and included directly in the sulfate-free tissue culture medium (see recipe) because tyrosine is insoluble at 10× the normal concentration.

Do not add antibiotics, as some (e.g., streptomycin) are sulfate salts.

RIPA lysis buffer

0.15 M NaCl
1% (v/v) Nonidet P-40 (NP-40)
0.5% (w/v) sodium deoxycholate
0.1% (w/v) SDS
0.05 M Tris · Cl, pH 8.0
Store up to 1 year at 4°C

SDS-PAGE sample buffer, 2×

20% (v/v) glycerol
4% (w/v) SDS
0.125 M Tris · Cl, pH 6.8 (*APPENDIX 2E*)
0.2 M DTT, added fresh
0.01% bromophenol blue
Store up to 6 months at −20°C

Sulfate-free tissue culture medium

For 1 liter:
265 mg $CaCl_2 \cdot 2H_2O$
0.10 mg $Fe(NO_3)3 \cdot 9H_2O$
400 mg KCl
168.75 mg $MgCl_2 \cdot 6H_2O$
6.4 g NaCl
125 mg $NaH_2PO_4 \cdot H_2O$
5 ml 20% (w/v) glucose
10 ml 11.0 mg/ml sodium pyruvate (added dropwise, see below)
49.3 ml 7.5% (w/v) $NaHCO_3$
72.0 mg tyrosine
40 ml 100× MEM vitamins (Sigma)
100 ml 10× low-Met/Cys amino acid mixture (see recipe)
3 ml 0.5% (w/v) phenol red
Mix the above ingredients (except phenol red) with 900 ml Milli-Q-purified water and stir for several hours at room temperature or overnight at 4°C to dissolve. Add phenol red and adjust pH to 7.2 with 1 M NaOH. Adjust volume to 1 liter with water. Filter sterilize solution through a 0.2-μm filter. Store up to 6 months at 4°C in the dark. Just before use, add L-glutamine (4 mM final).
NOTE: Sodium pyruvate is added drop-wise to avoid precipitation.

Wash buffer A

0.2% NP-40
150 mM NaCl
2 mM EDTA
10 mM Tris · Cl, pH 7.5 (*APPENDIX 2E*)
Store up to 1 year at 4°C

Wash buffer B

0.2% NP-40
500 mM NaCl
2 mM EDTA
10 mM Tris · Cl, pH 7.5 (*APPENDIX 2E*)
Store up to 1 year at 4°C

Wash buffer C

10 mM Tris · Cl, pH 7.5 (*APPENDIX 2E*)
Store up to 1 year at 4°C

Wash buffer D

5 mM NH_4HCO_3, pH 8
Store up to 1 year at 4°C

COMMENTARY

Background Information

Sulfation of tyrosine residues is a late post-translational modification that takes place in the trans-Golgi network and occurs in a wide spectrum of transmembrane and secretory proteins (Huttner, 1982). Tyrosine-sulfated proteins are found in all animals, and also exist in the plant kingdom. While occurring throughout metazoan evolution, tyrosine-sulfated proteins appear to be absent in unicellular eukaryotes and prokaryotes, implicating this post-translational modification in some aspect of multicellularity (Huttner and Baeuerle, 1988; Moore, 2003; Corbeil and Huttner, 2004). For most tyrosine-sulfated proteins, the physiological role of this post-translational modification is presently unknown. A review with a comprehensive list of tyrosine-sulfated proteins has recently been published (Moore, 2003). With regard to the cases in which the physiological relevance of tyrosine sulfation of a particular protein has been elucidated, the common denominator has emerged that tyrosine sulfation promotes extracellular protein-protein interactions occurring in various biological processes, ranging from the receptor binding of regulatory peptides to the interaction of viral envelope proteins with the cell surface (Huttner et al., 1991; Farzan et al., 1999; Kehoe and Bertozzi, 2000; Costagliola et al., 2002). Moreover, tyrosine sulfation has been reported to modulate the secretion kinetics of certain secretory proteins (Friederich et al., 1988) and to promote proteolytic processing (Bundgaard et al., 1995). Thus, the demonstration that a given protein undergoes tyrosine sulfation and the identification of the sulfated tyrosine residue are important aspects of the characterization of its physiological activity.

