ELSEVIER

Journal of Chromatography A, 949 (2002) 173–184

JOURNAL OF CHROMATOGRAPHY A

www.elsevier.com/locate/chroma

Global internal standard technology for comparative proteomics

Asish Chakraborty, Fred E. Regnier*

Department of Chemistry, Purdue University, West Lafayette, IN 47907, USA

Abstract

The work described in this paper tests the efficacy of a global isotope labeling (global internal standard technology, GIST) strategy for quantification in proteomics. Using GIST, overexpression of β-galactosidase in *Escherichia coli* was identified and quantified. The GIST protocol involved tryptic digestion of proteins from control and experimental samples followed by differential isotopic labeling of the resulting tryptic peptides, mixing the differentially labeled control and experimental digests, fractionation of the peptide mixture by reversed-phase chromatography, and isotope ratio analysis by mass spectrometry. *N*-Acetoxysuccinimide and *N*-acetoxy-[$^{2}H_{3}$]succinimide were used to differentially derivatize primary amino groups in peptides from experimental and control samples, respectively. The relative concentration of isotopically labeled peptides was determined by isotope ratio analysis with both matrix-assisted laser desorption ionization mass spectrometry and tandem mass spectrometry (MS–MS). Peptide masses and sequences obtained by MS–MS were used to identify proteins. MS–MS was found to be uniquely suited for isobaric peptide quantification. © 2002 Published by Elsevier Science B.V.

Keywords: Global internal standard technology; Internal standards; Enzymes; Peptides; Proteins

1. Introduction

Recent studies have shown that isotope labeling can provide an alternative to two-dimensional gel methods for quantification in proteomics [1–6]. All of these heavy isotope methods for quantitative proteomics are a derivative of the pioneering work of Schena et al. in the study of mRNA expression [7]. In the Schena et al. method, mRNA species in control and experimental samples were labeled with different fluorescent dyes, the two samples were mixed, individual mRNA species were isolated by hybridization, and the relative degree of change in mRNA concentration between the two samples determined by fluorescence ratio measurements. The isotope labeling methods for proteomics are similar except that isotopic labeling is used in place of fluorescent labeling. Quantification in the case of proteomics is achieved on isotopically labeled peptides and relative differences in concentration between samples measured by mass spectrometry (MS) as the peptides are being identified.

Although several analytical strategies based on isotope labeling have been used to quantify changes in protein concentration in complex mixtures via their tryptic peptides, these methods differ significantly. The popular isotope-coded affinity tagging (ICAT) method uses an alkylating agent coupled to biotin through an isotopically labeled coupling arm to derivatize sulfhydryl groups and select cysteine containing peptides from tryptic digests with immobilized avidin [4]. Specificity of chemical derivatization is a critical element in the ICAT method. Extension of ICAT beyond cysteine selection to

*Corresponding author. Tel.: +1-317-4941-648; fax: +1-317-4940-359.

E-mail address: fregnier@purdue.edu (F.E. Regnier).

0021-9673/02/$ – see front matter
PII: S0021-9673(02)00047-X

other features of peptides ranging from additional amino acids to post-translational modifications requires a different, highly specific derivatizing agent that is both affinity tagged and isotopically labeled.

A more global strategy is to use a single, universal labeling process to universally label peptides, independent of amino acid composition or post-translational modification. This approach has the advantage that any of a wide variety of derivatization and separation techniques may be applied in the selection, identification, and quantification of peptides [2,3]. It is even possible to employ multiple types of selection with the same sample using global internal standard technology (GIST) [3]. Of the several global derivatization strategies that have been examined, one is to acylate primary amino groups in peptides [3]. Another strategy is to incorporate isotopes into polypeptides during biosynthesis [8,9]. In vivo labeling is very attractive but is of limited utility with human subjects. Finally, there is the technique of incorporating ^{18}O from $H_2^{18}O$ into carboxyl groups during proteolysis [10] or deglycosylation [11]. This too is a simple, robust method. The problems in this case are that (1) trypsin exchanges ^{18}O into carboxyl groups of peptides with basic C-terminal amino acids in the absence of, or after proteolysis, (2) the rate of exchange can be structure specific [12], (3) it is not possible to vary the mass difference between labeled and unlabeled species, and (4) the analysis of more than two samples by isotopic multiplexing is precluded.

This paper will focus on quantification by acylating primary amino groups. Issues addressed in the paper are (1) the basic derivatization protocol, (2) uniformity of labeling in a complex mixture, (3) quantification of protein expression in *Escherichia coli*, and (4) quantification in the second dimension of mass spectrometry.

2. Experimental

2.1. Materials

Urea, monobasic sodium phosphate, dibasic sodium phosphate, sodium chloride, *N*-acetoxysuccinimide, *N*-hydroxysuccinimide, *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK)-treated trypsin, *N*-tosyl-L-lysyl chloromethyl ketone (TLCK), isopropyl β-thiogalactopyranoside (IPTG), tris(hydroxymethyl)aminomethane (Tris base), tris(hydroxymethyl)aminomethane hydrochloride (Tris acid), phenylmethylsulfonyl fluoride (PMSF), *N*-hydroxylamine, and α-cyano-4-hydroxycinnamic acid were purchased from Sigma (St. Louis, MO, USA). DL-Dithiothreitol (DTT) and 4-vinylpyridine were supplied by Bio-Rad. Yeast extract, tryptone, and Bacto agar were obtained from DIFCO (Sparks, MD, USA). Isopropanol and HPLC-grade acetonitrile (ACN) were acquired from Mallinckrodt Baker (Phillipsburg, NJ, USA). HPLC-grade trifluoroacetic acid (TFA) was purchased from Pierce (Rockford, IL, USA).

2.2. Instrumentation

Chromatographic analyses were performed on a BioCad Perfusion Chromatography Workstation (PerSeptive Biosystems, Framingham, MA, USA). Peptides were separated by reversed-phase chromatography using a 250×4.6 mm PepMap C_{18} column (PerSeptive Biosystems).

Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectral analyses were performed on a Voyager DE-RP Biospectrometry Workstation (PerSeptive Biosystems) equipped with an N_2 laser (337 nm). All spectra were obtained in the reflector mode using an accelerating voltage of 20 kV.

Electrospray ionization (ESI) MS analyses were carried out on a Finnigan MAT LCQ (Thermoquest, San Jose, CA, USA) mass spectrometer. The source and capillary voltages used were 3.5 kV and 300 V, respectively, while the temperature was set at 200 °C. Helium was used to improve the trapping efficiency and to act as the collision gas for MS–MS analysis. The collision energy was set at 37.5% of the maximum available for the tickle voltage.

