A novel method for site-determination of tyrosine O-sulfation in peptides and proteins

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Method Article

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Abstract

Introduction

Tyrosine O-sulfation is one of many posttranslational modifications described in nature that can impart critical functional properties to proteins that are independent of the genes encoding them[1, 2]. It plays a key role in regulating protein-protein interactions in the extracellular space[3] and is catalyzed by two closely related Golgi enzymes called tyrosylprotein sulfotransferases \(TPST-1 and TPST–2)[4, 5]. In this protocol, we describe a novel subtractive strategy to determine the sites of tyrosine sulfation in peptides and proteins[6]. Hydroxyl groups on unsulfated tyrosines are stoichiometrically acetylated by a one-step reaction using sulfosuccinimidyl acetate in the presence of imidazole at pH 7.0. The presence of sulfotyrosine is indicated by the detection of free tyrosine after tandem mass spectrometric analysis during which the sulfuryl group of sulfotyrosine is decomposed. Since phosphorylation and sulfation of tyrosine are isobaric, we used alkaline phosphatase treatment to distinguish these two modifications. This strategy combined with the subtractive methodology is used to unambiguously determine the tyrosine sulfation sites in proteins. Application of this methodology will substantially facilitate identification of additional tyrosine-sulfated proteins and determination of the sites of sulfation, and thus, provide the foundation for a broader understanding of the role of tyrosine sulfation in protein function.

Reagents

Cholecystokinin peptide \(Tyr9-CCK-8, DY\(SO_3H)MGWMDFY, Bachem) Recombinant mouse lumican \(R&D systems) Recombinant human tyrosylprotein sulfotransferase-1 and -2 Urea Proteomics grade modified trypsin \(Promega) Glu-C \(Roche Applied Science) Chymotrypsin \(Roche Applied Science) Alkaline phosphatase \(Roche Applied Science) Dithiothreitol DMSO Iodoacetamide Immidazole Sulfosuccinimidyl acetate \(Pierce) 3’-phosphoadenosine 5’-phosphosulfate peptide:N-glycosidase F \(Prozyme) Ammonium bicarbonate Ammonium acetate Ammonium hydroxide Formic acid Acetonitrile Methanol Organic solvents are HPLC grade or better. Reagents should be of the highest grade available

Equipment

Mass spectrometer with tandem MS capabilities HPLC Peptide sequencing software tools \(such as Waters MassLynx or UCSF ProteinProspector) Bench-top microcentrifuge Vacuum evaporator centrifuge Oasis HLB solid phase extraction cartridge \(Waters) Oasis WAX solid phase extraction cartridge \(Waters) Zorbax C8 HPLC column \(Agilent)

Procedure

Digestion of lumican and enrichment of the sulfated peptides 1. Incubate 100 µg of recombinant mouse lumican with 20 mM iodoacetamide in 350 µl PBS in the dark for 20 min 2. Dilute the solution to 1