The sulfate linked to tyrosine is present as an O^4-sulfate ester (Fig. 14.7.1). Such a modified tyrosine residue was first identified in 1954 in bovine fibrinopeptide B (Bettelheim, 1954). Remarkably, up to 1% of all tyrosine residues of the total protein in an organism can be sulfated, making this the most common post-translational modification of this particular residue (Huttner and Baeuerle, 1988). The sulfate transfer reaction to tyrosine residue is catalyzed by one of two trans-Golgi-associated tyrosylprotein sulfotransferases (TPSTs; EC 2.8.2.20) (Niehrs et al., 1994; Ouyang et al., 1998a,b; Beisswanger et al., 1998), which use the universal sulfate donor 3′-phosphoadenosine 5′-phosphosulfate (PAPS) as a co-substrate (Robbins and Lippmann, 1956) (Fig. 14.7.1). Biologically, tyrosine sulfation appears to be an irreversible modification in vivo due to the lack of a sulfatase capable of catalyzing the desulfation of tyrosine-sulfated proteins under physiological conditions. Extending previous attempts to detect serine sulfate and threonine sulfate in protein hydrolysates (Huttner, 1984; Huttner and Baeuerle, 1988), it has recently been demonstrated that, other than tyrosine residues, serine and threonine residues can be subject to sulfation (Medzihradszky et al., 2004).

Analysis of tyrosine sulfate

The method described in this unit is based on alkaline hydrolysis. Because the tyrosine sulfate ester is alkali-stable, but acid-labile, alkaline hydrolysis is the method of choice to release tyrosine sulfate from a polypeptide chain. For such hydrolysis, barium hydroxide is used because the inorganic radioactive sulfate, generated by hydrolysis of alkali-labile–sulfated carbohydrate residues of glycoproteins, is precipitated as barium sulfate, whereas tyrosine sulfate remains in solution (Huttner, 1984). The alkaline hydrolysis of [^{35}S]-labeled protein with barium hydroxide is not restricted to proteins that have been isolated by immunoprecipitation, but is also applicable to proteins contained in polyacrylamide gels, and to purified proteins (for details, see Huttner, 1984).

Several alternative analytical methods for studying tyrosine sulfation that are independent of radioisotope incorporation have been developed (Bundgaard et al., 2002). The sulfation of tyrosine residues in a protein of interest can be demonstrated using various chromatographic techniques, including reversed-phase high-performance liquid chromatography (*UNIT 8.7*). These methods are based on the chemical properties of the sulfate moiety, which introduces a highly acidic group into the protein of interest that alters its physical and chemical properties such as conformation and hydrophilicity. However, these analytical techniques require a pure protein or the use of specific tyrosine sulfate antisera, which are very difficult to generate. Nevertheless, an immunization protocol describing the generation of such antibody has been reported (Bundgaard et al., 2002). Tyrosine sulfate analysis can also be achieved using various techniques of mass spectrometry (MS), including matrix-assisted laser desorption/

ionization time-of-flight (MALDI-TOF) MS (*UNIT 16.5*) and electrospray ionization (ESI) MS (*UNIT 16.1*) (Önnerfjord et al., 2004). Although these techniques are very sensitive and precise, they require sophisticated and expensive instrumentation. Moreover, it is important to note that the tyrosine sulfate ester bond, which is more labile than peptide bonds, might be broken during the mass spectrometry acquisition, resulting in false-negative data (Wolfender et al., 1999).