2.3. Medium and growth condition for E. coli

The β-galactosidase gene was excised from pEBVHisLacZ plasma by digestion with restriction enzymes *Bam*HI and *Hind*III. The β-galactosidase gene was ligated into *Bam*HI and *Hind*III digested

pQE10. The resulting pHC construct was transformed into XL1 Blue *E. coli* to obtain $XLBp^{HC}$. The $XLBp^{HC}$ was plated on Luria-Bertani (LB) medium plate containing 50 μg/ml ampicillin and incubated overnight at 37 °C. A single colony was picked and incubated at 37 °C overnight in 250 ml LB medium containing 50 μg/ml ampicillin. Equal volumes of the overnight culture were added at 1:50 (v/v) ratio into two different flasks of equal volume containing LB media with 50 μg/ml ampicillin and grown at 37 °C until absorbance at 650 nm ($A_{650\ nm}$)=0.7. Cultures from flasks 1 and 2 will be referred to as experimental and control samples, respectively. IPTG was then added to a concentration of 2 m*M* in the experimental culture and 0.5 n*M* in the control culture, respectively. Growth was allowed to continue in the two cultures in parallel at 37 °C for 2 h.

2.4. Protocol for enzymatic assay

Two hours after the addition of IPTG, aliquots of the cultures were each placed into six 1.5-ml microcentrifuge tubes and centrifuged at 10 000 *g* for 1 min. Supernatants were removed. Each cell pellet was resuspended in 100 μl of Z buffer (Z buffer consists of 100 m*M* sodium phosphate, 10 m*M* potasium chloride and 1 m*M* magnesium sulfate, pH=7.0) [13] and was frozen in liquid nitrogen. Frozen samples were then placed in a 37 °C water bath to thaw. This freeze–thaw cycle was repeated two more times to ensure that the cells had been lysed. Samples were diluted with 700 μl of Z buffer containing mercaptoethanol at a final concentration of 50 m*M*. A blank tube with 100 μl of Z buffer was set up. Then 160 μl of ONPG at 4 mg/ml (*o*-nitrophenyl-β-D-galactoside) in Z buffer was added to the reaction and blank tubes and incubated at 30 °C until a yellow color developed. Reactions were stopped by adding 400 μl of 1 *M* Na_2CO_3. The $A_{420\ nm}$ of samples was measured relative to the blank to determine the amount of β-galactosidase present in both samples.

2.5. Preparation of E. coli lysate

Cultures were centrifuged in different tubes at 8000 *g* for 10 min. Pellets were washed with 0.85% NaCl. Cells were resuspended in 10 ml of Tris buffer, pH 8.0 with 100 m*M* PMSF and 1 m*M* DTT. Cells were lysated in a French Press at 1200 p.s.i. (1 p.s.i.=6894.76 Pa). The lysates were centrifuged for 15 min at 25 000 *g* to pellet the cellular debris, and supernatants were transferred to fresh tubes.

2.6. Proteolysis of experimental and control samples of E. coli and ovalbumin

E. coli lysates (20 mg of protein) from experimental and control samples were denatured individually by adding urea at a concentration of 8 *M*. 10 m*M* DTT was used to reduce the disulfide bonds in both samples. After 1 h incubation at 50 °C, vinyl pyridine was added at a concentration of 25 m*M* to alkylate the reduced disulfide bonds. Reactions were allowed to proceed at room temperature for 30 min. After dilutions with 50 m*M* Tris, pH 8.0 buffer to a final urea concentration of 1 *M*, TPCK-treated trypsin was added at a ratio of 1:50 (w/w) and incubated for 24 h at 37 °C.

Two equal amounts (5 mg each) of ovalbumin in 50 m*M* Tris, pH 8.0 buffer were denatured by adding urea at a concentration of 8 *M*. 10 m*M* DTT was added to reduce the disulfide bonds. After 1 h incubation at 50 °C, vinylpyridine was added at a concentration of 25 m*M* to alkylate the reduced disulfide bonds. Reactions were allowed to proceed at room temperature for 30 min. After dilutions with 50 m*M* Tris, pH 8.0 buffer to a final urea concentration of 1 *M*, TPCK-treated trypsin was added at a ratio of 1:50 (w/w) and incubated for 24 h at 37 °C.

2.7. Synthesis of N-acetoxy-[$^2H_3$]succinimide [3]

An 8.0-g (69.6 mmol) amount of *N*-hydroxysuccinimide in 21.4 g (105 mmol) of [$^2H_6$]C_1 acetic anhydride was stirred for 15 h at room temperature. White product crystals appeared gradually. Excess solvent was removed by rotary evaporation at room temperature. The white crystalline residue was treated with hexane and dried in vacuum. Product yield was 10.86 g (100%), m.p. 133–134 °C.

2.8. Isotope labeling of model peptides

A 50-fold molar excess of *N*-acetoxysuccinimide

and *N*-acetoxy-[$^2H_3$]succinimide was added individually to the two equal aliquots of 1 mg/ml peptides solutions in 50 m*M* phosphate buffer at pH 7.5 over the period of the first hour. The reactions were carried out at room temperature for 3 more hours with stirring. Then equal aliquots of the two samples were mixed and treated with excess amount of *N*-hydroxylamine at pH 11–12 for 20 min. The mixture of acylated peptides was fractionated on a C_{18} reversed-phase chromatography column. Collected fractions were then placed on a Speed-Vac and evaporated to dryness.

2.9. Isotope labeling of tryptic peptides

Tryptic digested peptides from experimental and control samples were dissolved separately in 50 m*M* phosphate buffer at pH 7.5. Fiftyfold molar excess of *N*-acetoxysuccinimide and *N*-acetoxy-[$^2H_3$]succinimide were added to the experimental and control samples, respectively. Labeling reagent was added in small amounts over the period of the first hour. The reactions were carried out at room temperature for three more hours with constant mixing. Equal aliquots of labeled experimental and control samples were mixed and treated with an excess of hydroxylamine at pH 11–12. Two equal amounts of tryptic digested peptides of ovalbumin were dissolved in 50 m*M* phosphate buffer at pH 7.5 separately. One portion was derivatized with *N*-acetoxysuccinimide and the other with *N*-acetoxy-[$^2H_3$]succinimide. The resultant mixture was treated as mentioned above.

