Concurrent mass spectrometric analysis of multiple samples using Py-Tag reagents

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Keywords: Proteomics, Py-Tag, LC-MS, Stable isotope

Abstract

The quantification of amino acids, peptides and proteins are important in fundamental science to understand intravital event, as well as in applied fields such as biological, medical, and pharmaceutical science.

Py-Tags (Py0, Py6 and Py12) are primary amine-reactive isotope-labelled tagging reagents. Py-Tags can selectively and efficiently react with the primary amino group which has more than a two-carbon chain from the alpha-carbon of the target sample by the reaction of pyrylium salts with nucleophiles under alkaline conditions, thereby increasing the mass by 175.15, 181.17, and 187.19 Da, respectively, and generating positive ions.

We used Py-Tags to label not only the amino acids but also peptides with a lysine residue(s), fragment peptides with a lysine(s), digested proteins, and intact proteins. All Py-Tag-labelled peptide sets showed similar mass differences. Thus, we could easily analyze expression ratio by single measurement. Furthermore, the ratio of the measured ion intensities was representative of the relative amount of each material.

Introduction

The exploration of disorder biomarkers is important for proteome analysis-based drug discovery. Protein sequences and protein expression levels are often determined by investigating the RNA expression level of that protein using microarrays or real time PCR. Post-translational protein modification, such as glycosylation, phosphorylation, and polyglutamylation[1] affect protein structure and function. However, sometimes there is no difference in a protein expression level between normal and abnormal samples, making quantitative and exhaustive protein analysis particularly challenging. Stable isotope-labelled biological samples have been analyzed by mass spectrometry (MS) to understand protein expression[2-8]. The other common stable isotope tags are ICAT (isotope coded affinity tag)[9] and stable isotope labelling by amino acids in cell culture (SILAC)[10]. A third approach allows analysis of multiplex protein profiling using iTRAQ (isobaric tags for relative and absolute quantitation). In conventional method, samples are labelled using four independent reagents with the same mass, and each reagent consists of three groups, which are called the reporter, balance, and peptide reactive groups. iTRAQ modified-peptides provide four reporter ions (mass-to-charge ratio (*m/z*) 114-117) in MS/MS

analysis that are needed to quantify that peptide in different samples. Indeed, these are tactically designed to eliminate any interference from other fragment ions and to retain the additive mass[11,12]. These approaches provide fidelity and accuracy, but may require higher sensitivity and multi-stage procedures. In addition, there is essentially need for tandem MS techniques that provide quantitative protein profiles. When a mass difference is conferred to the same sample, such as a peptide, by using two or more labelling compounds with different masses due to labelling with an isotope, the mass difference is typically small. This causes interference between different peaks, complicating analysis.

Thus, the development of new isotope-labelled tagging reagents exhibiting improved analytical quantity, sensitivity, accuracy, the largest possible mass difference, and easy analysis are required. In this research, we developed and evaluated a new stable isotope tag called to Py-Tag. Py-Tag is a pyrylium-based compound (Figure 1A) that can specifically react with primary amino groups (e.g., the amino group of the lysine side chain: Figure 1B). Py-Tag did not react with the alpha-amino group of amino acids. Py-Tag reacts with primary amines by nuclear attack but requires the presence of a free amino group at a geometrical distance longer than a two-carbon chain from the alpha-carbon of

the amino acid to generate a pyridinium ion adduct. Thus, Py-Tags could specifically modify amino acids with a long carbon chain such as taurine and lysine, but not with the primary amine of the N-terminus. Thus, only lysine in the essential amino acid can be react with Py-Tag. Almost all proteins contain numerous lysine residues which react preferentially with Py-Tag reagents. The data indicate was simple that provide easily analysis for us. Py-Tag allows high labelling efficiency, efficient peptide quantification due to wide mass differences of 6 Da (using ^{13}C as the sole stable labelling isotope), and is chemically stable. Py-Tag causes no interference between the monoisotopic peak and the labelled peak with a higher mass, allowing facile quantitative analysis of the target sample such as amino acids, peptide and proteins. The target sample labelled with a different Py-Tag shows a different *m/z*, even when different samples are mixed (Figure 1C). Herein we demonstrate the usefulness of Py-Tag reagent using free amino acids, standard peptides, digested proteins, and an intact protein.

