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Linear ion-trap mass spectrometric characterization of human pituitary nitrotyrosine-containing proteins

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Abstract

The nitric oxide-mediated Tyr-nitration of endogenous proteins is associated with several pathological and physiological processes. In order to investigate the presence – and potential roles – of Tyr-nitration in the human pituitary, a large-format two-dimensional gel separation plus a Western blot against a specific anti-3-nitrotyrosine antibody were used to separate and detect nitroproteins from a human pituitary proteome. The nitroproteins were subjected to in-gel trypsin digestion, and high-sensitivity vacuum matrix-assisted laser desorption/ionization (vMALDI) linear ion-trap tandem mass spectrometry was used to analyze the tryptic peptides. Those MS/MS data were used to determine the amino acid sequence and the specific nitration site of each tryptic nitropeptide, and were matched to corresponding proteins with Bioworks TuboSEQUEST software. Compared to our previous study, 16 new nitrotyrosine-immunoreactive positive Western blot spots were found within the area pI 3.0–10 and M_r 10–100 kDa. Four new nitroproteins were discovered: the stanniocalcin 1 precursor—involved in calcium and phosphate metabolism; mitochondrial co-chaperone protein HscB, which might act as a co-chaperone in iron–sulfur cluster assembly in mitochrondria; progestin and adipoQ receptor family member III—a seven-transmembrane receptor; proteasome subunit alpha type 2—involved in an ATP/ubiquitin-dependent non-lysosomal proteolytic pathway. Those data demonstrate that nitric oxide-mediated Tyr-nitration might participate in various biochemical, metabolic, and pathological processes in the human pituitary.

Keywords: Human pituitary; Nitroproteomics; Two-dimensional Western blotting; Tandem mass spectrometry

1. Introduction

Reactive nitrogen species (RNS)-mediated protein nitration is an important post-translational modification that is associated with many pathologies and physiological processes [1–5]. Our studies [1,5] and others [6–11] have indicated that nitric oxide and protein nitration play important roles in the human pituitary. A two-dimensional Western blot against an anti-3-nitrotyrosine antibody is a sensitive method to detect nitroproteins in a human pituitary tissue proteome [1]. We had previously discovered four nitroproteins in a human pituitary post-mortem control tissue with a two-dimensional Western blot on a 2D gel within the area pI 4.3–6.0 and M_r 18–60 kDa. The amino acid sequence of those nitroproteins were determined with liquid chromatography–electrospray ionization–quadrapole-ion-trap mass spectrometry (LC–ESI–Q-IT-MS) [1]. Because the amount of each nitroprotein was very low, a high-sensitivity mass spectrometer was needed to characterize each nitroprotein. Although we had previously used an LC–ESI–Q-IT-MS (LCQ^{Deca}) to characterize several high-abundance 2D-separated nitroproteins, we could not characterize several

Abbreviations: AGC, automatic gain control; ASF, auto spectrum filter; BCIP/NBT, 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium; CHCA, α-cyano-4-hydroxycinnamic acid; CPS, crystal-positioning system; vMALDI, vacuum matrix-assisted laser desorption/ionization; MS, mass spectrometry; M_r, relative molecular mass (dimensionless); MS/MS, tandem mass spectrometry; *m/z*, mass-to-charge ratio; pI, isoelectric point; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis; 2DGE, two-dimensional gel electrophoresis; LTQ, linear ion-trap; S/N, signal-to-noise ratio; RNS, reactive nitrogen species; Th, Thomson (*m/z* unit)

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extremely low-abundance nitroproteins. In this present study, a high-sensitivity (ca. 1 fmol) vMALDI-LTQ mass spectrometer was used to characterize several extremely low-abundance nitroproteins. Sixteen new immune-positive nitroprotein-spots against an anti-3-nitrotyrosine antibody were detected; four new nitroproteins were discovered, and their nitration-sites were determined; a consensus sequence for nitration is suggested; and photochemical-induced fragments confirmed the presence of a nitrotyrosine.

2. Materials and methods

2.1. Separation of human pituitary nitroproteins

A normal (control) human pituitary post-mortem tissue (male, 45-years old, drowning) was obtained from the Memphis Regional Medical Center. The extracted protein mixture was separated with two-dimensional gel electrophoresis (2DGE) with an 18 cm IPGstrip (pH 3–10 NL) and 12% sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The 2DGE-separated proteins were transferred onto a PVDF membrane, and a Western blot reaction was performed against a rabbit anti-human nitrotryrosine antibody. The nitroproteins on that Western blot membrane were visualized with 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT). The proteins on the 2DGE gel were visualized with silver staining. The visualized Western blot membrane and the 2DGE gel were both digitized and analyzed with PDQuest software (Bio-Rad, version 7.1, Hercules, CA). Each positive Western blot spot was matched to its corresponding 2DGE gel-spot. The detailed experimental procedures have been described [1]. The 2DGE-separated nitroproteins, which corresponded to the positive Western blot spots, were subjected to trypsin treatment and MS/MS analysis.

2.2. Trypsin-digestion of 2DGE-separated nitroproteins

The silver-stained 2D gel-spots that corresponded to the positive Western blot spots were excised, and the proteins were subjected to in-gel trypsin digestion [12]. The tryptic peptide mixture was purified with a ZipTipC18 micro-column according to the manufacturer's instructions. The purified tryptic peptides were eluted directly from the microcolumn onto a vMALDI 96-well plate with 2 μl of a α-cyano-4-hydroxycinnamic acid (CHCA) solution (2.5 mg/ml in 50% (v/v) acetonitrile/0.1% (v/v) TFA) (seven cycles); the matrix was dried in ambient air.