2.10. Reversed-phase separation of peptide mixtures

Derivatized peptides mixture was injected onto the reversed-phase column pre-equilibrated with mobile phase A containing 0.1% TFA and 1% ACN in deionized (ddI) water. Gradient elution of peptides was achieved using 100% mobile phase A to 100% mobile phase B containing 0.1% TFA and 5% ddI water in ACN. Column eluents were monitored at 215 nm using an on-line UV detector. As the peptide fragments were eluted from the reversed-phase column, they were manually collected and dried on a Speed-Vac.

2.11. Sample preparation for mass spectrometry analysis

Peptides were reconstituted by dissolving peptides in water–acetonitrile (50:50, v/v). A 1-μl volume of reconstituted sample solution was mixed with 2 μl of matrix solution. A 1-μl volume of the resultant mixture was spotted on the MALDI plate and allow to air dry. The matrix was a saturated solution of α-cyano-4-hydroxycinnamic acid in ACN–water (50:50, v/v), 0.1% TFA, prepared fresh daily. The reconstituted sample solution in water–acetonitrile (50:50, v/v) was used directly for ESI-MS analysis.

2.12. MALDI-TOF-MS analysis

Two hundred and fifty six laser shots from 10 different areas within a sample spot on the MALDI plate were averaged to obtain a spectrum to overcome any sample inhomogenity that occurred during crystal formation. Peptide masses and amino acid sequences were used to identify protein from DNA databases [14].

3. Results and discussion

Proteolysis is a basic component of proteomics, primarily because peptides are easier to separate and analyze than proteins. The fact that both carboxyl groups and amino groups are generated during proteolysis is propitious. This means these functional groups will be present in all tryptic peptides except those derived from amino- or carboxy-terminal blocked proteins. Initial reports indicate that quantification can be achieved by both amino acylation with an isotopically label reagent [2,3] and incorporation of ^{18}O from $H_2^{18}O$ into carboxyl groups during proteolysis [10].

3.1. Global derivatization through acylation

Similar to the procedure described by Schena et al. [7], control and experimental samples were derivatized individually with a labeling agent and mixed before analyses. As a prerequisite to proteolysis, disulfides in all proteins were reduced and alkylated before trypsin digestion. Reduction and alkylation

substantially enhances the digestion of proteins resistant to proteolysis [15]. After proteolysis, primary amino groups in samples were amino acylated with *N*-acetoxysuccinimide (NAS) in water. It has been established by mass spectrometry that acetylation with this reagent occurs at the amino-terminus of peptides and ϵ-amine groups of lysine [3]. This means that tryptic peptides with a carboxy-terminal (C-terminal) arginine are acetylated only once and increase in mass by 42 u after derivatization with NAS. Those with both a free amino-terminus and a C-terminal lysine are acetylated twice and increase by 84 u. Exceptions to this were amino-terminally blocked peptides and incompletely digested tryptic fragments. It is also known that in the case of proteins with adjacent basic amino acids, more than one lysine residue can be present in a tryptic peptide. When multiple lysine residues are present in a peptide they are all acylated by NAS. Quantitative acetylation of peptides with five lysine residues was observed with a synthetic peptide (data not shown) (see Fig. 1). Quantification with this reagent will be addressed below.

N-Acetoxysuccinimide showed little propensity to acylate hydroxyl groups and imidazol groups when used in an aqueous environment, but exceptions were observed. Low levels of acetylation were observed in glycopeptides and serine–threonine peptides at high concentrations of NAS (data not shown). Instances of histidine acetylation were also noted. As a precaution to exclude the possibility of acetate esters being present in samples, treatment with *N*-hydroxylamine was examined. *N*-Hydroxylamine was found to hydrolyze acetate esters in a few minutes at basic pH. Alkylation of sulfhydryl groups prior to acylation precluded acetylation of cysteine residues. Although amino groups in peptides and proteins have been isotopically labeled with a variety of acylating

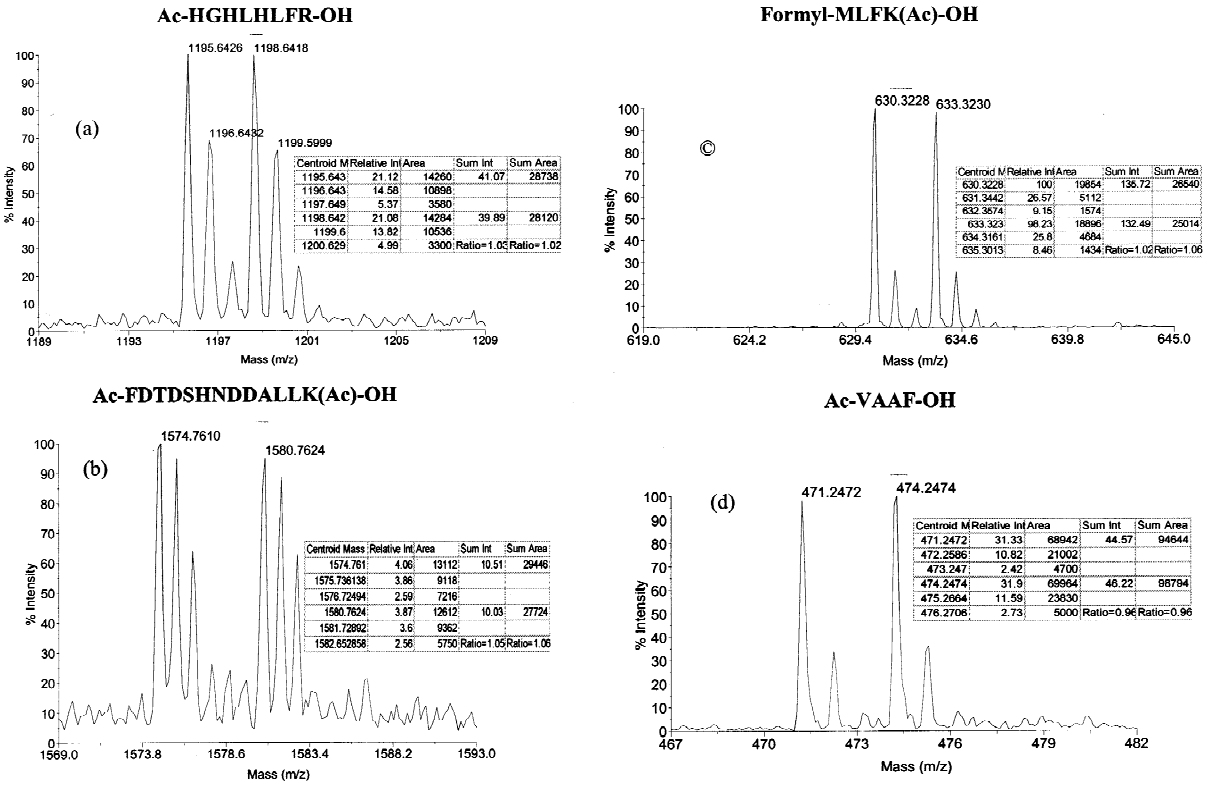

Fig. 1. MALDI mass spectra of differentially acetylated peptides; (a) C-terminal arginine-containing peptide, (b) C-terminal lysine-containing peptide, (c) a peptide containing a C-terminal lysine that is formylated at the N-terminus, (d) a C-terminal peptide without lysine or arginine.

agents [17–19], acetylation was chosen for these studies because acetic anhydride is commercially available in a multiplicity of labeled forms and the requisite *N*-acetoxysuccinimide acylating agent is easily synthesized with acetic anhydride [3].