ml using 25 mM NH_4OAc \(pH 7.8) and add 20 µl of 100 mM $CaCl_2$. 3. Add 2 µg of trypsin \(Promega, Madison, WI) and 2 µg of Glu-C \(Roche Applied Science, Indianapolis, IN) followed by incubation overnight at RT with gentle rocking. 4. Remove the N-linked glycans by addition of 5 mU peptide:N-glycosidase F \(Prozyme, San Leandro, CA) and incubation at RT for 3 hrs. 5. Load the resulting solution onto a weak-anion exchange cartridge \(Waters, Milford, MA) pre-conditioned with 1 ml methanol and 1 ml 25 mM NH_4OAc \(pH 4.2). 6. Wash the cartridge three times, each using 1 ml 100 mM NH_4OAc in methanol and elute using 2 ml of 8 M NH_4OAc in methanol containing 5 % NH4OH. **Critical step: Since sulfated peptides are highly acidic \(a result of both the sulfate group and the acidic residues in the flanking sequences), they bind tightly to the anion-exchange resin. Thus a weak anion-exchange SPE cartridge should be used to avoid irreversible binding and the resulting sample loss**. 7. Extensively lyophilize the eluate to remove NH_4OAc. 8. Inject the lyophilized peptides onto a Zorbax C8 column and perform RP-HPLC at 1 ml/min using solvent A \(20 mM NH_4OAc, pH 6.8) and solvent B \(20 mM NH_4OAc, pH 6.8 in 80/20 acetonitrile/H_2O). **Critical step: Tyrosine sulfation is unstable under acidic conditions. Therefore, acidic ion-pairing reagent such as formic acid and trifluoroacetic acid should be avoided**. 9. Monitor the chromatograms by UV absorbance at 215 nm and manually collect the fractions. 10. Split the fractions into two Eppendorf tubes and lyophilize them. 11. Resuspend one half of the fraction in methanol. Analyze by electrospray ionization mass spectrometry. Switch between positive and negative ion mode to identify sulfated peptides. **Critical step: Although tyrosine sulfate is relatively stable during negative electrospray ionization process, loss of sulfate could still occur. It is important to tune the mass spectrometer using a model sulfated peptide to derive parameters allowing for gentle ionization**. 12. Resuspend the other half in 100 mM NH_4OAc \(pH 6.8) and add 2 U of alkaline phosphatase and incubate at RT for 2 hrs. Analyze the resulting solution by electrospray ionization mass spectrometry using negative ion mode detection. 13. Compare the results of step 11 and 12. Loss of 80 Da or multiples of 80 Da during negative/positive ion mode switch indicates a sulfated peptide while loss of 80 Da or multiples of 80 Da as a result of phosphatase treatment indicates a phosphorylated peptide. 14. Resuspend the fraction corresponding to the N-terminal peptide \(pyroGlu19-Lys57, containing a total of four sulfotyrosine residues) in 100 mM NH_4OAc. 15. Add chymotrypsin at an enzyme:substrate ratio of approximately 1:100 and incubate at RT for 2 hrs, which generates two smaller fragments, pyroGlu19-Phe28 \(tri-sulfated at positions 20, 21 and 23) and Met29-Tyr52 \(singly sulfated). 16. Reduce the disulfide bonds by adding 2 mM DTT followed by incubation at 50ºC for 30 min 17. Cool to RT, add iodoacetamide to a final concentration of 5 mM and incubate in the dark for 30 min. 18. Separate these two peptides by using HPLC and collect the fraction corresponding to Met29-Tyr52. Extensively lyophilize the fraction and determine the site of tyrosine sulfation using steps from 27-36. **Sulfation of peptides using human tyrosylprotein sulfotransferases** 19. Use the following protocol for determination the sulfotyrosine sites of peptides generated _in vitro_. 20. Add the peptide substrate, for example the peptide modeled onto the residues 12-20 of CCR8 \(VTDYYYPDI), at a final concentration of 10 µM, to a solution of 80 µM PAPS in 100 µl 20 mM MOPS, pH 7.5, 100 mM NaCl, 10% glycerol. 21. Initiate the reaction by addition of 13 µg human TPST-1 or TPST-2. 22. Incubate the mixture at RT for 1 h. 23. Inject the reaction mixture onto a Zorbax C8 column and perform RP-HPLC at 1 ml/min using solvent A \(20 mM NH_4OAc,

pH 6.8) and solvent B \(20 mM NH_4OAc, pH 6.8 in 80/20 acetonitrile/H_2O). 24. Monitor the chromatograms by UV absorbance at 215 nm and manually collect the fractions. 25. Extensively lyophilize the fractions to remove both the organic solvents and ammonium acetate. 26. Acetylate the unsulfated tyrosines and determine the sites of sulfation using the following procedure. **Derivatization of tyrosine residues** 27. Resuspend the tyrosine sulfated peptide in 100 µl of 200 mM HEPES \(pH 7.0). 28. Add 3 mM imidazole and incubate the mixture at 4 ºC \(Figure 1). 29. Quickly weigh sulfosuccinimidyl acetate \(S-NHSAc), dissolve in 5 µl DMSO and add immediately to the mixture to a final concentration of 30 mM. **Critical step: Since sulfosuccinimidyl acetate readily hydrolyzes, it is important to avoid moisture condensation during handling and storage of this chemical. Prepare fresh solution immediately before use and discard the unused materials. In addition, sulfosuccinimidyl acetate reacts with primary amines. Therefore, it is important not to use any amine-containing buffers. Ammonium salts \(e.g. ammonium acetate and ammonium bicarbonate) from the previous steps of sample workup should also be removed by extensive lyophilization**. 30. Vortex the reaction mixture and incubate at 4 ºC overnight. 31. Desalt the mixture by using Oasis HLB solid-phase extraction cartridges. 32. Elute peptides using methanol and analyze by electrospray ionization mass spectrometry. **Site-determination of tyrosine sulfation by tandem mass spectrometry** 33. Perform mass spectrometric analysis on a mass spectrometer with tandem MS capabilities, for example, LTQ linear ion trap mass spectrometer \(Thermo Electron, San Jose, CA). 34. First analyze the modified peptide using negative ion mode detection. Infuse the peptides in a solution of methanol at a flow rate of 2 µl/min. Maintain the capillary temperature and the spray voltage at 200ºC and 3.6 kV, respectively. Check for complete acetylation of the sulfated peptide. 35. Switch to positive ion mode and perform tandem mass spectrometry on the desulfated peptide ion. Select the precursor ions using an isolation width of 3 Da and fragment them by setting the normalized collision energy to 25% using helium as the collision gas. 36. Use a peptide sequencing software, for example, MassLynx \(Waters, Milford, MA), to generate the _in silico_ tandem MS spectra of the acetylated peptide. When there are several possible sites of sulfation \(as a result, several potential sequences of the acetylated peptide), generate the theoretical fragment ions for each individual sequence. Compare with the experimental MS/MS spectrum and determine the site of sulfation.