2.3. vMALDI-LTQ linear ion-trap tandem mass spectrometry

The tryptic peptide mixture was analyzed with a vacuum matrix-assisted laser desorption/ionization-linear ion-trap mass spectrometer (vMALDI-LTQ) (ThermoFinnigan, San Jose, CA, USA) in the "Nth-order double-play" data-dependent experiment mode to obtain the amino acid sequence of each peptide. The instrument parameters were: (1) for an MS scan, wide range (m/z 600–4000), normal scan rate, full scan, polarity, profile data type, and five microscans of each experiment were used; (2) for an MS^2 scan, the 50 most-intense peaks in the full-MS spectrum (m/z 50–4000), normal scan rate, polarity, profile data type, isolation width 3.0 Th, normalized collision energy 40%, default charge-state 1, minimal signal threshold 100 counts, activation Q-value 0.25, activation time 30 ms, and 5 microscans of each experiment were used; (3) for the vMALDI source, the crystal-positioning system (CPS) and auto spectrum filter (ASF) were enabled. The ASF threshold was 500 counts for an MS scan, and 250 counts for an MS^2 scan; (4) automatic gain control (AGC) was enabled to allow the vMALDI software to automatically adjust the number of laser shots to maintain the quality of the spectra. The vMALDI-LTQ MS/MS data analysis was performed with Xcalibur software. A number ($n = 20$–200) of MS^2 spectra of each precursor ion were acquired to improve significantly the signal-to-noise ratio (S/N) and the quality of each MS^2 spectrum. The nitration site of each nitroprotein was determined from an MS^2 spectrum.

2.4. Database analysis of MS/MS data

An MS/MS spectrum was used to identify each nitroprotein by searching the SWISS-PROT and NCBInr databases with Bioworks 3.2 software. Mass modifications of +45 Da ($+NO_2$–H) at Tyr and of +57 Da ($+NH_2COCH_2$–H) at Cys were considered in the search. Each positive search result (K or R at the C-terminus; K or R preceding the N-terminus; 0 or 1 missed trypsin cleavage sites; singly charged *b*-, *y*-, and *a*- product ions, *homo sapiens* database, and high-quality MS and MS^2 spectra) was confirmed with a manual interpretation of the MS and MS^2 data. The function of each nitroprotein was obtained from the literature data that are indexed on the SWISS-PROT annotation page.

3. Results and discussion

3.1. Nitroproteins detected with a 2D Western blot

Fig. 1A shows a representative silver-stained 2D gel image (pI 3–10; M_r 10–100 kDa) that contained ca. 1000 protein spots before the transfer of proteins onto a PVDF membrane. Fig. 1B shows the corresponding silver-stained 2D gel image after the transfer of proteins onto a PVDF membrane to demonstrate that most (ca. 99%) of the proteins were transferred onto the PVDF membrane. Fig. 1C shows the Western blot image against an anti-3-nitrotyrosine antibody; each positive-stained Western blot spot represents a nitroprotein that was transferred onto the PVDF membrane. Fig. 1D shows a parallel negative-control experiment in order to determine any cross-reactivity of the secondary antibody with a protein. Each 2D gel in Fig. 1 encompasses the area pI 3–10 and M_r 10–100 kDa. A total of 32 positive Western blot spots that were labeled in Fig. 1C were determined on the 2D image with PDQuest software by comparing the digitized Western blot image (Fig. 1C) to the negative-control (Fig. 1D). Spots 1–7 in Fig. 1C demonstrated a cross-reactivity of the secondary antibody with a protein in Fig. 1D. However, a semi-quantitative analysis showed that the intensity of spots 1–7 in Fig. 1C was

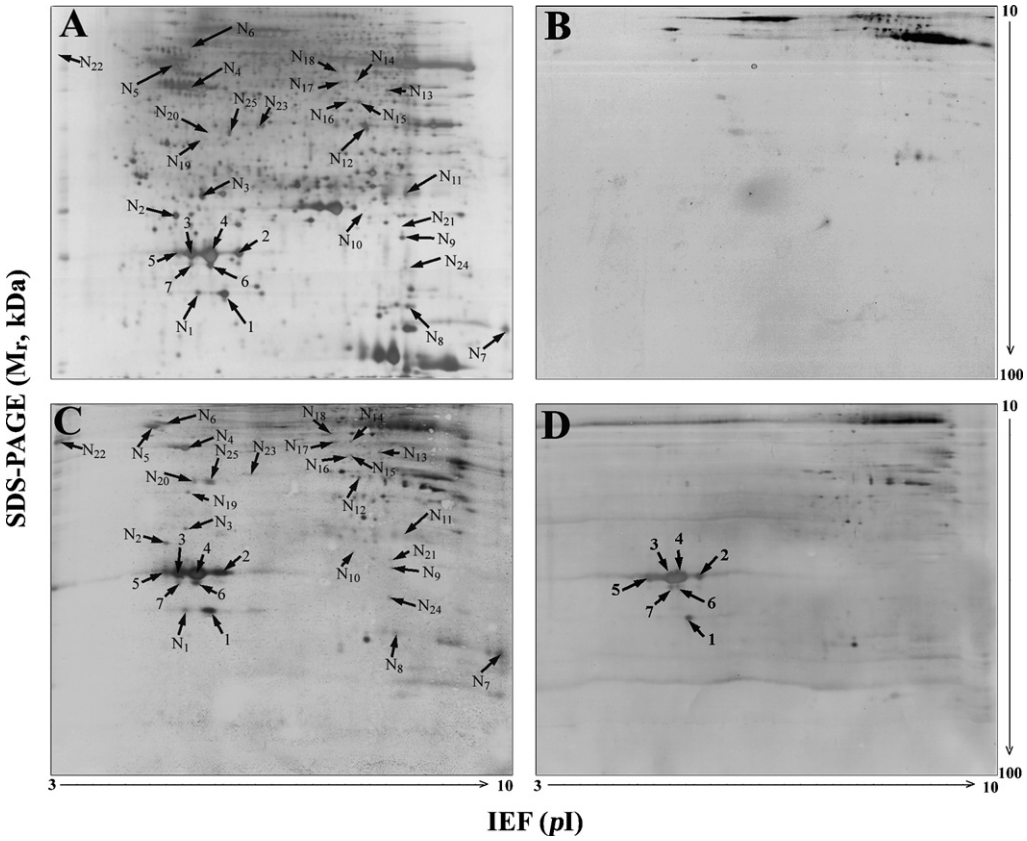

Fig. 1. Two-dimensional Western blot analysis of anti-3-nitrotyrosine-positive proteins in a human pituitary (70 μg protein per 2D gel). (A) Silver-stained image on a 2D gel before the transfer of proteins onto a PVDF membrane. (B) Silver-stained image on a 2D gel after the transfer of proteins onto a PVDF membrane. (C) Western blot image of anti-3-nitrotyrosine-positive proteins (anti-3-nitrotyrosine antibodies + secondary antibody). (D) Negative control of a Western blot to show the cross-reaction of the secondary antibody (only the secondary antibody; no anti-3-nitrotyrosine antibody).

much higher than that intensity of the corresponding negative-control spot. Those data suggested that nitroproteins were also contained in those seven spots in Fig. 1C, and by extension also in the other spots in Fig. 1A and C. The positive Western blot spots in Figure 1C were matched to the corresponding silver-stained 2D gel-spots in Fig. 1A.