3.2. Uniformity of peptide labeling in a complex mixture

Isotope ratios may be determined either by using peak intensity or peak area. Isotope ratios in MALDI-MS studies were determined by the summed relative intensities of the acetylated form of a peptide divided by the summed intensities of the trideuteroacetylated form. ESI-MS data were treated in a similar fashion. It is important to note however, that acylation of C-terminal lysine peptides not containing histidine or arginine diminishes the limit of detection in positive ion mode MALDI-MS unless they carry a sodium or a potassium ion.

A tryptic digest of ovalbumin was used to further examine the GIST approach to quantification. Table 1 lists a series of labeled peptides and their measured isotope ratios, as seen in column 3. Again, the relative intensities in each isotope cluster were summed and the intensities of the acetylated and trideuteroacetylated forms compared. Column 4 shows the percent difference between the expected and experimentally determined ratios. Although the differences ranged from 0% to 20% in ovalbumin, the difference in the mean, i.e., the mean of observed and expected ratios, was 6% with standard deviations of 0.08 in intensity-based and 0.06 in area-based calculations. It was also found that taking 2–3 spectra per spot in MALDI-MS analyses and averaging them decreased the ratio difference (data not shown).

3.3. Quantitative analysis of protein expression

The objective here was to determine the accuracy of GIST in quantifying changes in protein concentration relative to a conventional reference method. The enzyme β-galactosidase was chosen because the activity of this enzyme can be precisely assayed. An inducible plasmid was used to test the GIST strategy for quantification based on the fact that protein (enzyme) synthesis could be readily initiated with a low-molecular-mass inducer. The *E. coli* vector XLBpHC used in this study contained an IPTG inducible genetic construct for the expression of β-galactosidase. The relative change in β-galactosidase concentration after induction with IPTG was determined both by enzymatic assays and by isotope ratio analysis of tryptic peptides. Tryptic digests were fractionated by reversed-phase chromatography prior to MALDI-MS and MS–MS of the column fractions (Fig. 2).

Table 1
Quantitative analysis of representative peptides from ovalbumin

Sequence identified	Expected ratio (E)	Observed ratio (O)	Error between E and O (%)	Mean±SD of O ratio	Error*** (%)
Ac-DILNQITKPNDVYSFSLASR	1.0	1.10*/1.14	10**/14		
Ac-ISQAVHAAHAEINEAGR	1.0	1.08*/1.07	8**/7		
Ac-GGLEPINFQTAADQAR	1.0	1.0*/1.06	0**/6		
Ac-ELINSWVESQTNGIIR	1.0	1.0*/1.0	0**/0		
Ac-AFK(Ac)DEDTQAMPFR	1.0	1.10*/1.06	10**/6		
Ac-LTEWTSSNVMEERK-Ac	1.0	0.99*/1.0	1**/0	1.06±0.08-I	6
Ac-LTEWTSSNVMEER	1.0	0.94*/1.06	6**/6	1.06±0.06-A	6
Ac-YPILPEYLQCVK-Ac	1.0	1.20*/1.19	20**/19		
Ac-ADHPFLFCIK-Ac	1.0	1.06*/1.10	6**/10		
Ac-LYAEER	1.0	1.12*/0.99	12**/1		
Ac-CVSP	1.0	0.99*/0.96	1**/4		
Ac-ELYR	1.0	1.20*/1.12	20**/12		
Ac-SLASR	1.0	1.01*/1.04	1**/4		
Ac-LTEW	1.0	1.03*/1.0	3**/0		

The symbols * and ** represent the observed ratio and percent error between expected and observed ratio in intensity based calculation, respectively. The symbol *** indicates the percent error of the mean of observed ratios.