Materials and methods

Sample preparation

Standard amino acids and standard peptides

The following aqueous solutions (4 mM) were prepared: taurine (sulfated amino acid), lysine, leucine, isoleucine, valine, glycine, glutamine, asparagine (WAKO Pure Chemicals, Japan) and four model peptides (Sigma-Aldrich, USA) (Table 1).

Table 1 Synthetic peptide

Peptide	Exact mass	Sequence
Substance P	1346.728	RPKPQFFGLM-NH_2
Substrate for hepatocyte growth factor activator	971.555	Ac-KTKQLR-MCA
Mastoparan	1478.007	INLKALAALAKKIL-NH_2
Angiotensin I	1295.677	NRVYIHPFHL-NH_2

Ac: Acetylation, MCA: 4-methylcoumaryl-7-amide

A 2.5 μL aliquot of mixed amino acid or peptide solution was mixed with 7.5 μL of reaction 50 mM borate buffer (pH 9.5) in a sealed 0.2 mL PCR test tube. A 2.5 μL aliquot of 200 mM Py0, 6 or 12 (Taiyo Nippon Sanso Corp. Japan) was added, and then the mixture was heated at 55 °C for 4 hours. A 0.5 μL aliquot of formic acid was then analyzed by MALDI-MS (autoflex : Bruker Daltonics, Billerica, MA) .

Protein samples

Bovine serum albumin (BSA, 100 μg; Sigma-Aldrich, USA) was solubilized in 50 μL water, then 50 μL of 8 M guanidium hydrochloride, 10 mM EDTA and 100 mM Tris-HCl (pH 8.0) was added, followed by the addition of 4 μL of 500 mM dithiothreitol (DTT) and incubation at 55 °C for 60 min. Then, 8 μL of 500 mM iodoacetamide was added and the mixture was incubated in the dark for 60 min at room temperature. The buffer was changed by centrifuging the sample using an Amicon Ultra (10 kDa, Merck Millipore, Germany) to 20 μL, then 62 μL of 0.1 M Tris-HCl (pH 8.0) and 18 μL of 6 M urea were added, followed by 100 ng of Lys-C endopeptidase/trypsin mix (MS Grade, Promega, USA). The solution was incubated at 25 °C for 3 hours and 37 °C overnight, followed by desalting with a GL-Tip SDB (GL Science, Japan). Py0, 6 or 12 reagent solution and borate buffer were added to each of the three dispensed recovered solutions, followed by heating at 50 °C for 4 hours. Excess Py-Tag reagent was removed by centrifugation using a GL-Tip SDB. Each peptide fragment solution was analyzed by LC-MS (Orbitrap mass spectrometer : Thermo Scientific, San Jose, CA). For GAPDH, transferrin, lactoferrin, glutamic dehydrogenase and lysozyme as other sample protein, the same procedures were

adopted to prepare the samples for LC-MS analysis.

Whole BSA was modified with Py-Tag by solubilizing 30 μg of BSA in 30 μL of water, and 63 μL of 7 M guanidium hydrochloride (Gu-HCl), 10 mM EDTA and 50 mM Tris-HCl (pH 9.5) were added to the 0.2 mL PCR tube, followed by the addition of 8 μL of 200 mM Py0, 6 or 12 reagent solution. The labelling reaction was conducted at 55 °C for 4 h, and Py-labelled BSA was digested by Lys-C/Trypsin as described above.

Samples with ratio of 1: 1: 1 or 1: 2: 3 in protease digested peptide mix were prepared and labeled with Py0, Py6 or Py12, the resulting labeled peptide samples were mixed and analyzed by LCMS. Each sample was diluted 10-fold with Milli-Q water, then injected into an ODS column for nano-LC (5 μm particle size, 0.1 × 200 mm). Mobile phase A consisted of 0.1% formic acid in water (v/v) and mobile phase B consisted of 0.1% formic acid in acetonitrile (v/v). The gradient conditions (B%) were 0-3 min = 10%, 3-73 min = 83%, and 73-74 min = 100%. The flow rate and injection volume were 300 nL/min and 1 μL, respectively.