Even though the abundance of a nitroprotein in a human tissue proteome is very low, 2DGE separates and enriches each nitroprotein to improve its immunodection and MS-characterization.

3.2. vMALDI-MS/MS characterization of nitroproteins

The mass spectrometric characterization of low-abundance nitroproteins is limited by various confounding factors between in-gel trypsin digestion and database analysis: (a) because a visualized 2D gel-spot could include several proteins, non-nitrated proteins and nitroproteins could also co-exist in a positive Western blot spot. Three potential factors could result in multiple proteins in a 2D gel-spot: poor electrophoretic focusing (some proteins cannot be separated effectively in the pI direction), poor SDS-PAGE (some proteins cannot be separated effectively in the M_r direction), and proteins with an extremely similar pI and M_r (those proteins cannot be separated effectively on a 2D gel); (b) no uniform database analysis method and evaluation criteria are available for MS/MS data-derived database search results; (c) some unavoidable contaminants often interfere with the identification of low-abundance nitroproteins. Those contaminants usually include trypsin-autodigestion fragments, keratins from skin and hair, matrix (e.g., CHCA), and other unknown contaminants. The intensity of trypsin autodigestion fragments and of the peptides from contaminated keratins is much higher than the intensity of tryptic nitropeptides and, therefore, interfere seriously with the detection of tryptic nitropeptides in a mass spectrum.

Commonly occurring "background" ions (Fig. 2) from a blank region of a human pituitary 2D gel occur in an MS spectrum at *m/z* 665.2, 780.3, 843.2, 879.4, 992.2, 1121.1, 1180.2, 1422.0, 1476.2, 1549.2, 1697.0, 1810.1, 1924.2, 1942.0, 1995.2, 2065.82, 2084.1, 2212.1, 2222.2, 2240.1, 2300.2, 2384.9, 2421.2, 2686.2, 2915.3, 3097.2, 3113.3, 3332.2, and 3348.2. Some of those interfering ions often also appeared in the MS spectrum of an analyzed sample. If any one of those "background" ions is selected as a precursor ion for MS/MS analysis, then careful attention must be taken to the *b*-, *y*-ion series to determine whether they derive from a "real" peptide from the analyzed sample.

To improve the quality of an MS^2 spectrum, an accumulation of up to hundreds of MS^2 spectra for a precursor ion improved significantly the S/N of an MS/MS spectrum, and commensurately increased the confidence in the database analysis result.

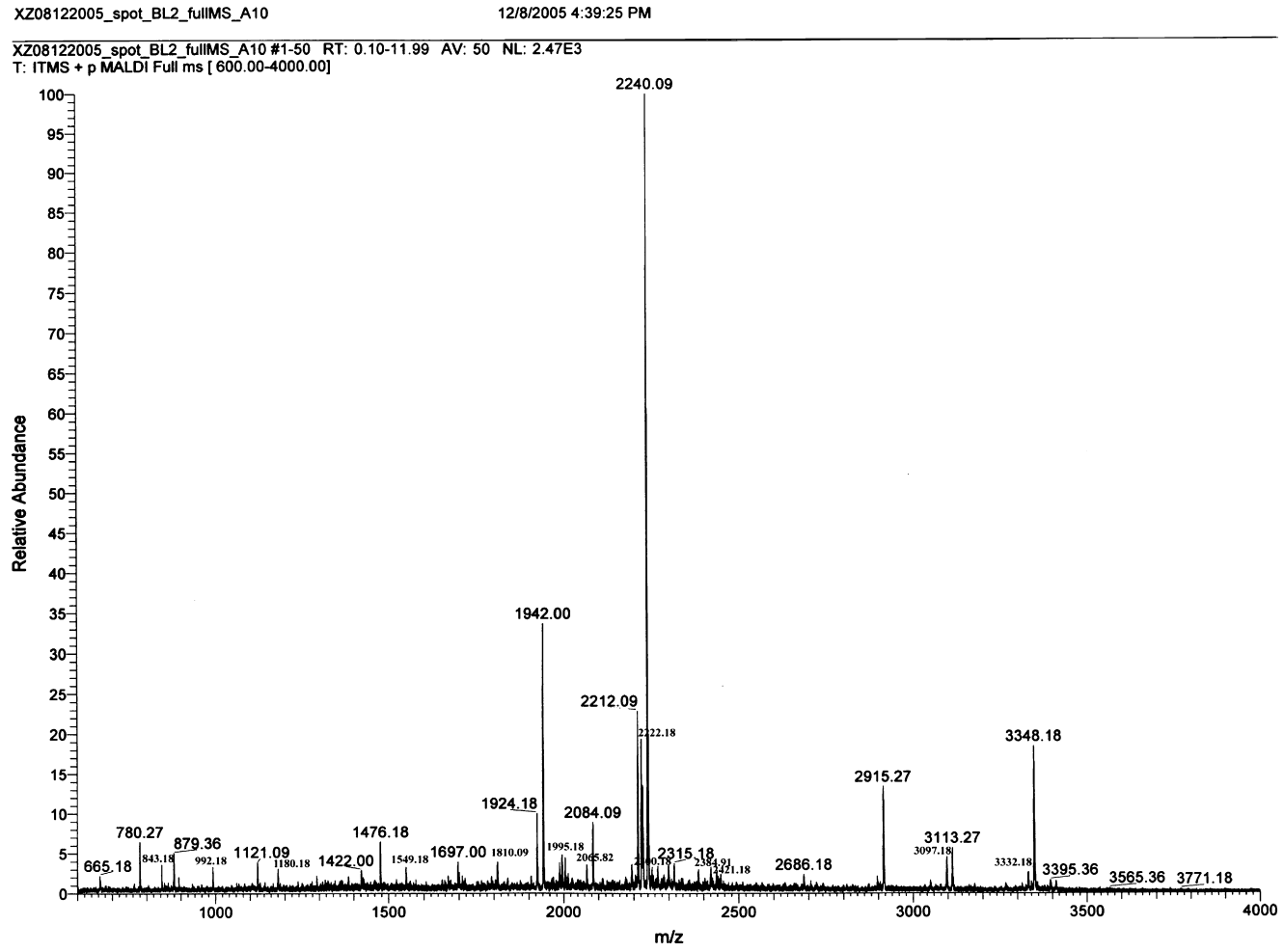

Fig. 2. A representative vMALDI-LTQ MS "background" spectrum from a human pituitary 2D gel (spectrum taken from a "blank" region near the margin of the gel).