Timing

Digestion of lumican and enrichment of the sulfated peptides \(Steps 1-18): 4-5 days Sulfation of peptides using human tyrosylprotein sulfotransferases \(Steps 19-26): 1 day Derivatization of tyrosine residues \(Steps 27-32): 1 day Site-determination of tyrosine sulfation by tandem mass spectrometry \(Steps 33-36): 1 day

Anticipated Results

A dominant feature of most known tyrosine sulfation sites is the acidic residues in the flanking sequence. Therefore, sulfated peptides can be enriched from protein digests using anion-exchange chromatography.

As shown in Figure 2, a highly acidic peptide \(at 14.3 min) can be isolated from trypsin and Glu-C digests of mouse lumican. This peptide corresponds to the N-terminal peptide \(pyroGlu19-Lys57) \(Figure 2b), which contains four tyrosine sulfates, two disulfide bonds and one pyroglutamic acid. The lumican 19-57 peptide then is digested with chymotrypsin to generate two smaller peptides, pyroGlu19-Phe28 and Met29-Tyr52. The peptide pyroGlu19-Phe28 corresponds to pQY\(SO_3H)Y\(SO_3H)DY\(SO_3H)DIPLF \(Δ = 0.77 ppm). While peptide Met29-Tyr52 contains three tyrosine residues at positions 30, 47 and 52, only one of them was sulfated. This peptide is purified by HPLC and is modified by using S-NHSAc and imidazole. Positive MS/MS experiments then determine that the N-terminal amine, Tyr47 and Tyr52 are acetylated, which indicates that it corresponds to MY\(SO_3H)GQISPNCAPECNCPHSYPTAMY. Collectively, the N-terminal region of mouse lumican contains four sulfotyrosine residues at positions 20, 21, 23 and 30 \(Figure 3).

References

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Figures

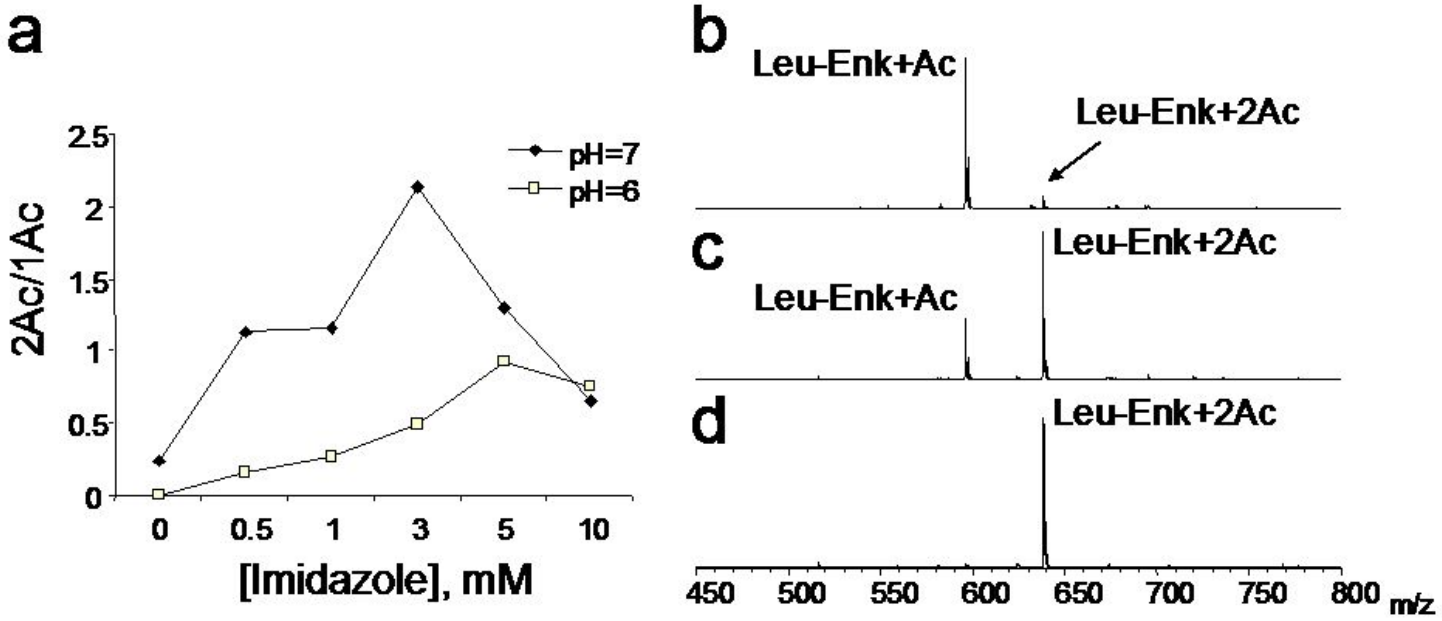

Figure 1

Effect of imidazole concentration on the reactivity of Leu-enkephalin toward S-NHSAc. (a) Leu-enkephalin (YGGFL) has two reactive sites, the N-terminal amino group which reacts preferentially with S-NHSAc, and the tyrosine hydroxyl group. The y axis represents the ratio of the di-acetylated product (both N-terminal amino and tyrosine hydroxyl acetylation) to the singly acetylated product (N-terminal amino acetylation). Reaction was performed in 200 mM phosphate buffer. (b)-(d) ESI-MS spectra of the products of the reaction between Leu-enkephalin and S-NHSAc. (b) Reaction performed in 200 mM phosphate buffer (pH 7.0) at RT using 10 mM S-NHSAc. (c) Reaction performed in 200 mM phosphate buffer (pH 7.0) at RT using 10 mM S-NHSAc in the presence of 3 mM imidazole as a catalyst. (d) reaction performed in 200 mM HEPES (pH 7.0) at 4 ºC overnight using 3 mM imidazole as a catalyst.