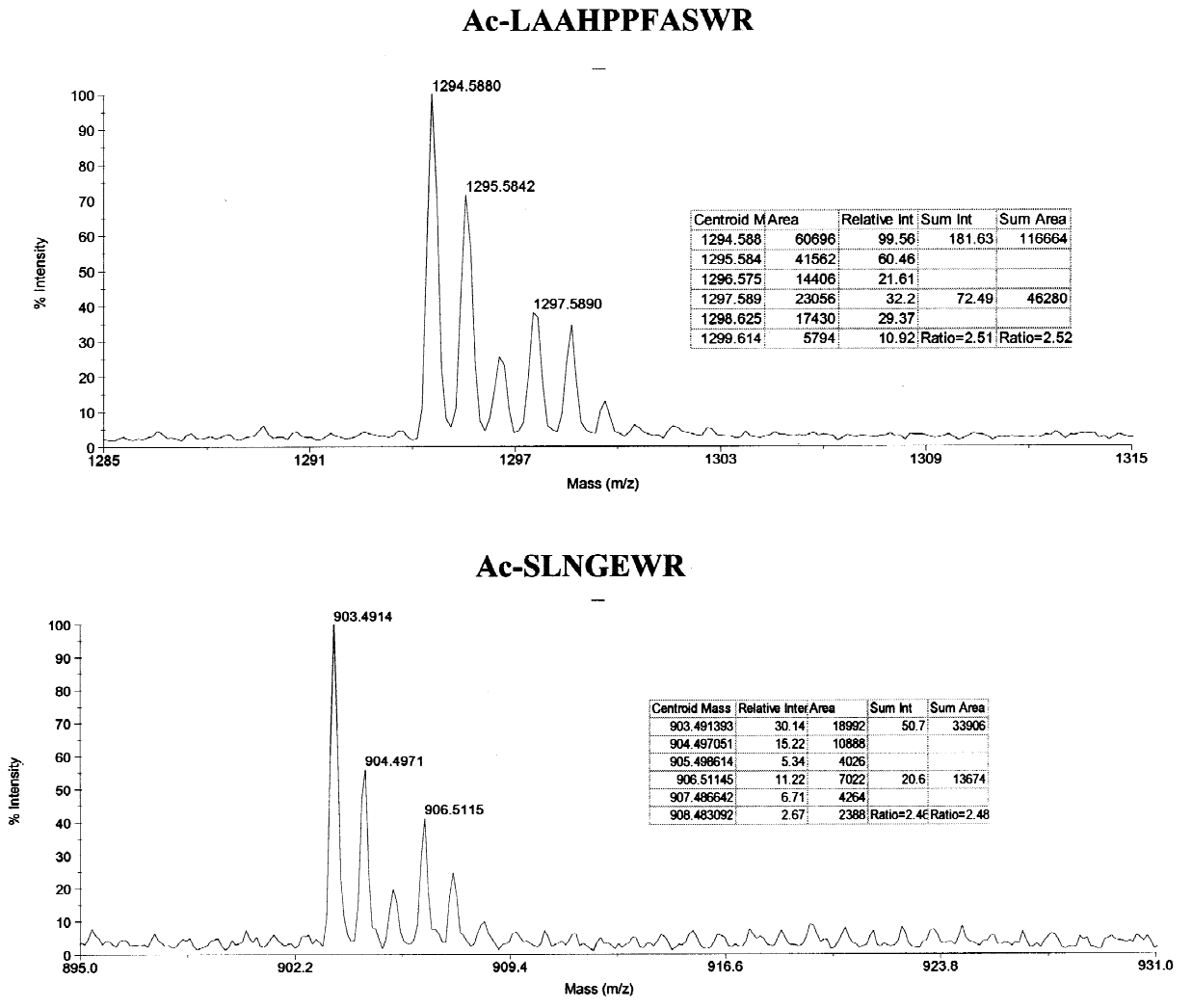

Fig. 2. MALDI mass spectra of representative peptides from over expressed β-galactosidase in *E. coli*.

Expression of β-galactosidase in experimental and control samples was determined enzymatically using the substrate *O*-nitrophenolgalactoside (ONPG). According to data from these enzyme assays, the concentration of β-galactosidase was up-regulated 2.5-fold.

Isotope ratios of the peptides from β-galactosidase are listed in Table 2. Column 2 shows the ratios based on summed intensities denoted by the letter ''I'' as well as ratios based on summed areas denoted by the letter ''A''. The identified peptides in β-galactosidase yield a mean ratio of 2.52±0.04 (in area-based calculation) and 2.49±0.04 (in intensity-based calculation) with a ±95% confidence interval. The error was less than 7% (Table 2). The answers from isotope ratio analysis and enzyme assay are essentially the same. It may be concluded that quantification of relative change in protein concentration can be achieved by differential labeling with acetate.

3.4. Quantification by tandem mass spectrometry

In silico analyses indicate that when proteins in the *E. coli* or yeast proteome are tryptic digested, GIST labeled, and the resulting mixture of peptides

Table 2
Statistical analysis of peptide ratios derived found in β-galactosidase

Sequence identified	Observed ratio ($^1H_3/^2H_3$)	Mean	Mean±SD	Error (%)
Ac-GSHHHHHHTDPMIDPVVLQR	2.57 I/2.61 A			3.21 I/3.4A
Ac-LAAHPPFASWR	2.51 I/2.52 A			1.20 I/0 A
Ac-HQQQFFQFR	2.39 I/2.4A			3.61 I/4.8 A
Ac-YSQQQLMETSHR	2.56 I/2.5 A			2.81 I/0.8 A
Ac-SLNGEWR	2.46 I/2.48 A			1.19 I/1.6 A
Ac-YHYQLVWCQK-Ac	2.57 I/2.61A	2.52A	2.52±0.08	3.21 I/3.4 A
Ac-IDPNAWVER	2.44 I/2.43A	2.49I	2.49±0.08	2.0 I/3.6 A
Ac-APLDNDIGVSEATR	2.51 I/2.54A			0.8 I/0.8 A
Ac-WQFNR	2.63I/2.58 A			5.62 I/2.38 A
Ac-QLLTPLR	2.44 I/2.48 A			2.0 I/1.59 A
Ac-LPSEFDLSAFLR	2.45 I/2.57 A			1.61 I/1.98 A
Ac-MSGIFR	2.32 I/2.36 A			6.75 I/6.35 A
Ac-QNNFNAVR	2.55 I/2.62 A			2.41 I/3.97 A

The letters ''A'' and ''I'' represent the ratios based on summed areas and summed intensities, respectively.

separated by reversed-phase chromatography that multiple tryptic peptides of the same unit mass would co-elute [20]. When peptides of the same mass co-elute in the final separation step before mass spectrometry, the observed isotope ratio (R_{ob}) for the mixture can be described by the equation:

$$R_{ob} = \sum n_i R_i$$

where n_i is the mole fraction of each specific peptide and R_i is the isotope ratio for the two isotopic forms of that peptide. Because n_i is almost never known in the analysis of tryptic peptides obtained from biological extracts, R_{ob} by itself is useless when the objective is to determine the isotope ratio for specific peptides. The question to be examined below is whether this problem can be solved in the second dimension of multidimensional mass spectrometry.

When the mass selection window in the first dimension of tandem mass spectrometry is opened sufficiently to select several isotopic forms of an amino acid it has been shown that deuterated amino acids can be used as internal standards for the quantification of their non-deuterated analogue in the second dimension of mass spectrometry [21]. Because the global internal standard labeling process produces species similar to this example, it is anticipated that fragment ions of GIST labeled peptides will appear as doublets in the tandem mass spectrum carrying the isotope ratio of their precursor ions.

The peptides Ac-HLGLAR-OH (1) and Ac-HLAGLR-OH (2) were chosen to examine the issue of differentiating between isobaric peptides in isotope ratio measurements (Fig. 3). When these peptides were acetate labeled in the GIST format, they appear in mass spectra at m/z 708.4/711.4 as doublet clusters separated by 3 mass units. Again, the mass selection window is opened to include both of these isotopic forms in the second dimension of mass spectrometry. It was seen in the specific case of these two peptides that the mass of all the fragment ions from both peptides is the same except for fragment ions b3 and b4, a4, and y1 through y3 that are unique to the specific peptide from which they were derived. The C-terminal fragment ions (y-ions) are not acetate labeled and cannot be used to determine the ratios from the GIST protocol. In contrast, the b4, b3 and a4 ions are labeled and can potentially be used for isotope ratio analysis (Table 3).