MS/MS data and a database search were used to analyze each positive Western blot sample. Most of those analyzed samples demonstrated very weak spots in a 2D gel (Fig. 1A and C). A total of four nitropeptides, which were matched to four different proteins, respectively, were characterized (Table 1). Table 1 contains the nitroprotein name, SWISS-PROT accession number, nitropeptide amino acid sequence, Tyr-nitration site in the nitroprotein, spot number, and X_{corr} score.

A representative vMALDI-LTQ MS/MS spectrum of a nitropeptide that contained 24 amino acids is shown in Fig. 3. The $[M+H]^+$ ion at $m/z = 2730.18$ of a tryptic peptide in the MS spectrum (Fig. 3A) was selected as the precursor ion, and Fig. 3B contains the MS/MS spectra of that $[M+H]^+$ precursor ion. The product ions labeled in Fig. 3B include the singly charged *b*-, *y*-, and *a*-ions (see Table 2). The corresponding amino acid sequence, HSTLVNDAY*KTLLAPLSRGLYLLK (Y* = nitrotyrosine), of that peptide is shown in Table 2. That nitropeptide matched the amino acid sequence 120–143 of human mitochondrial co-chaperone protein HscB (SWISS-PROT number = Q8IWL3) (Table 1). The Tyr-nitration was assigned to Tyr-128 in the nitroprotein (position 9 in the peptide—Table 2). Moreover, the loss of NH_3 from the singly

Table 1
Human pituitary nitroproteins characterized with vMALDI-LTQ tandem mass spectrometry

Protein name (SWISS-PROT number)	Nitropeptide	Spot number	Tyr nitration site	X_{corr}
Stanniocalcin 1 precursor (P52823)	K.RNPEAITEVVQLPNHFSNRY*YNR.L	N5	159	2.51
Co-chaperone protein HscB, mitochondrial precursor (Q8IWL3)	K.HSTLVNDAY*KTLLAPLSRGLYLLK.L	N4	128	2.53
Progestin and adipoQ receptor family member III (Q6TCH7)	R.LYTY*EQIPGSLKDNPYITDGYRAYLPSR.L	N10, N14	33	2.85
Proteasome subunit alpha type 2 (P25787)	K.DY*LAAIA.-	N3, N6, N10, N19, N22	228	1.65

Y*, nitrotryrosine. The period in each nitropeptide amino acid sequence refers to the trypsin cleavage site.

charged b_{11}, b_{12}, b_{13}, b_{17}, y_{20} ions, the loss of H_2O from the singly charged b_{14}, b_{18}, b_{21}, b_{22}, y_{23} ions, and the addition of a Na to the singly charged b_{19}, b_{21}, b_{22}-NH_3 ions were also detected (Table 2). Each MS/MS spectrum was interpreted manually to confirm the amino acid sequence.

3.3. Expanded data in this present study

Our overall goal is to develop a high-throughput, specific, and sensitive analytical method to discover nitroproteins in the human pituitary in order to provide a database of pituitary tumor-related nitroproteins, to characterize the human pituitary on a molecular level, and to elucidate molecular mechanisms that contribute to the formation of a pituitary adenoma [13]. A two-dimensional Western blot based on a specific anti-3-nitrotyrosine antibody is an effective method to array and detect nitroproteins in a human pituitary tissue proteome [1]. Compared to our previous study [1], (1) in this current study we expanded the area of study of a 2D Western blot from pI 4.3–6.0 and M_r 18–60 kDa to pI 3–10 and M_r 10–100 kDa. More anti-nitrotyrosine positive Western blot spots (n = 16; spots N7–N18, N21–N24) were found (Fig. 3C). The positive Western blot spots 1–7, N1–N6, N19, N20, and N25 were the same as the spots in our previous study; (2) a vMALDI-LTQ MS/MS instrument and Bioworks software were used here instead of the previous LCQ^{Deca} LC–ESI–Q-IT-MS/MS instrument and SEQUEST software. The sensitivity of a vMALDI-LTQ instrument is significantly higher than that of an LCQ^{Deca}; for example, some extremely low-abundance proteins (such as spot N10) were characterized in this present study. Compared to an LCQ^{Deca}, another advantage of a vMALDI-LTQ is that the sample on a MALDI plate can be kept up to several weeks for reanalysis; that possibility is always useful for a precious human pituitary sample [14,15]. Also, an accumulated MS/MS spectrum was obtained to improve significantly the quality of each MS/MS spectrum and to increase the confidence in each result; (3) the four new nitroproteins discovered here in a human pituitary post-mortem tissue extended our database of the human pituitary nitroproteome. Those four new nitroproteins were the stanniocalcin 1 precursor that is involved in the metabolism of calcium and phosphate [16],