Critical Parameters and Troubleshooting

A critical factor in the [^{35}S]-sulfate labeling of mammalian cells is the optimization of sulfate incorporation into the protein to be analyzed. The efficiency of sulfation is the most variable parameter and relies on maintaining the cells in a medium that is compatible with continued protein synthesis and with sulfate labeling conditions. The labeling medium that the authors use is a modified DMEM. Other media, such as RPMI, do not allow efficient sulfate incorporation (for additional information, see *Current Protocols in Cell Biology*, *UNIT 7.3*). The advantage of using the isotope at high specific activity should be balanced against the potential disadvantage that may result from the use of dialyzed serum. It is therefore recommended to ascertain that the use of sulfate-free medium and dialyzed serum during long-term [^{35}S]-sulfate labeling does not reduce the viability of the cells under investigation.

To facilitate the detection of [^{35}S]-labeled protein, it is important that most, if not all, cells used express the protein of interest. If the efficiency of the cell transfection is low, establishing a permanent cell line expressing the protein of interest is strongly recommended. NIH 3T3 cells (Niehrs et al., 1992), CHO cells (Weigmann et al., 1997), and PC12 cells (Glombik et al., 1999) are excellent host cells.

To determine if the protein of interest is sulfated on a tyrosine residue, control experiments utilizing a mutant form of the protein should be performed in parallel to substantiate any positive results. A phenylalanine residue can substitute the presumably sulfated tyrosine residue (Friederich et al., 1988). Negative controls with cells lacking the protein of interest should also be performed.

To exclude the possibility that the incorporation of [^{35}S]-sulfate occurs on N- and O-linked carbohydrates, the protein of interest recovered after immunoprecipitation can be treated with N-glycosidase F, neuraminidase, and/or O-glycosidase, prior to SDS-PAGE and detection of [^{35}S]-sulfated protein (Farzan et al., 1999). Likewise, it can be informative to perform [^{35}S]-labeling in the absence or presence of tunicamycin, an inhibitor of N-glycosylation. If, upon such treatments, the protein of interest displays a similar pattern of [^{35}S]-labeling, i.e., without any loss of signal intensity, it can be concluded that the protein contains sulfated tyrosine residues. Final proof that [^{35}S]-sulfate is indeed incorporated into tyrosine residues is provided by thin-layer electrophoresis of a $Ba(OH)_2$ hydrolysate of the immunoprecipitated protein of interest.

Anticipated Results

Typically, on thin-layer electrophoresis at pH 3.5, tyrosine sulfate migrates ~1.5 times as fast toward the anode as phenol red. The non-sulfated tyrosine residue remains near the origin, whereas serine sulfate migrates ~1.5 times as fast as tyrosine sulfate (see Fig. 14.7.2).

Time Considerations

After the cells have grown to the desired density, the [^{35}S]-labeling experiment can be completed within 1 day, unless an overnight labeling is needed. Harvesting the cells, preparing the cell lysate and/or concentrating the culture medium as well as the immunoprecipitation require at least 2 days. An additional day is needed for SDS-PAGE and fixing the gel. If transfer of labeled proteins from the gel to nitrocellulose membrane is to be performed, several additional hours are required depending upon the blotting technique used. Exposure time to X-ray film or phosphorimaging must be determined on an experiment-to-experiment basis. Tyrosine sulfate analysis (alkaline hydrolysis, thin-layer electrophoresis) requires ~1 day, and exposing the cellulose thin-layer sheet containing tyrosine [^{35}S]-sulfate another 2 to 5 days.

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Key References

Huttner, 1984. See above.

Describes methods for sulfate labeling and various procedures to detect tyrosine sulfate in proteins. The determination of the stoichiometry of tyrosine sulfation of proteins is also discussed.

Bundgaard et al., 2002. See above.

Describes several analytical methods for tyrosine sulfate analysis.

Contributed by Denis Corbeil,
Christoph Thiele, and
Wieland B. Huttner
Max-Planck-Institute of Molecular Cell Biology and Genetics
Dresden, Germany