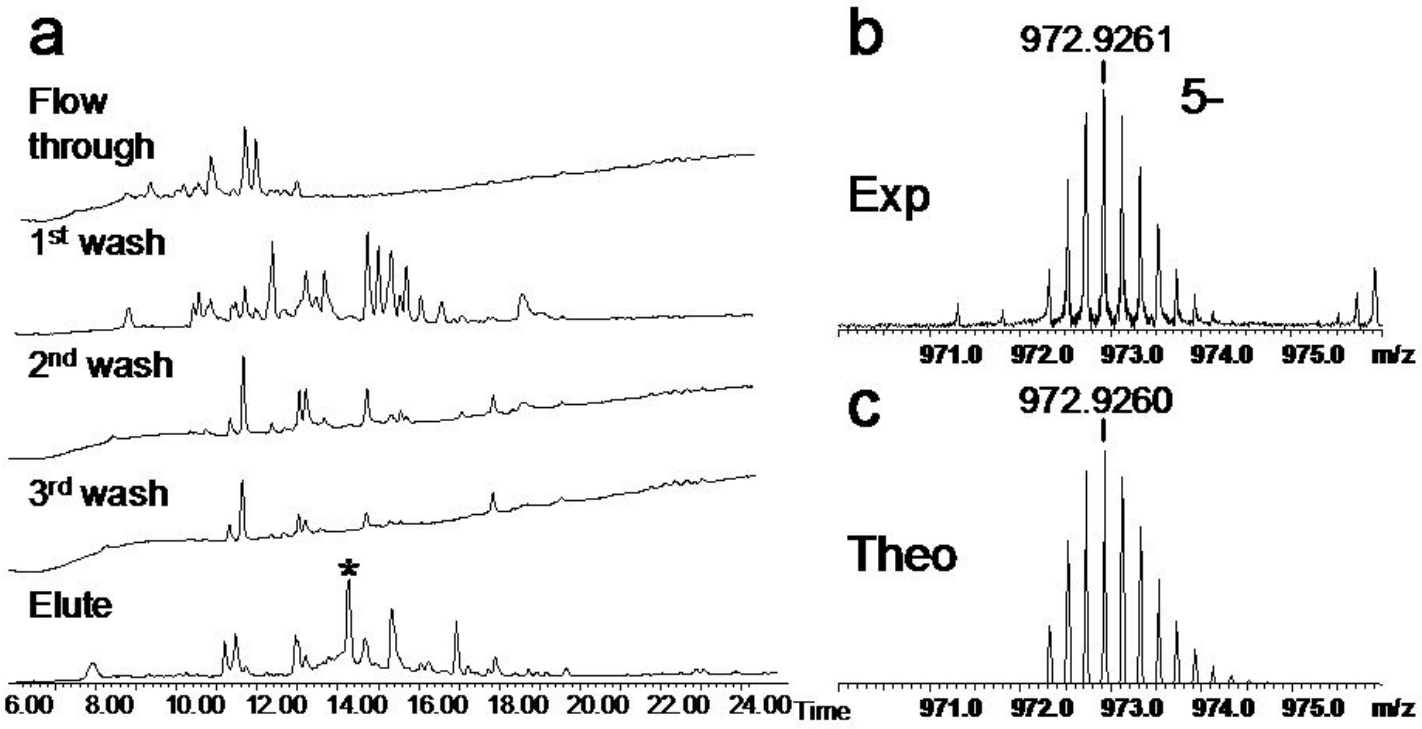

Figure 2

Purification of the tyrosine sulfated peptide from mouse lumican. (a) Purified mouse lumican was digested with a combination of trypsin and Glu-C and weak anion-exchange (WAX) chromatography was employed to enrich the sulfated, N-terminal peptide. The fraction represented by the asterisk was analyzed by negative ESI FT-ICR mass spectrometry and was identified to be pyroGlu19-Lys57, which contained four tyrosine sulfates, two disulfide bonds and one pyroglutamic acid. (b) experimental and (c) theoretical isotopic distribution of the 5 – charge state of this peptide.