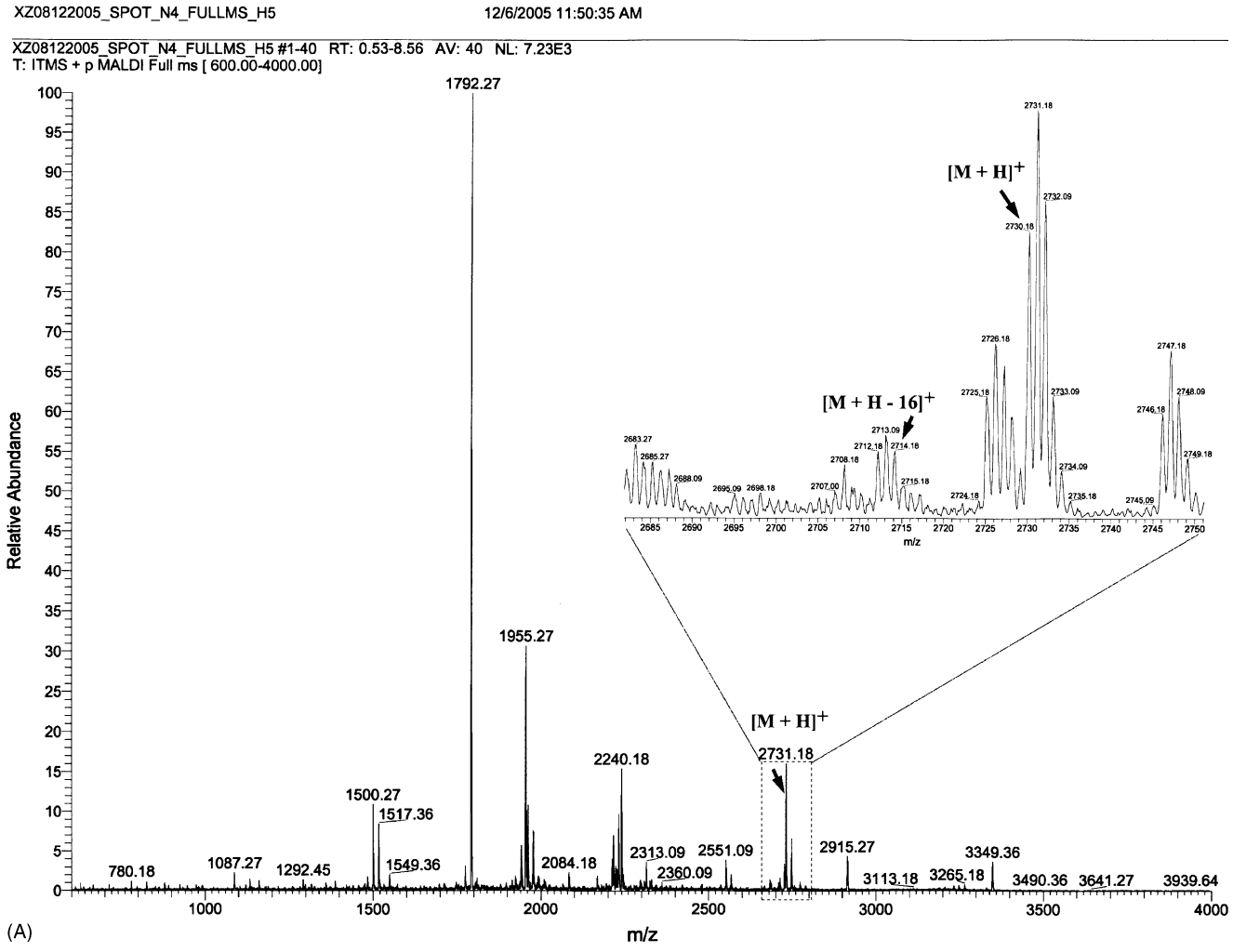

Fig. 3. MS spectrum and precursor ion (A; m/z = 2731.2) and MS^2 spectrum (B) of the nitropeptide, ^{120}HSTLVNDAY*KTLLAPLSRGLYLLK^{143} (nitration site = Y^{128} in the protein; Y^9 in the peptide), from human mitochondrial co-chaperone protein HscB (SWISS-PROT number = Q8IWL3). *The loss of NH_3; #the loss of H_2O; M − 14 = M + 2H − O; M − 16 = M − O; M − 32 = M − 2O; M − 45 = M + H − NO_2.