In the case of the peptide Ac-HLGLAR-OH, the isotope ratios of all doublets were determined by the relative abundance (peak heights) of the non-deuterated form divided by the relative abundance of the deuterated form. As an example, the relative abundance of m/z 463.1 divided by the relative abundance of m/z 466.1 showed the ratio of the b4 ion of peptide 1 to be 1.07 (Table 3). The mean ratio of fragment ions b4, b3, and a4 in both peptides (1 and 2) is 1.09 with a standard deviation of 0.03. The

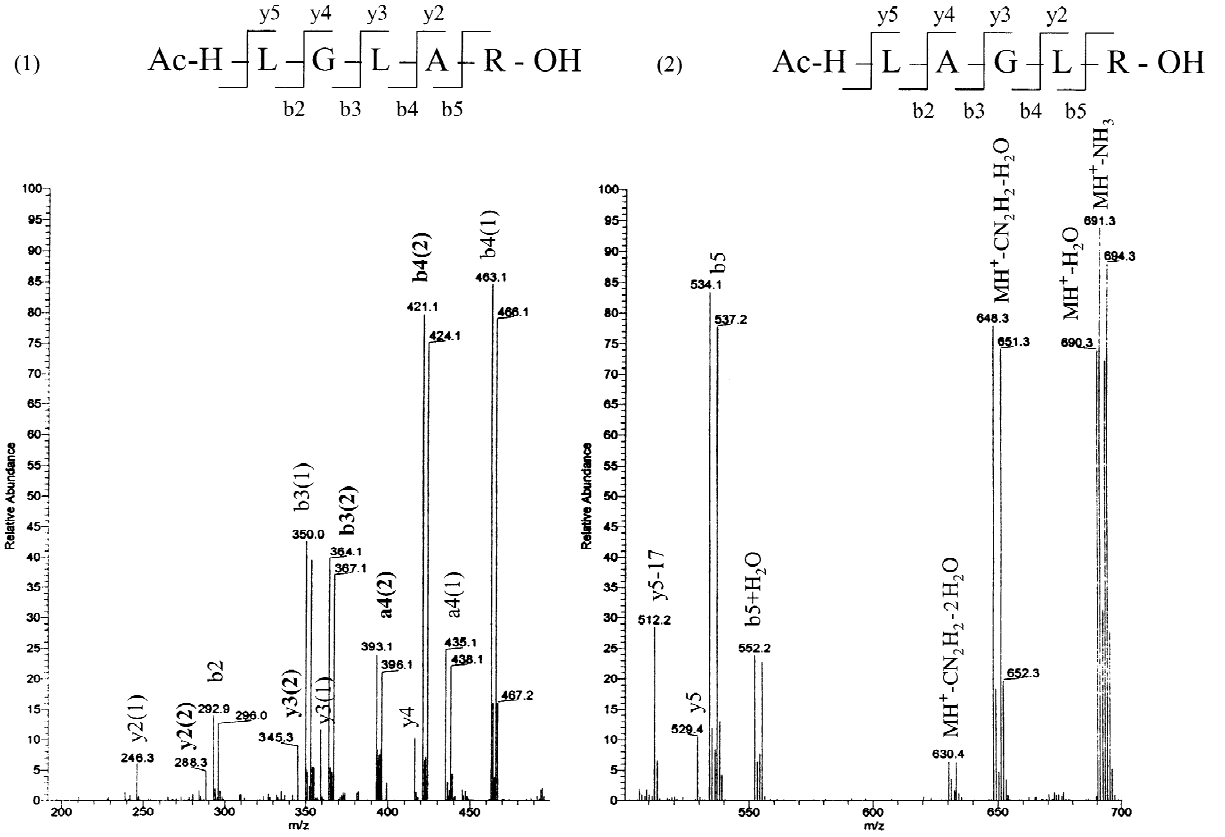

Fig. 3. Tandem mass spectra of differentially acetylated C-terminal arginine terminated isobaric peptides 1 and 2. CAD was performed on the singly charged precursor ion isotope cluster m/z 708.4/711.4 $(M+H)^+$.

error between the experimental mean and expected mean of b4, b3, and a4 ions was 9% in both peptides. However, the mean ratio of fragment ions listed in this table is 1.07 with a standard deviation of 0.03. The error between the experimental mean and expected mean was 7%.

The peptide Ac-VAAF-OH showed similar results. Again, isotope ratios of all doublets were determined

Table 3
Quantitative analysis of differentially labeled isobaric peptides, Ac-HLGLAR-OH (1) and Ac-HLAGLR-OH (2)

Fragment ion	Experimental ratio	Mean±SD	Expected ratio	Error (%)
MH^+-NH_3	1.07		1.0	
MH^+-H_2O	1.02		1.0	
$MH^+-CN_2H_2-H_2O$	1.05		1.0	
$MH^+-CN_2H_2-2H_2O$	1.0		1.0	
$b5+H_2O$	1.04	1.07±0.030	1.0	7.0
b5	1.07		1.0	
b4	$1.07^1/1.06^2$		1.0	
b3	$1.07^1/1.08^2$		1.0	
b2	1.12		1.0	
a4	$1.12^{1,2}$		1.0	

Superscripts 1 and 2 in column 2 are representative of peptides 1 and 2, respectively.

Table 4
Quantitative analysis of fragment ions of the differentially labeled peptide, Ac-VAAF-OH

Fragment ion	Experimental ratio	Mean±SD	Expected ratio	Error (%)
$MNa^+ - H_2O$	1.02		1.0	
b3+Na−H+H_2O	1.03		1.0	
b3+Na−H	1.06		1.0	
b3+Na−H+H_2O	0.97		1.0	1
b2−H+Na	1.0	1.01±0.03	1.0	
a3−H+Na	1.02		1.0	
a2−H+Na	1.0		1.0	

from the relative abundances (peak heights) of non-deuterated and deuterated forms of the fragment ions. The relative abundance of m/z 306.2 divided by the relative abundance of m/z 309.2 showed the ratio of the b3+Na−H ion to be 1.06 (Table 4 and Fig. 4). Although the differences between the expected and experimentally determined ratios ranged from 0 to 6%, difference in the mean of expected and observed ratio of all fragment ions listed in this table was 1.01 with a standard deviation of 0.03. The