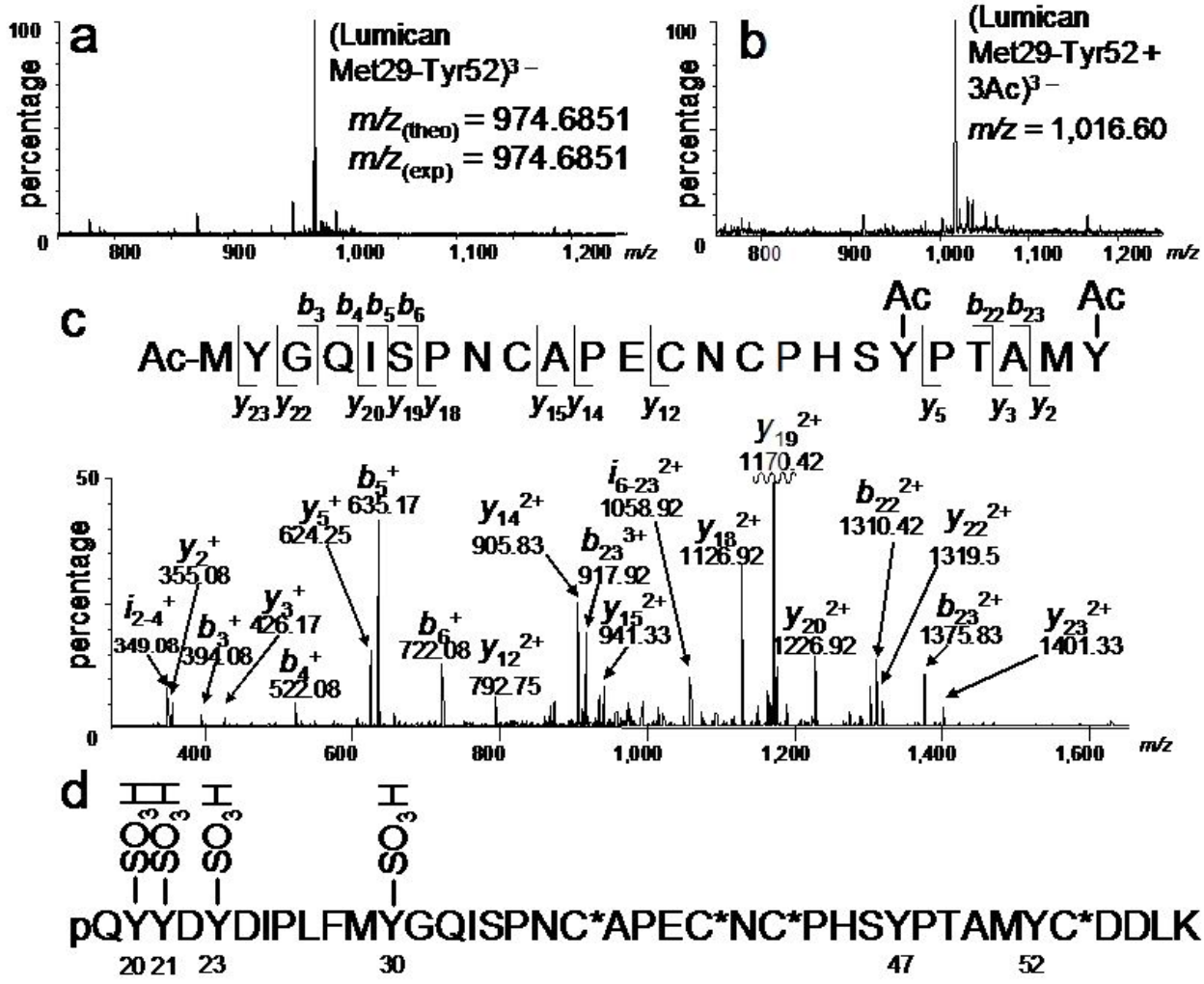

Figure 3

Identification of the sites of sulfation in lumican peptide Met29-Tyr52. (a) Met29-Tyr52 generated from chymotrypsin digest of pyroGlu19-Lys57, which was then reduced by DTT and alkylated by iodoacetamide. (b) after reaction with S-NHSAc and imidazole (c) positive MS/MS spectrum of the modified, desulfated Met29-Tyr52, in which N-terminal amine, Tyr47 and Tyr52 were determined to be acetylated. (d) Primary sequence of the lumican N-terminal peptide, pyroGlu19-Lys57. Cys labeled with asterisks form two disulfide bonds. The positions of tyrosines are indicated. Spectrum (a) was acquired on an ESI FT-ICR mass spectrometer and spectra (b), (c) were acquired on an LTQ ion trap mass spectrometer, using the conditions as indicated in the methods.