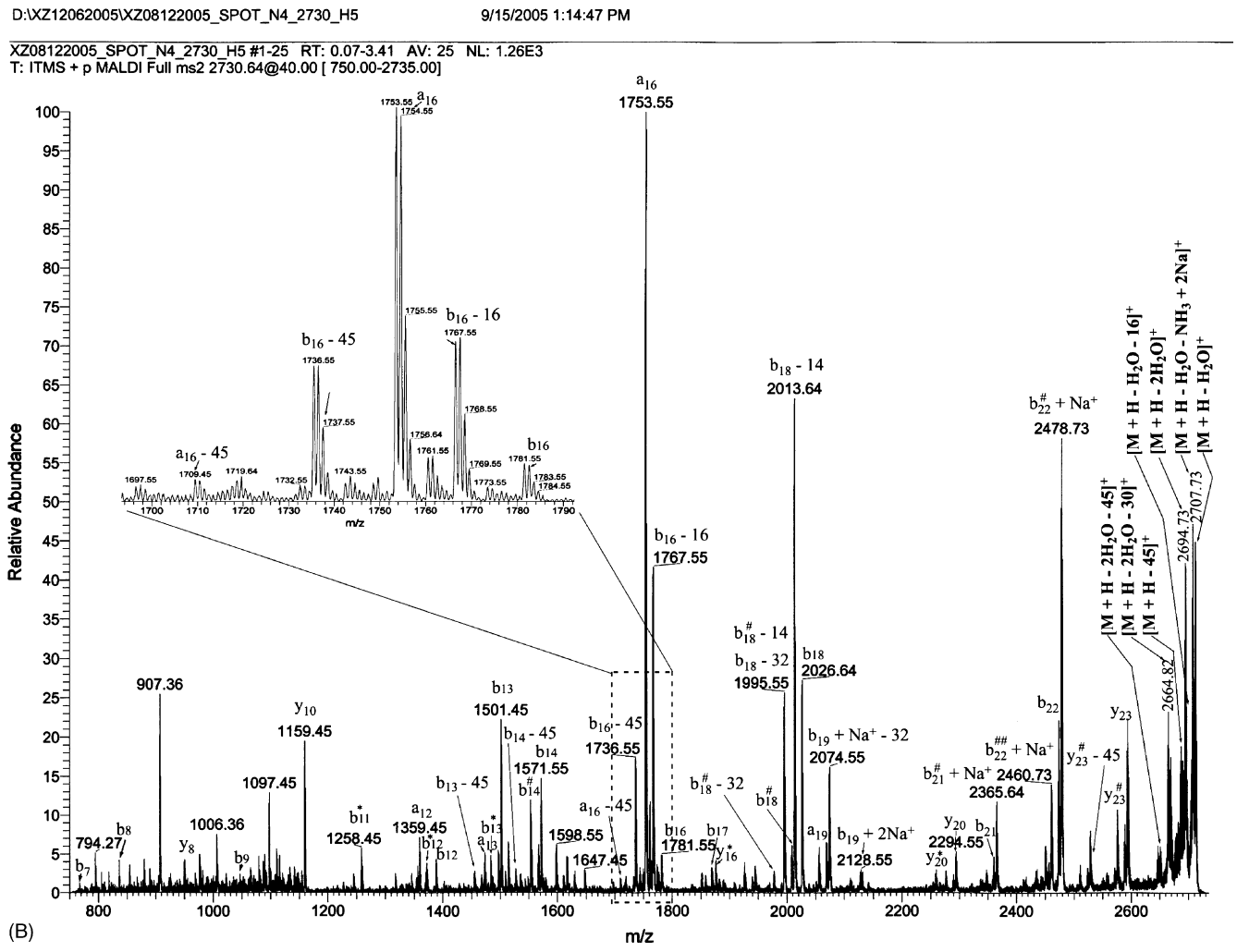

Fig. 3. (*Continued*).

the mitochondrial co-chaperone protein HscB that might act as a co-chaperone in iron–sulfur cluster assembly in mitochrondria [17], the progestin and adipoQ receptor family member III that is a seven-transmembrane receptor [18], and the proteasome subunit alpha type 2 that is a multicatalytic proteinase complex that is involved in an ATP/ubiquitin-dependent nonlysosomal proteolytic pathway [19,20]. The presence of those newly discovered nitroproteins in the human pituitary demonstrated that nitric oxide-mediated Tyr-nitration might participate in various biochemical, metabolic, and pathological processes in the pituitary; (4) the nitration of mitochondrial co-chaperone protein HscB that participates in the iron–sulfur cluster assembly in mitochondria is an interesting phenomena in the human pituitary because the iron–sulfur (Fe–S) cluster assembly that is involved in electron-transfer processes is an important post-translational modification in the mitochondrial complex I that is responsible for superoxide anion generation that is frequently linked to disease pathophysiology [21,22]. That result indicates that the reactive nitrogen species (RNS) system is associated with the reactive oxygen species (ROS) system, and that nitration and denitration [23,24] might play roles in superoxide anion generation in the human pituitary.

3.4. Sensitivity, MS/MS analysis of endogenous nitropeptides, and diagnostically important photochemical-induced decomposition products of nitrotyrosine

Four endogenous nitroproteins and their sites of nitration were characterized from eight 2D gel spots that had a positive immunoreactivity against an anti-3-nitrotyrosine antibody (Table 1). However, the nitroproteins (and their sites of nitration) in the other 24 ($32-8=24$) immunopositive 2D gel spots were not identified—perhaps due to several factors: (1) extreme low-abundance. The vMALDI-LTQ instrument has a 1 fmol sensitivity under our experimental conditions. The amount of most of the endogenous nitroproteins in the 2D gel spots was very low—probably lower than 1 fmol. (2) MALDI-mediated photochemical fragmentation of nitrated tyrosine. Photochemical-induced decomposition of nitrotyrosine under MALDI conditions has been demonstrated in *in vitro* studies [25–28]. The photochemical-induced decomposition of a Tyr-nitrated peptide includes the loss of one or two oxygen and hydrogen atoms from the nitro ($-NO_2$) group to form $[M+H-14]^+$, $[M+H-16]^+$, $[M+H-30]^+$, and

Table 2
Singly charged MS^2 product ions of the precursor ion ($[M+H]^+$, *m/z* 2730) of a nitropeptide, corresponding to Fig. 3B

Number	AA	*a*-Ion	*b*-Ion	*y*-Ion	Number	$-H_2O(^{\#})$	$-NH_3(^{*})$	+Na	−14	−16	−30	−32	−45
1	H	110.1	138.1		24								
2	S	197.1	225.1	2594.5	23	y_{23}							$y^{\#}_{23}$
3	T	298.2	326.2	2507.4	22								
4	L	411.2	439.2	2406.4	21								
5	V	510.3	538.3	2293.3	20		y_{20}			y_{20}, y^{*}_{20}		y_{20}	
6	N	624.4	652.3	2194.2	19								
7	D	739.4	767.4	2080.2	18								
8	A	810.4	838.4	1965.2	17								
9	Y*	1018.5	1046.5	1894.1	16		y_{16}						
10	K	1146.6	1174.6	1686.1	15								
11	T	1247.6	1275.6	1557.9	14		b_{11}		b^{*}_{11}		b^{*}_{11}	b^{*}_{11}	
12	L	1360.7	1388.7	1456.9	13		b_{12}		a_{12}	b_{12}		b_{12}	
13	L	1473.8	1501.8	1343.8	12		b_{13}			b_{13}, b^{*}_{13}	b^{*}_{13}		b_{13}
14	A	1544.8	1572.8	1230.8	11	b_{14}							b_{14}, $b^{\#}_{14}$
15	P	1641.9	1669.9	1159.7	10								
16	L	1754.9	1782.9	1062.6	9					b_{16}			b_{16}, a_{16}
17	S	1841.9	1869.9	949.6	8		b_{17}						
18	R	1998.1	2026.1	862.6	7	b_{18}			b_{18}, $b^{\#}_{18}$		b_{18}, $b^{\#}_{18}$	b_{18}, $b^{\#}_{18}$	
19	G	2055.1	2083.1	706.5	6			b_{19} + Na	b_{19}	a_{19}		b_{19} + Na, b_{19}	
20	L	2168.2	2196.2	649.4	5								
21	Y	2331.3	2359.3	536.3	4			$b^{\#}_{21}$		$b^{\#}_{21}$ + Na			
22	L	2444.3	2472.3	373.3	3	$b^{\#}_{22}$ + Na		$b^{\#}_{22}$	$b^{\#}_{22}$ + Na	$b^{\#}_{22}$ + Na			
23	L	2557.4	2585.4	260.2	2								
24	K			147.1	1								

Y*, nitrotyrosine; #loss of H_2O; *loss of NH_3; −14, +2H − O; −16, −O; −30, +2H − 2O; −32, −2O; −45, +H − NO_2. The underlined ion means that it was found in the MS/MS spectrum.