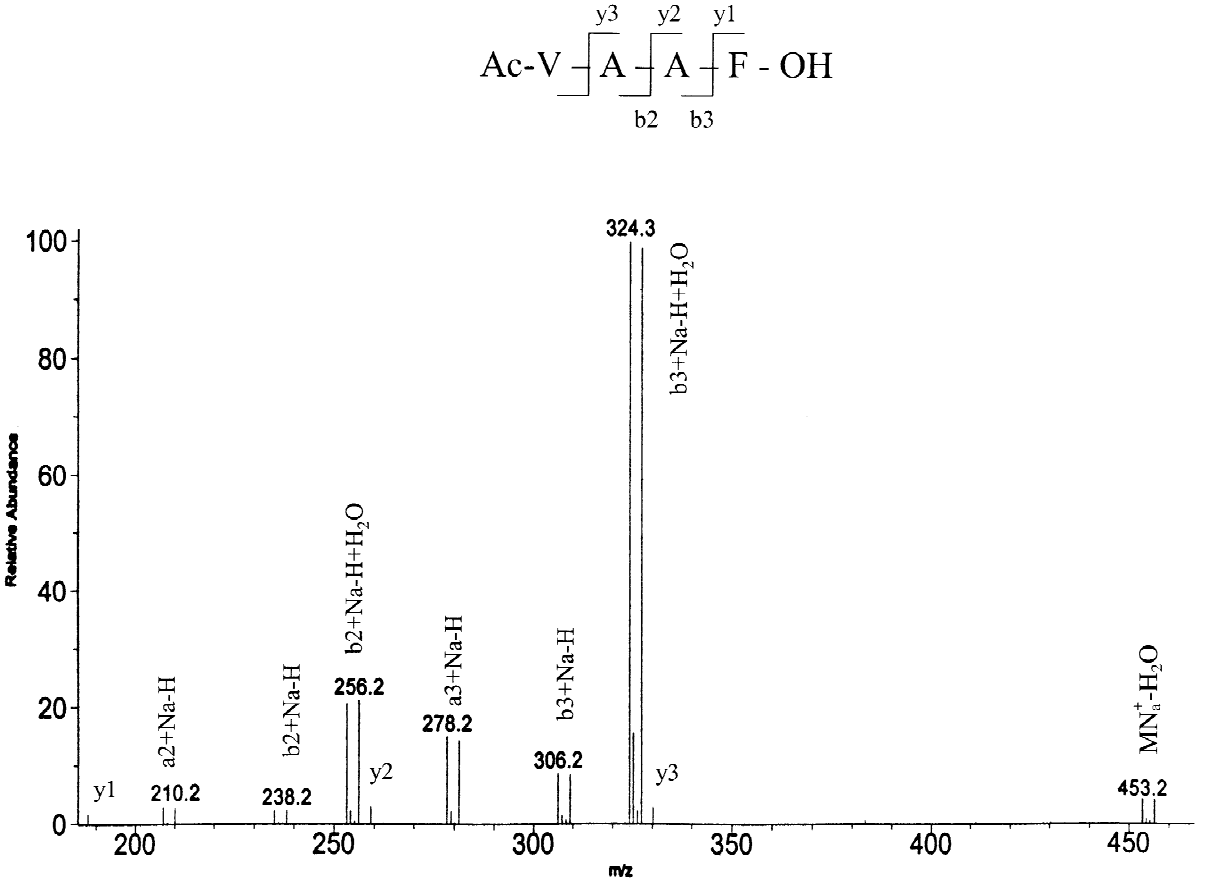

Fig. 4. Tandem mass spectrum of a differentially acetylated C-terminal peptide with no lysine or arginine present. CAD was performed on the precursor ion at m/z 471.3/474.3 $(M+Na)^+$.

error between the experimental mean and expected mean was 1%.

Similar results were found with the peptides Ac-HDMNK(Ac)VLDL-OH, formyl-MLFK(Ac)-OH, Ac-HGHLHLHFR-OH, and Ac-FDTDSHNDDALLK-(Ac)-OH. The difference between the expected and observed ratios ranged from 1 to 12% in the case of Ac-HDMNK(Ac)VLDL-OH. However, the mean ratio was 1.03 with a standard deviation of 0.07 and an error between the experimental mean and expected mean of 3%. Differences of 2–10% between the expected and observed ratios are seen in the case of formyl-MLFK(Ac)-OH. The mean ratio was 0.99 with a standard deviation of 0.08 and a 1% error between the experimental mean and expected mean. When all the fragment ions were examined together the differences between the expected and observed ratios ranged from 0 to 11% in the case of Ac-HGHLHLHFR-OH. But the mean ratio was 1.02 with a standard deviation of 0.07 and a 2% error between the experimental mean and expected mean. Values for the same variables in the cases of Ac-FDTDSHNDDALLK(Ac)-OH were 5 to 15%, 1.07, 0.09, and 7%, respectively.

The data above was for peptides present at an isotope ratio of one. But it has been shown in Table 2 that the objective in quantitative proteomics is to identify peptides from proteins that have been up- or down-regulated, i.e., the isotope ratio would not be one. This means the strategy for recognizing peptides from proteins in regulatory flux would be to search for doublet ion clusters in the MS–MS spectra with a ratio other than one. The hypothetical mass spectrum in Fig. 5 showing only the diagnostic ions from a set of isobaric peptides provides an illustration of how ratio discrimination can be achieved in the second