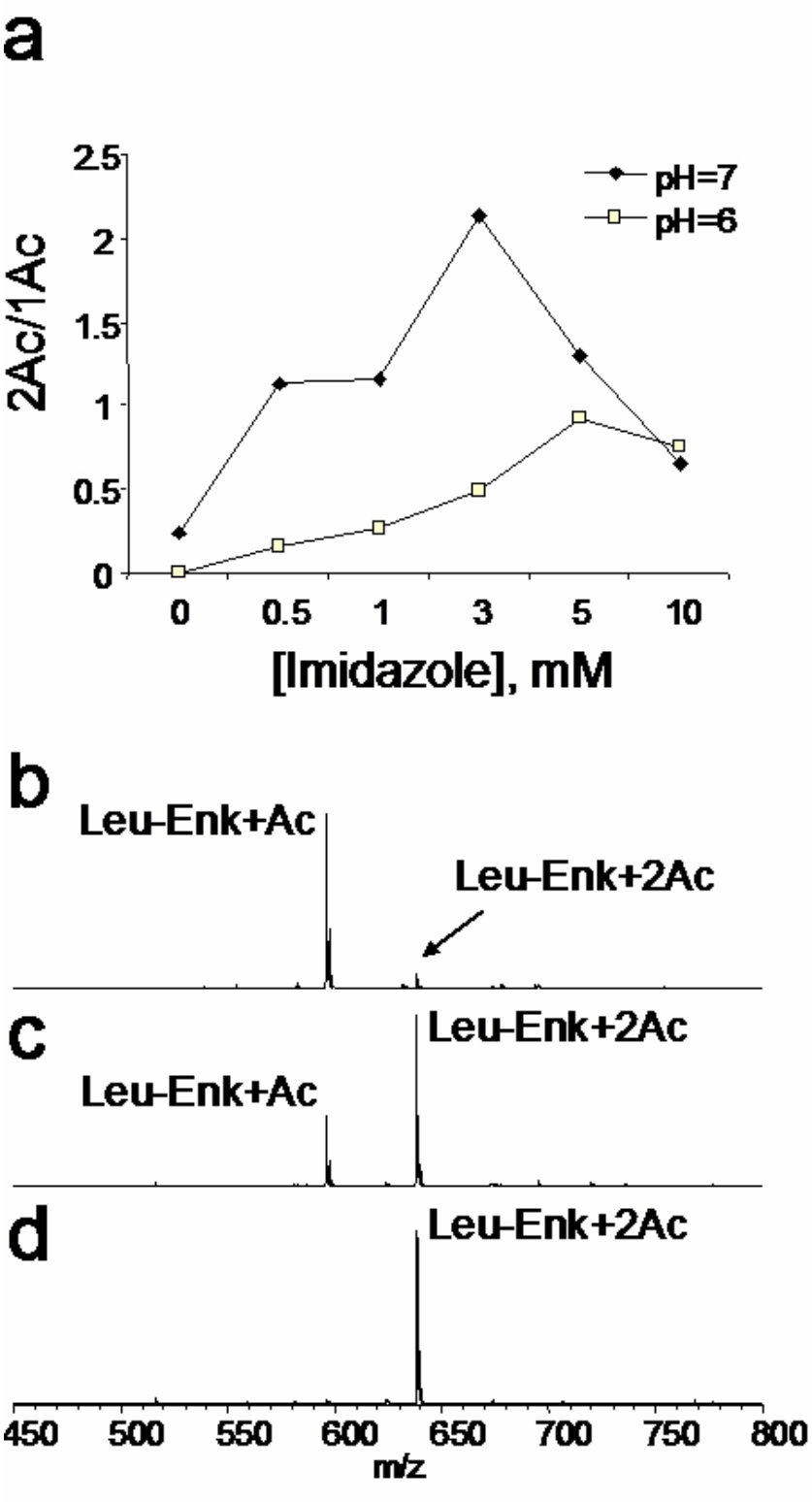
a
2.5
2
1.5
1
0.5
0
2Ac/1Ac
pH=7
pH=6
0
0.5
1
3
5
10
[Imidazole], mM
b
Leu-Enk+Ac
Leu-Enk+2Ac
c
Leu-Enk+2Ac
Leu-Enk+Ac
d
Leu-Enk+2Ac
450
500
550
600
650
700
750
800
m/z