$[M+H-32]^+$ ions. In addition, a low-mass immonium ion was formed. That triad (immonium ion; loss of one oxygen atom; loss of two oxygen atoms) provides a unique signature of a nitrotyrosine peptide, and provides unequivocal evidence for the presence of a nitrotyrosine-containing protein [26]. Our MS data also contained those diagnostic ions; for example, in Fig. 3A, the peak at *m/z* 2714.18 (2730.18 − 16 = 2714.18), plus many others (see Table 2). Photochemical-induced decomposition could reduce the MS signal of nitrotyrosine-containing ions and might hamper a protein identification. We also found

Table 3
Consensus sequence for nitration of a tyrosine residue in a protein

−1 Position	Y*	+1 Position
K.GQC@KDALEI	Y*	K.R
D.	Y*	TTQNLIR.M
K.DL	Y*	ANNVLSGGTTMYPGIADR.M
K.GE	Y*	FGEKALI.S
K.RNPEAITEVVQLPNHFSNR	Y*	YNR.L
K.HSTLVNDA	Y*	KTLLAPLSRGLYLLK.L
R.LYT	Y*	EQIPGSLKDNPYITDGYRAYLPSR.L
K.D	Y*	LAAIA-
K.HIGFV	Y*	HPTKETCLSGQNCTDIK.V
R.SSNP	Y*	LLSHPSEPLELVVSGATETLNPAQKK.S
K.DLYRDVMLEI	Y*	SNLLSMGYQVSKPDALSK.L
K.GC@EL	Y*	VQLHGIQQVLKDC@IVHLCISK.P
K.YGY*APKGSSLVL	Y*	SDKK.Y
K.LM#CELGNVIINQI	Y*	EARVEAM#AVKK.P
K.D	Y*	LAAIA-
K.DIMDL	Y*	NQPEPVKSFLFYHSQSGR.N
R.AAGVLN	Y*	LK.D

Amino acid sequences of the 17 endogenous nitrotyrosine peptides discovered in our human pituitary studies [[1,5], present]. Those nitrotyrosine-containing peptides are tryptic peptides. However, the amino acid residue that precedes the N-terminus of Y*TTQNLIR is the acid-labile D residue; the purified tryptic peptides were kept in an acidic solution [1]. The amino acid residue at the C-terminus of GEY*FGEKALI is the residue I; that peptide might have been produced because no protease inhibitors were used to extract proteins; for example, some endogenous proteinases might cleave I at its C-terminus; that nitropeptide was confirmed by *de novo* analysis [1]. Those nitropeptides are collected from our three studies [[1,5], present study]. Y*, nitrotyrosine; M#, oxidized Met; C@, Cys-CAM. The "period" refers to the cleavage site at N-terminus and C-terminus of the nitrated peptide. – refers to the C-terminus of a nitrated protein. These amino acid sequences are aligned to the nitrotyrosine residue.

a characteristic decomposition cluster pattern for *b*-/*y*-ions in the MS^2 spectrum (Fig. 3B; Table 2) for those product ions that contained a nitro group: $y_{20}-16$, $y^*_{20}-16$, $y_{20}-32$; $b^*_{11}-14$, $b^*_{11}-30$, $b^*_{11}-32$; $b_{12}-16$, $b_{12}-32$; $a_{12}-14$; $b_{13}-16$, $b^*_{13}-16$, $b^*_{13}-30$; $b_{16}-16$ (Fig. 3B, top-left); $b_{18}-14$, $b_{18}-30$, $b_{18}-32$; $b^\#_{18}-14$, $b^\#_{18}-30$, $b^\#_{18}-32$; $b_{19}-14$, $b_{19}-32$, $a_{19}-16$, etc. (*, loss of ammonia; $^\#$, loss of water). Furthermore, for a precursor ion that contained a nitrotyrosyl group, the neutral loss of a nitro group might occur in the MS/MS spectra. In the high-mass region of Fig. 3B, the peak at *m/z* 2685.64 (2730.18 − 45 = 2685.18) corresponded to the neutral loss of a nitro group from the $[M+H]^+$ ion at *m/z* 2730.64. The loss of a nitro group was found in the product ions at $b_{13}-45$, $b_{14}-45$, $b^\#_{14}-45$, $b_{16}-45$, and $a_{16}-45$ (Fig. 3B; Table 2). In the present study, amino acid sequence data were not obtained from the photochemical-induced decomposition ions ($[M+H-14]^+$, $[M+H-16]^+$, $[M+H-30]^+$, and $[M+H-32]^+$), or the neutral loss $[M+H-45]^+$ ions of nitropeptides because those ions from those endogenous nitroproteins were of very-low abundance.

In vitro studies [25,26] also indicated that the immonium ion [^+H_2N=CH–CH_2–C_6H_3(OH)(NO_2)] at *m/z* 181.06 from nitrotyrosine (Tyr-NO_2), at *m/z* 165 for nitrosotyrosine (Tyr-NO), at *m/z* 149 from nitrenetyrosine (Tyr-N), and at *m/z* 151 from aminotyrosine (Tyr-NH_2) might occur in an MS/MS spectrum. The immonium ion [^+H_2N=CH–CH_2–C_6H_3(OH)] from tyrosine (Tyr) occurs at *m/z* 136.10. Therefore, screening the precursor ions that produce those immonium ions would be an effective method to identify nitropeptides in a mixture. However, in the present study, the low-end cut-off (<*m/z* 200) of our ion trap did not allow that analysis.

3.5. Consensus sequence for nitration of a tyrosine residue in a protein

Table 3 collects the amino acid sequences of the 17 endogenous nitropeptides (18 nitrotyrosine residues) that we have discovered in our three studies of the human pituitary [[1,5]; present study]. Those amino acid sequences are aligned to the 3-NT residue in each nitropeptide. The amino acids at the −1 position include 12 aliphatic (I, L, A, V, P, N, G), five possible (depends on pH) ionic (D, R, E), and one hydroxylic (T) amino acids. The amino acids at the +1 position include eight aliphatic (L, A, V, N), five possible (pH-dependent) ionic (E, K, H), 3 hydroxylic (S, T), and two aromatic (F, Y) amino acids. That limited dataset suggests that the consensus sequence. . .aliphatic-Y-aliphatic. . .is required for the nitration of a tyrosine residue in a protein.