Results and discussion

Standard amino acids

All analyses were performed six times to confirm the reproducibility of the technique. Py-Tag labelled taurine and lysine each provided three signals: taurine (*m/z* 300.2, 306.2 and 312.2) and lysine (*m/z* 321.3, 327.3 and 333.3) (Figure 2A). Triplet signals were detected by MALDI-MS and both intervals between the three signals were 6 Da, indicating successful modification by Py-Tag. The ion signal with the lowest mass corresponded to the Py0-modified target, and the second and third ion signals indicated that both targets were also modified with Py6 and Py12 reagents, respectively. No signals corresponding to Py-Tagged leucine, isoleucine, valine, glycine, glutamine and asparagine could be detected, indicating that Py-Tag did not react with the alpha-amino group of amino acids. Thus, Py-Tags could specifically modify amino acids with a long carbon chain such as taurine and lysine, but not with the primary amine of the N-terminus. Notably, labelling with Py-Tag results in a positive ion. Generally, MALDI-MS analysis of non-labelled taurine showed a negative ion and therefore decreased ionization efficiency compared to general amino acids which provide positive ions. Py-Tags introduce a pyridinium cationic group and thus all fragments were positively charged and

all derivatives could be monitored with high efficiency in positive MS mode.

Tandem MS confirmed the fragmentation ions of Py-Tags. Py0-modified taurine (Figure 2B) or lysine (Figure 2C) cleaved at the pyridine ring moiety and provided *m/z* 192.2. Modification with Py6 and Py12 also provided *m/z* 198.2 and 204.2 (data not shown). The detection of fragmented Py-Tags unequivocally demonstrates modification of the target molecule by Py-Tag and the amenability of the tagged molecule to quantitative analysis.

Standard peptides

To confirm the labelling efficiency of Py-Tag derivatization, four peptides with different numbers of lysine residues, positions of the lysines and exact mass were used (Table 1). And different molar ratios of Py-Tag to peptide were tested. Unlabelled peptides were observed when the ratio was 1-5 fold, whereas when it was 10-fold or more, only Py-Tag-labelled peptides were observed (supporting information 1A, B). In other experiment, we prepared 50-fold Py-Tag against sample to unfailingly obtain Py-Tag-labelled sample. Thus, Py-Tag could efficiently react with target molecule.

The linearity of Py labelling was investigated using SP. Standard solutions containing 0, 0.12, 0.3, 0.6, 1.2, 3.0, or 6.0 nmol of peptide were prepared in 9 μL of 50 mM borate buffer (pH 9.5) and mixed with 10 μL of 30 nmol/μL Py0 solution. The reaction was stopped with 1 μL of formic acid. A 2 μL aliquot was analyzed by HPLC, corresponding to 0.01-1.0 μg per injection (100% labelling efficiency). The peak area was measured using HPLC system (LC10Avp: Shimadzu Corporation, Japan). Each experiment was performed four times, and the peak area versus Py0-labelled peptide amount was plotted to confirm the linearity of the labelling (supporting information 1C). The correlation coefficient (R^2) was 0.9995. To conform reproducibility, a different ratio of Py-Tag for sample peptide (1:2:1, 1:2:2 and 1:2:4) was achieved. The intensity ratio for peptide indicated the 1:2:1, 1:2:2 and 1:2:4 for the mixed sample at a same mix ratio labelled with Py0, 6 and 12, respectively (supporting information 2).

Several peptide samples modified with Py-Tags were analyzed by ESI MS and their multivalent ions were detected. Four model peptides were used (Table 1): substance P (SP), substrate for hepatocyte growth factor activator (HGFA), and mastoparan (which contain one, two, and three lysine residues, respectively), and angiotensin I, which has no

lysine residue and was used as a negative control. Figure 3A shows the mass spectrum of Py-Tag-modified SP, which has a cationic group. Theoretically, the mass of Py0, 6 and 12-modified SP should show 1521.8, 1527.8 and 1533.9, respectively. Signals were observed at *m/z* 761.443, 764.452 and 767.461. The theoretical mass difference was 6 Da but the observed interval between signals was 3 Da due to modification with Py-Tag and the tagged peptide provided bivalent ions. HGFA provided signals at *m/z* 440.952, 444.965 and 448.978. When two Py-Tags modified the HGFA sequence, the theoretical mass difference was 12 Da but the observed mass difference was 4 Da, indicating a trivalent ion (Figure 3B). From these results, the mass-to-charge ratio (*m/z*) was obtained using Eq. (1):

$$m/z = [\mathrm{M}+x\mathrm{Py}+n\mathrm{H}]^{x+n} \quad (1)$$

M: Peptide mass

x: number of K residues

n: number of cationic amino acids (i.e., W, R)

SP and HGFA contain an arginine residue as a cationic amino acid, and thus provided multivalent (x+1) ions.