Figure 4

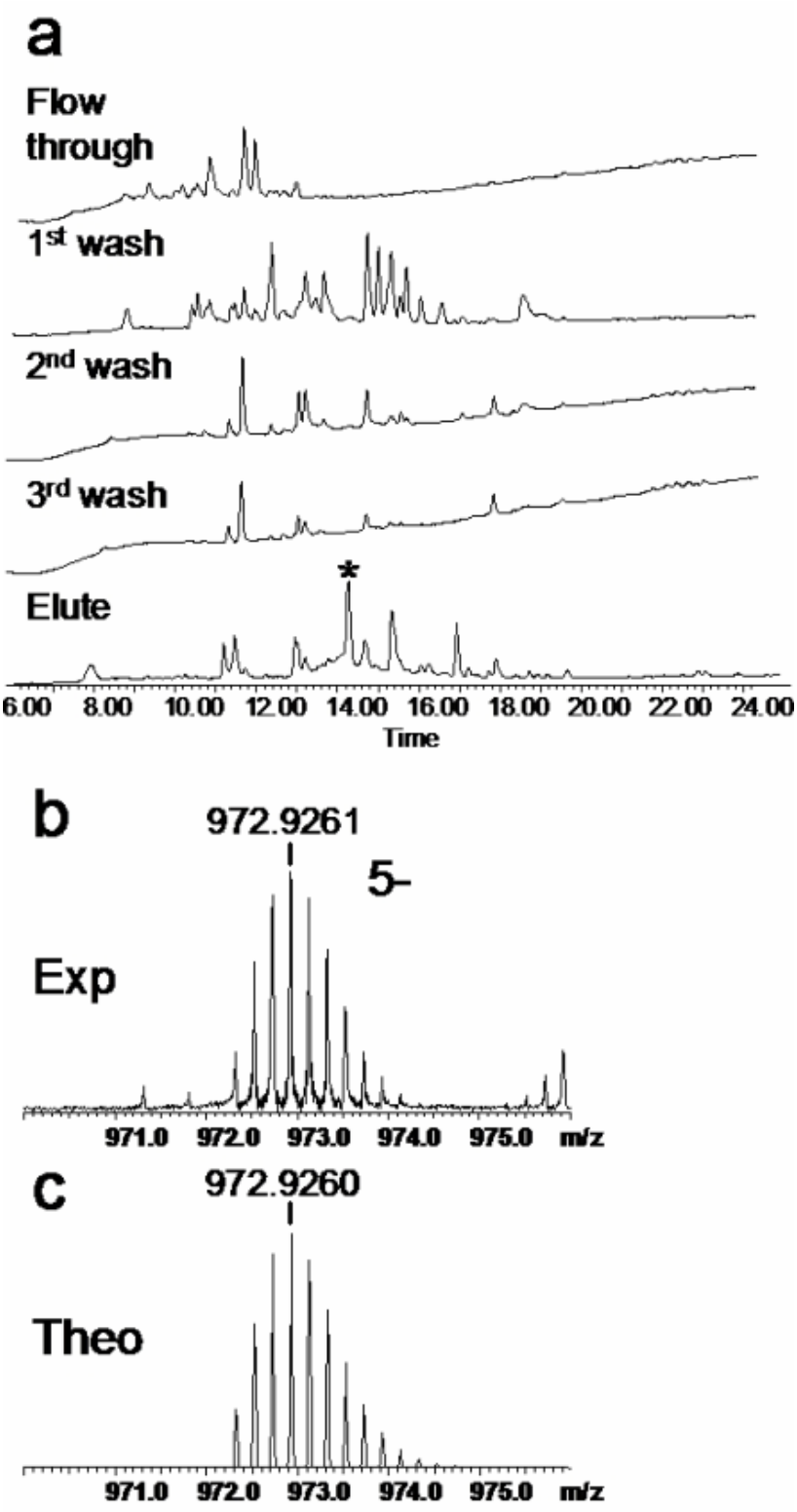
a
Flow through
1st wash
2nd wash
3rd wash
Elute
*
6.00 8.00 10.00 12.00 14.00 16.00 18.00 20.00 22.00 24.00
Time
b
972.9261
5-
Exp
971.0 972.0 973.0 974.0 975.0 m/z
c
972.9260
Theo
971.0 972.0 973.0 974.0 975.0 m/z