4. Conclusions

The mapping and characterization of the human pituitary nitroproteome is a crucial first step to elucidate the role of protein nitration in human pituitary physiological and pathological processes. The large-format (18 cm × 20 cm) two-dimensional Western blot against a specific anti-3-nitrotyrosine antibody is an effective approach to array and detect nitroproteins in a human pituitary tissue proteome. A total of 32 immunoreactive positive nitroprotein-spots were detected. Tandem mass spectrometry (MS/MS) was used to characterize each 2D gel-separated nitroprotein and to determine each nitration-site. A high-sensitivity vMALDI-LTQ mass spectrometer characterized each low-abundance nitroprotein. Four new nitroproteins were discovered, and each nitration-site was identified. Those data indicate that protein nitration could play an important role in the human pituitary, and extend our database of the human pituitary nitroproteome. We observed several photochemical-induced decomposition ions [25,26] that confirmed the presence of a nitrated tyrosine residue in the endogenous nitrotyrosine-containing peptides that derived from a human pituitary.

This present study differs significantly from most previous nitroprotein studies. Many previous nitroprotein studies [28] were performed on *in vitro* systems of nitroproteins; this present study used a human tissue, endogenous nitroproteins were located and characterized, and amino acid sequence data were obtained.

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References

[1] X. Zhan, D.M. Desiderio, Biochem. Biophys. Res. Commun. 325 (2004) 1180.
[2] B. Halliwell, K. Zhao, M. Whiteman, Free Radic. Res. 31 (1999) 651.
[3] M. Miyagi, H. Sakaguch, R.M. Darrow, L. Yan, K.A. West, K.S. Aulak, D.J. Stuehr, J.G. Hollyfield, D.T. Organisciak, J.W. Crabb, Mol. Cell. Proteomics 1 (2002) 293.
[4] I.Y. Haddad, G. Pataki, P. Hu, C. Galliani, J.S. Beckman, S. Matalon, J. Clin. Invest. 94 (1994) 2407.
[5] X. Zhan, D.M. Desiderio, Anal. Biochem. 354 (2006) 279.
[6] Y. Ueta, A. Levy, M.P. Powell, S.L. Lightaman, Y. Kinoshita, A. Yokota, I. Shibuya, H. Yamashita, Clin. Endocrinol. (Oxf.) 49 (1998) 29.
[7] S.M. McCann, S. Karanth, C.A. Mastronardi, W.L. Dees, G. Childs, B. Miller, S. Sower, W.H. Yu, Arch. Med. Res. 32 (2001) 476.
[8] R.V. Lloyd, L. Jin, X. Qian, S. Zhang, B.W. Scheithauer, Am. J. Pathol. 146 (1995) 86.
[9] W. Riedel, Z. Rheumatol. 59 (II) (2002) 36.
[10] S.M. McCann, G. Haens, C. Mastronardi, A. Walczewska, S. Karanth, V. Rettori, W.H. Yu, Curr. Pharm. Des. 9 (2003) 381.
[11] B.H. Duvilanski, C. Zambruno, A. Seilicovich, D. Pisera, M. Lasaga, M.C. Diaz, N. Belova, V. Rettori, S.M. McCann, Proc. Natl. Acad. Sci. U.S.A. 92 (1995) 170.
[12] X. Zhan, D.M. Desiderio, Proteomics 3 (2003) 699.
[13] C.S. Moreno, C.O. Evans, X. Zhan, M. Okor, D.M. Desiderio, N.M. Oyesiku, Cancer Res. 65 (2005) 10214.
[14] X. Zhan, C.O. Evans, N.M. Oyesiku, D.M. Desiderio, Pituitary 6 (2003) 189.
[15] X. Zhan, D.M. Desiderio, Mass Spectrom. Rev. 24 (2005) 783.
[16] H.S. Olsen, M.A. Cepeda, Q.Q. Zhang, C.A. Rosen, B.L. Vozzolo, G.F. Wagner, Proc. Natl. Acad. Sci. U.S.A. 93 (1996) 1792.
[17] G. Sun, J.J. Gargus, D.T. Ta, L.E. Vickery, J. Hum. Genet. 48 (2003) 415.

[18] Y.T. Tang, T. Hu, M. Arterburn, B. Boyle, J.M. Bright, P.C. Emtage, W.D. Funk, J. Mol. Evol. 61 (2005) 372.
[19] T. Tamura, D.H. Lee, F. Osaka, T. Fujiwara, S. Shin, C.H. Chung, K. Tanaka, A. Ichihara, Biochim. Biophys. Acta 1089 (1991) 95.
[20] P. Kristensen, A.H. Johnsen, W. Uerkvitz, K. Tanaka, K.B. Hendil, Biochem. Biophys. Res. Commun. 205 (1994) 1785.
[21] J. Carroll, I.M. Fearnley, J.M. Skehel, M.J. Runswick, R.J. Shannon, J. Hirst, J.E. Walker, Mol. Cell. Proteomics 4 (2005) 693.
[22] Y.R. Chen, C.L. Chen, L. Zhang, K.B. Green-Church, J.L. Zweier, J. Biol. Chem. 280 (2005) 37339.
[23] T. Koeck, X. Fu, S.L. Hazen, J.W. Crabb, D.J. Stuehr, K.S. Aulak, J. Biol. Chem. 279 (2004) 27257.
[24] K.S. Aulak, T. Koeck, J.W. Crabb, D.J. Stuehr, Am. J. Physiol. Heart Circ. Physiol. 286 (2004) H30.
[25] A.S. Petersson, H. Steen, D.E. Kalume, K. Caidahl, P. Roepstorff, J. Mass Spectrom. 36 (2001) 616.
[26] A. Sarver, N.K. Scheffler, M.D. Shetlar, B.W. Gibson, J. Am. Soc. Mass Spectrom. 12 (2001) 439.
[27] B.A. Petre, N. Youhnovski, J. Lukkari, R. Weber, M. Przybylski, Eur. J. Mass Spectrom. 11 (2005) 513.
[28] I.V. Turko, F. Murad, Methods Enzymol. 396 (2005) 266.