We investigated a mastoparan peptide containing 3 lysine residues. Triplet signals at *m/z* 668.152, 674.171 and 680.190 corresponded to py0, 6 and 12-modified peptide (Figure 3C). The mass difference was 6 Da, indicating a trivalent ion. Since the theoretical mass difference is 18 Da (modification with three Py-Tags), the sequence has no positive amino acid residues except K. Thus, modification with 3 Py-Tags provided a single trivalent cationic ion. The mass-to-charge ratio (*m/z*) was calculated using Eq. (2):

$$m/z = [\mathrm{M}+x\mathrm{Py}]^{x} \quad (2)$$

M: Peptide mass

x: number of K residues

The obtained MS intensities were similar using a 1:1:1 ratio of Py0, 6 and 12-labelled peptides. This findings showed that Py-Tag did not affect ionization efficiency and thus Py-Tag can be utilized for quantitative analysis using just precursor ions, without tandem MS measurements.

We also conducted tandem MS analysis to confirm the influence of the Py-Tag on the tandem MS behavior of peptides. The observed fragment ions *m/z* 192.2, 198.2 and 204.2 correlated with Py0, 6 and 12, respectively, indicating that Py-Tags successfully modified

the target peptide. Combining with the linearity after Py-Tag labelling, Py-Tag can also use quantitative experiment by tandem MS. Angiotensin I peptide has no lysine residues and was used as a negative control. Angiotensin I provided a mass signal only at *m/z* 649.090, corresponding to the diprotonated form of the peptide without a pyridinium adduct. Thus, Py-Tag reagent can modify only lysine residues in peptides and not the free *N*-terminus of peptides, nor the guanidino- and carbamino-groups of arginine and glutamine, respectively.

Protein samples

The usefulness of Py-Tags for the quantitative analysis of proteins was examined using peptides from digested BSA and intact BSA. First, the BSA peptide mixture digested with the protease was adjusted to a ratio of 1: 1: 1 or 1: 2: 3, and then labeled with Py0, 6 or 12, finally mixed and analyzed by LCMS. BSA contains 59 lysine residues. We analyzed large data sets using *Proteome Discoverer* 2.1^{TM}. The digested BSA sample was analyzed by LC coupled to tandem MS: 84% of the peptides were assigned, and 56 fragment peptides (including 26 Py-Tagged peptides) were detected. In this experiment, we

measured the mass of sample over *m/z* 350. Almost detected ions were multivalent ion, thus, some short fragment sequences which include lysine residue were not detected due to small mass. 19 fragment sequences were correlated with short sequence. Thus, 65% in BSA were modified with Py-Tags except short fragment (supporting information 3), indicating that Py-Tags is sustainable to the analysis of protein sample as well as small molecules. Representative mass spectra of the peptide fragments are shown (Figure 4A and B). Three signals (*m/z* 818.926, 821.936, 824.945) were observed as bivalent ions, and thus their mass difference was 3 Da. The cationic group of Py-Tag and a protonation reaction allowed the detection of a multivalent ion identified as $[\text{Py-Tag-M} + \text{H}]^{2+}$. The intensity ratio of this peptide was 1:1:1 (Figure 4A) or 1:2:3 (Figure 4B) for the mixed sample labelled with Py0, 6 and 12 at ratios of 1:1:1 or 1:2:3, respectively. The results for other labelled peptides were similar (Figure 4C and D). The averaged intensity values for all labelled peptides showed 1:1:1(Figure 4E) or 1:2:3 (Figure 4F) for the mixed sample labelled with Py0, 6 and 12 at ratios of 1:1:1 or 1:2:3, respectively.

Py-Tag reagents were evaluated for the quantitative analysis of other proteins by measuring fragment peptides of pre-digested GAPDH, transferrin, lactoferrin, glutamic

dehydrogenase, and lysozyme, which contain 25, 57, 55, 32, and 6 lysine residues, respectively. Mass spectra of a representative peptide sequence (RVIISAPSADAPMFVMGVNHEK) for GAPDH are shown Supporting information 4A-D). Triplet signals (*m/z* 868.126, 870.131, 872.138) were observed as trivalent ions differing in mass by 2 Da and were assigned to $[\text{Py-Tag-M} + 2\text{H}]^{3+}$. The intensity ratio of the average for all peptides was the same or 1:2:3 for the mixed sample at a mix ratio 1:1:1 and 1:2:3 labelled with Py0, 6 and 12, respectively.