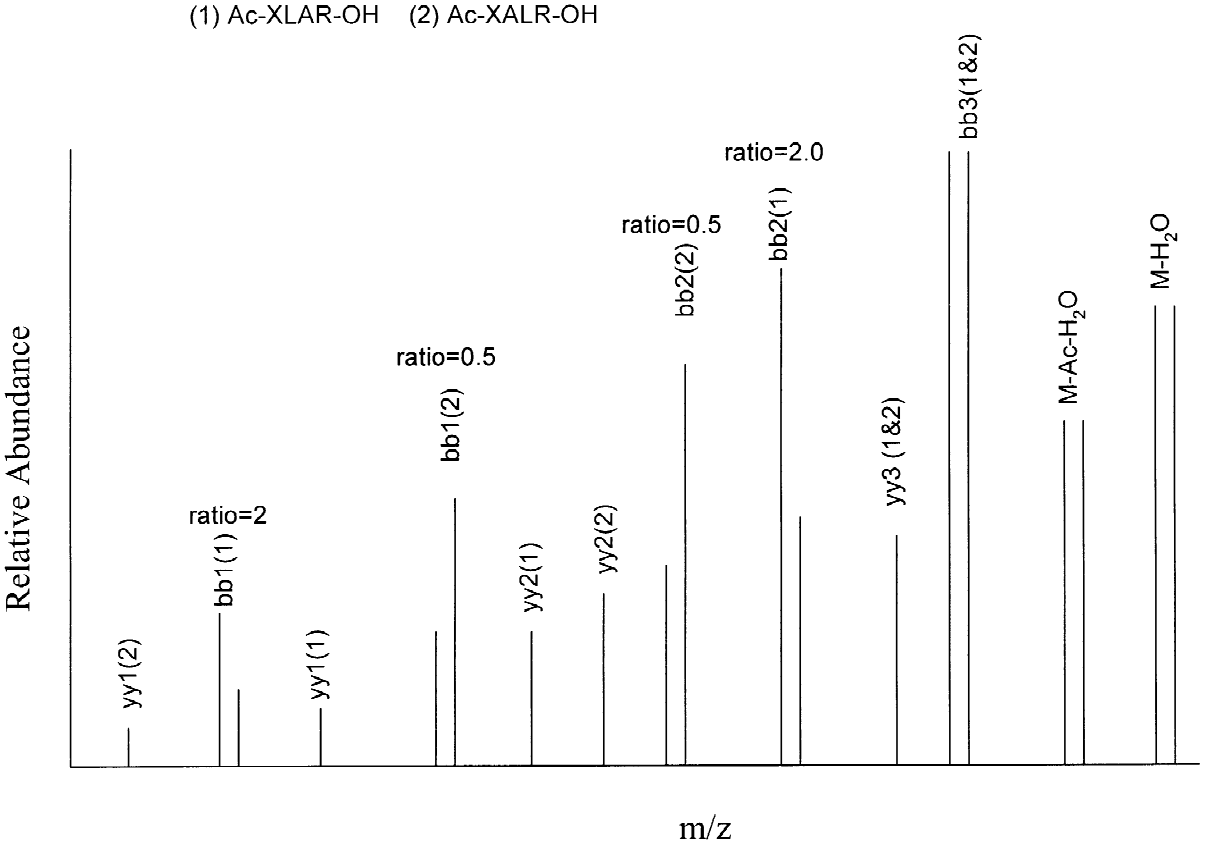

Fig. 5. Tandem mass spectra of differentially acetylated isobaric peptides 1 and 2 that are present in different isotopic ratios. ''X'' represents any amino except lysine and bb1(1) and bb1(2) are the representative of b1 ions of peptides 1 and 2, respectively.

dimension of tandem mass spectrometry. Two things should be noted in this Figure. One is that the isotope ratio in the b ions from the two peptides is different. The second is that the isotope ratio alone can be used to group b ions that originated from the same peptide. These two facts will be of great valuable in both quantitative and qualitative proteomics.

4. Conclusions

Based on the data present here it may be concluded that the global internal standard labeling strategy (1) uniformly labels all peptides in a tryptic digest, (2) accurately predicts the degree of change in protein expression in a biological system, and (3) can be used with either MALDI-MS or ESI-MS. It is further concluded that the problem of determining the isotope ratio of isobaric peptides may be solved by quantification in the second dimension of MS–MS. This will permit multiple parent ions having the same mass to be readily distinguished and quantified after CAD of the precursor ion in a tandem mass spectrometer.

The great value of the GIST described here is that it will enable the study a variety of regulated changes in cells involving post-translational modifications, in addition to changes in expression.

5. Further reading

[16]

Acknowledgements

The authors gratefully acknowledge support from the National Institute of Health (GM 59996) and Applied Biosystems. The authors would like to express their appreciation to Ms. Hari (Purdue University) for the generous gift of the *E. coli* $XLBp^{HC}$. The authors would also like to thank Deb Miles for her assistance in obtaining the ESI-MS–MS data used in this paper.

References

[1] A. Chakraborty, F. Regnier, presented at the 100th ACS Conference on Genomics and Proteomics, Washington, DC, 18–24 August 2000.
[2] M. Geng, J. Ji, F. Regnier, J. Chromatogr. A 870 (2000) 295.
[3] J. Ji, A. Chakraborty, M. Geng, X. Zhang, A. Amini, M. Bina, F. Regnier, J. Chromatogr. B 745 (2000) 197.
[4] S.P. Gygi, B. Rist, S.A. Gerber, F. Turecek, M.H. Gelb, R. Aebersold, Nat. Biotechnol. 17 (1999) 994.
[5] Y. Oda, K. Huang, F.R. Cross, D. Cowburn, B.T. Chait, Proc. Natl. Acad. Sci. USA 96 (1999) 6591.
[6] T.P. Conrads, K. Alving, T.D. Veenstra, M.E. Belov, G.A. Anderson, D.J. Anderson, M.S. Lipton, L. Pasa-Tolic, H.R. Udseth, W.B. Chrisler, B.D. Thrall, R.D. Smith, Anal. Chem. 73 (2001) 2132.
[7] D. Schena, D. Shalon, R. Heller, A. Chai, P.O. Brown, R.W. Davis, Science 270 (1995) 467.
[8] Y. Oda, K. Hunag, F.R. Cross, D. Cowburn, B.T. Chait, Proc. Natl. Acad. Sci. USA 96 (1999) 6591.
[9] T.D. Veenstra, S. Martinovic, G.A. Anderson, L. Tasa-Tolic, R.D. Smith, J. Am. Soc. Mass Spectrom. 11 (2000) 78.
[10] M. Schnolzer, P. Jedrzejewski, W.D. Lehmann, Electrophoresis 17 (1996) 945.
[11] B. Kuster, M. Mann, Anal. Chem. 71 (1999) 1431.
[12] M. Schnolzer, P. Jedrzejewski, W.D. Lehmann, Electrophoresis 17 (1996) 945.
[13] Current protocols in molecular biology, www.clontech.com.
[14] http://expasy.cbr.nrc.ca/sprot/
[15] M. Friedman, L.H. Krull, J.F. Cavins, J. Biol. Chem. 245 (1970) 3868.
[16] K. Biemann, Annu. Rev. Biochem. 61 (1992) 977.
[17] S. Wang, F. Regnier, J. Chromatogr. A, in press.
[18] X.H. Chen, Y.H. Chen, V.E. Anderson, Anal. Biochem. 273 (1999) 192.
[19] D.F. Hunt, A.M. Buko, J.M. Ballard, J. Shabanowithz, A.B. Giordani, Biomed. Mass Spectrom. 8 (1981) 397.
[20] X. Zhang, Ph.D Thesis, Purdue University, West Lafayette, IN, June, 2001.
[21] X. Zhu, J.T. Robertson, H.S. Sacks, F.C. Dohan, J. Tseng, D.M. Desiderio, Peptides 16 (1995) 1097.