Figure 5

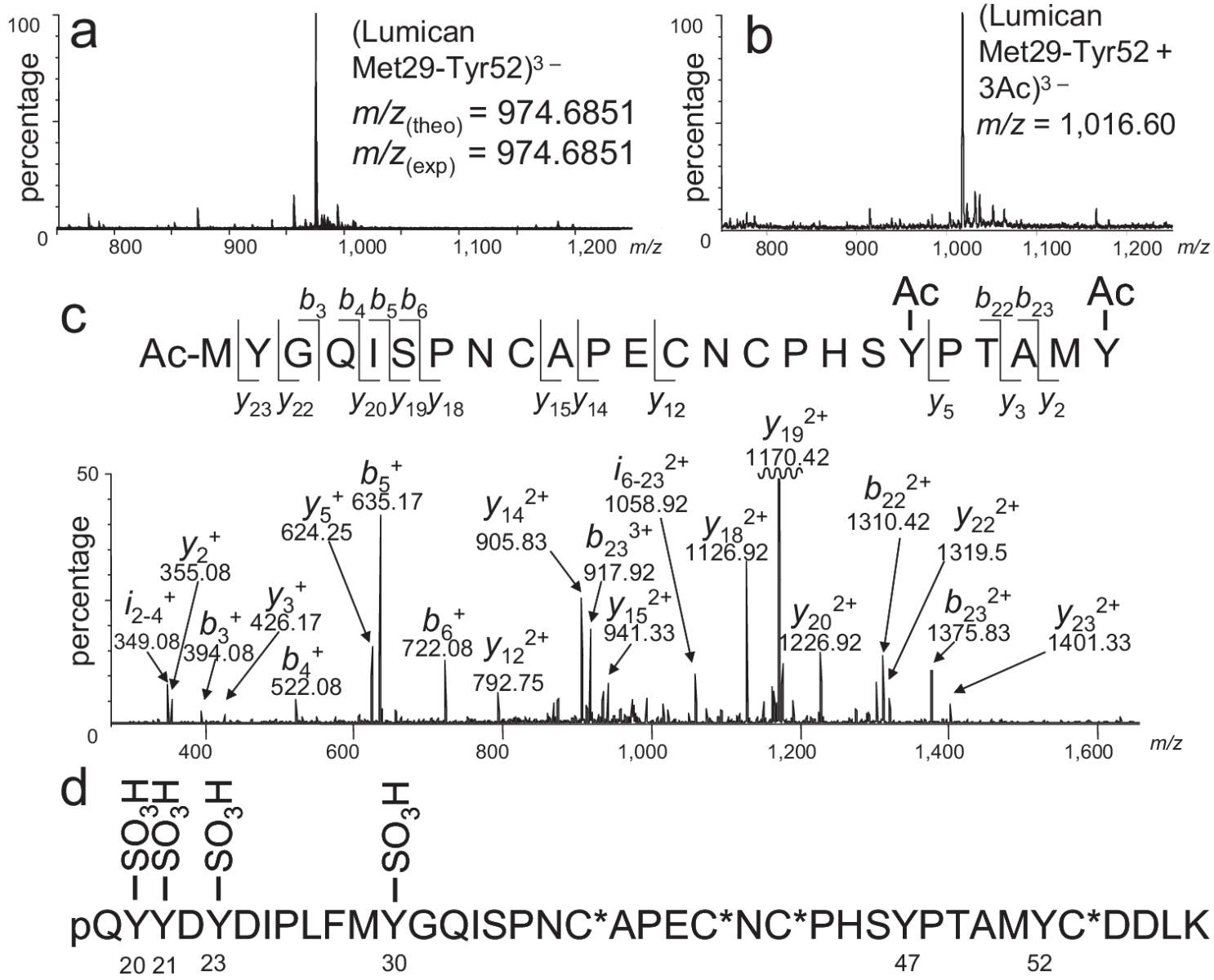
a
(Lumican Met29-Tyr52)3 −
m/z(theo) = 974.6851
m/z(exp) = 974.6851
percentage
m/z
b
(Lumican Met29-Tyr52 + 3Ac)3 −
m/z = 1,016.60
c
Ac-MYGQISPNCAPECNCPHSYPTAMY
Ac
Ac
d
pQYYDYDIPLFMYGQISPNC*APEC*NC*PHSYPTAMYC*DDLK
SO3H
20 21 23 30 47 52

Figure 6

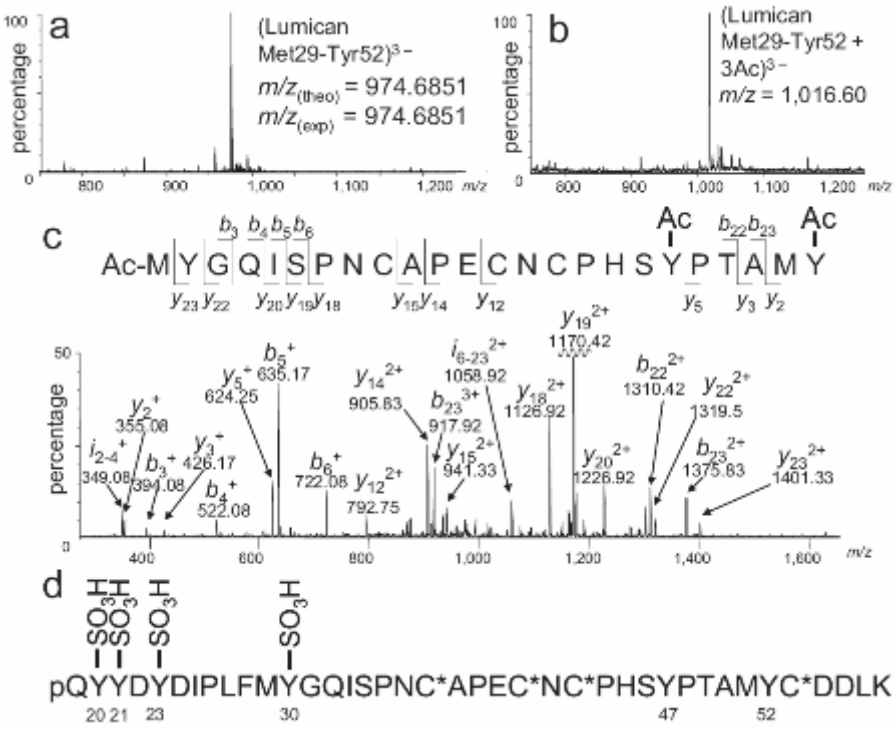
a
(Lumican Met29-Tyr52)3−
m/z(theo) = 974.6851
m/z(exp) = 974.6851
b
(Lumican Met29-Tyr52 + 3Ac)3−
m/z = 1,016.60
percentage
m/z
c
Ac-MYGQISPNCAPECNCPHSYPTAMY
Ac
Ac
d
pQYYDYDIPLFMYGQISPNC*APEC*NC*PHSYPTAMYC*DDLK
SO3H
20 21 23 30 47 52

Figure 7