Approximately 50% of the lysine residues in the other proteins tested (transferrin, lactoferrin, glutamic dehydrogenase and lysozyme) were modified with Py-Tags. In addition, the intensity ratios of these fragment peptides were 1:1:1 for the sample mixed at a ratio of 1:1:1 (Supporting information 4E-F). The above results indicate that Py-Tag reagents are useful for the analysis of essentially any protein sample because almost all proteins contain numerous lysine residues which react preferentially with Py-Tag reagents.

We prepared whole BSA independently pre-labelled under denaturing solvent conditions (7M Gu·HCl) with the three Py-Tag reagents prior to mixed protease digestion. LC coupled tandem MS identified BSA, and 60% of the theoretical peptide fragments were

assigned. Three Py-Tagged peptides were detected. The number of identified Py-adduct-containing peptides was dramatically reduced due to decreased reactivity of the reagents caused by steric hindrance between the numerous side chains in the denatured protein. Furthermore, Py-labelled lysine in a protein cannot be recognized by ~~mixed~~ proteases, and therefore Py-labelled peptides are longer and more complex, in contrast to post Py-labelled peptides. The intensity ratio of each set of Py-labeled peptides detected was 1: 1: 1 (Figure 4G) and the average of these peptides was also 1: 1: 1 (Figure 4H). This result indicated a merit of Py reagents: namely, that Py-Tag can be used to derivatize whole protein, followed by subsequent quantitative analysis without the need for complicated procedures and analyses.

Conclusion

We succeeded in covalently labelling lysine residues with Py-Tags. Py-Tagged small molecules, peptides, and whole proteins could be detected with high sensitivity by their pyridinium positive ions formed by modification with pyrylium ion. For monovalent ions, the mass difference of Py-Tag (6 Da) is larger than that of other derivatization reagents.

Py-Tag allows the analysis of multivalent ions because it provides a clear mass difference. If a sample provides a bivalent ion, Py-Tag labelling of this sample will result in a 3 Da mass difference. However, it is important to analyze mass differences to one decimal point using other derivatization reagents. The reproducibility of this semi- quantitation technique is due to the use of a stable isotope-labelled reagent. Py-Tag reagents can easily confer a large mass difference (6 Da/Lysine) to molecules such as lysine-containing bioactive peptides and proteolytic peptides derived from protein samples, as well as amine-containing small molecules such as active amines. We will examine protein expression analysis using Py-Tag in future work.

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Science and Technology (to S. T).

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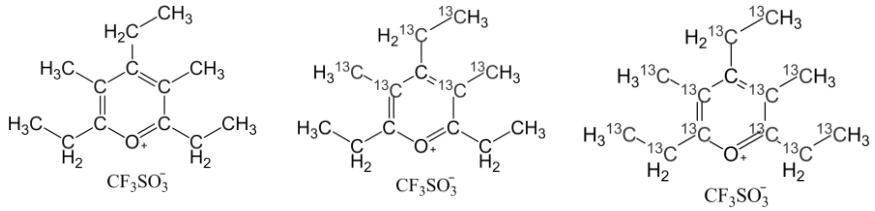
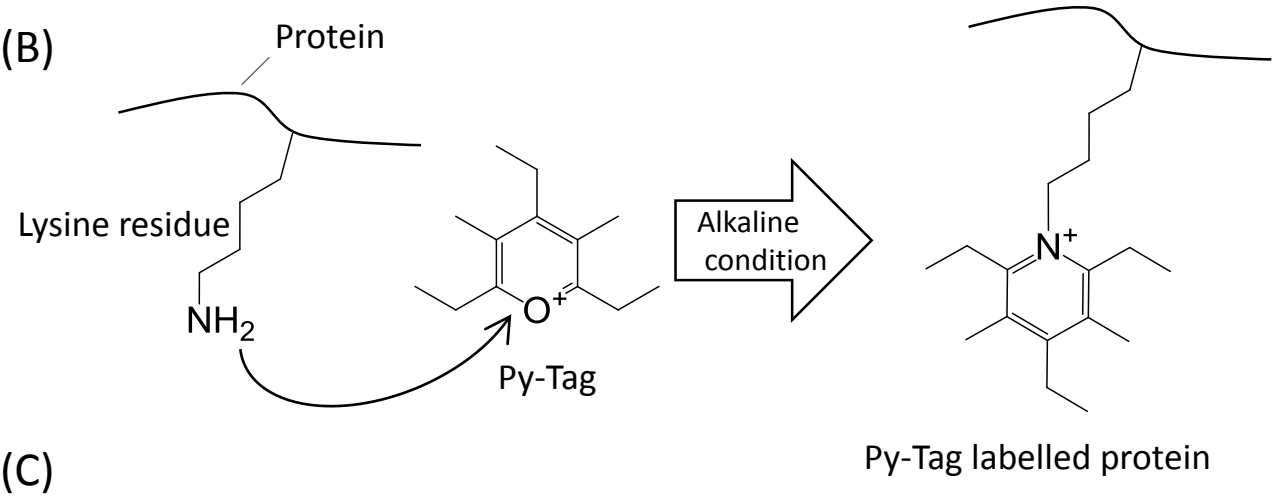
(A)



	Py0	Py6	Py12
Composition formula adducts	$C_{13}H_{19}$	$C_7{}^{13}C_6H_{19}$	$C_1{}^{13}C_{12}H_{19}$
Mw	175.15	181.17	187.19
Δ mass		6	6

(B)



(C)

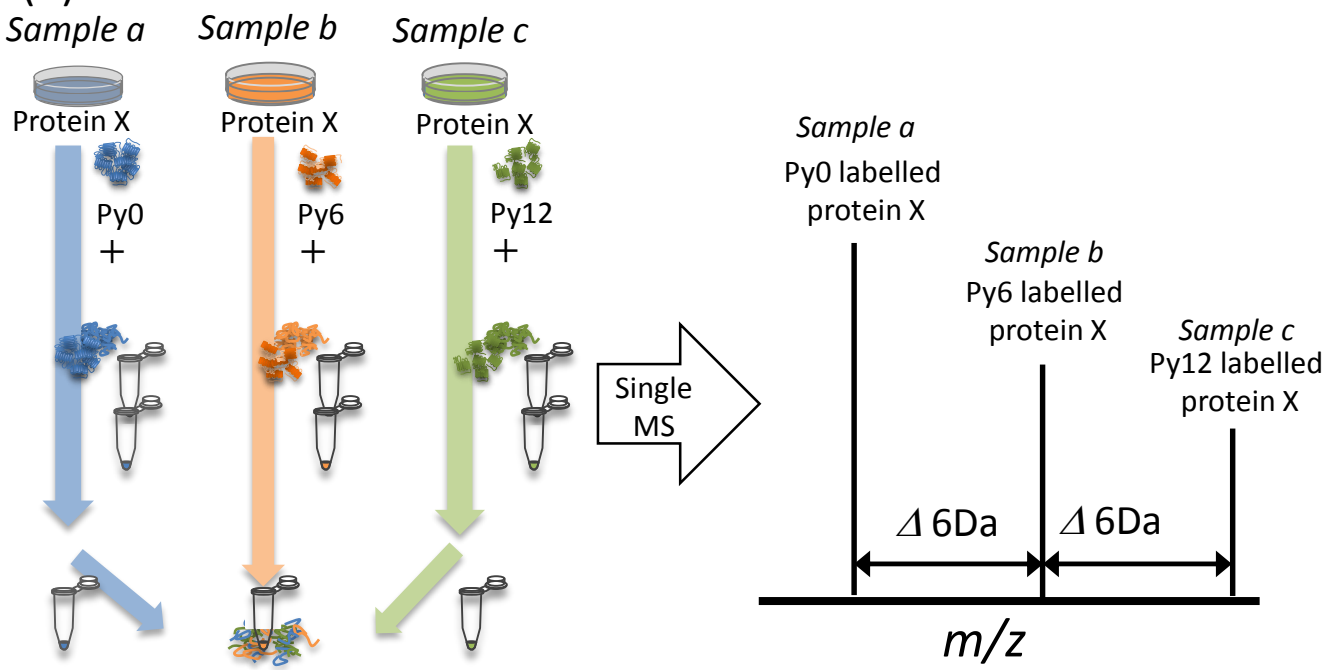


Figure 1 Structure and composition of Py0, 6 and 12 (A), Reaction principle behind Py-Tag labelling of protein (B), schematic illustration of the comparative proteomics approach using Py-Tag (C).

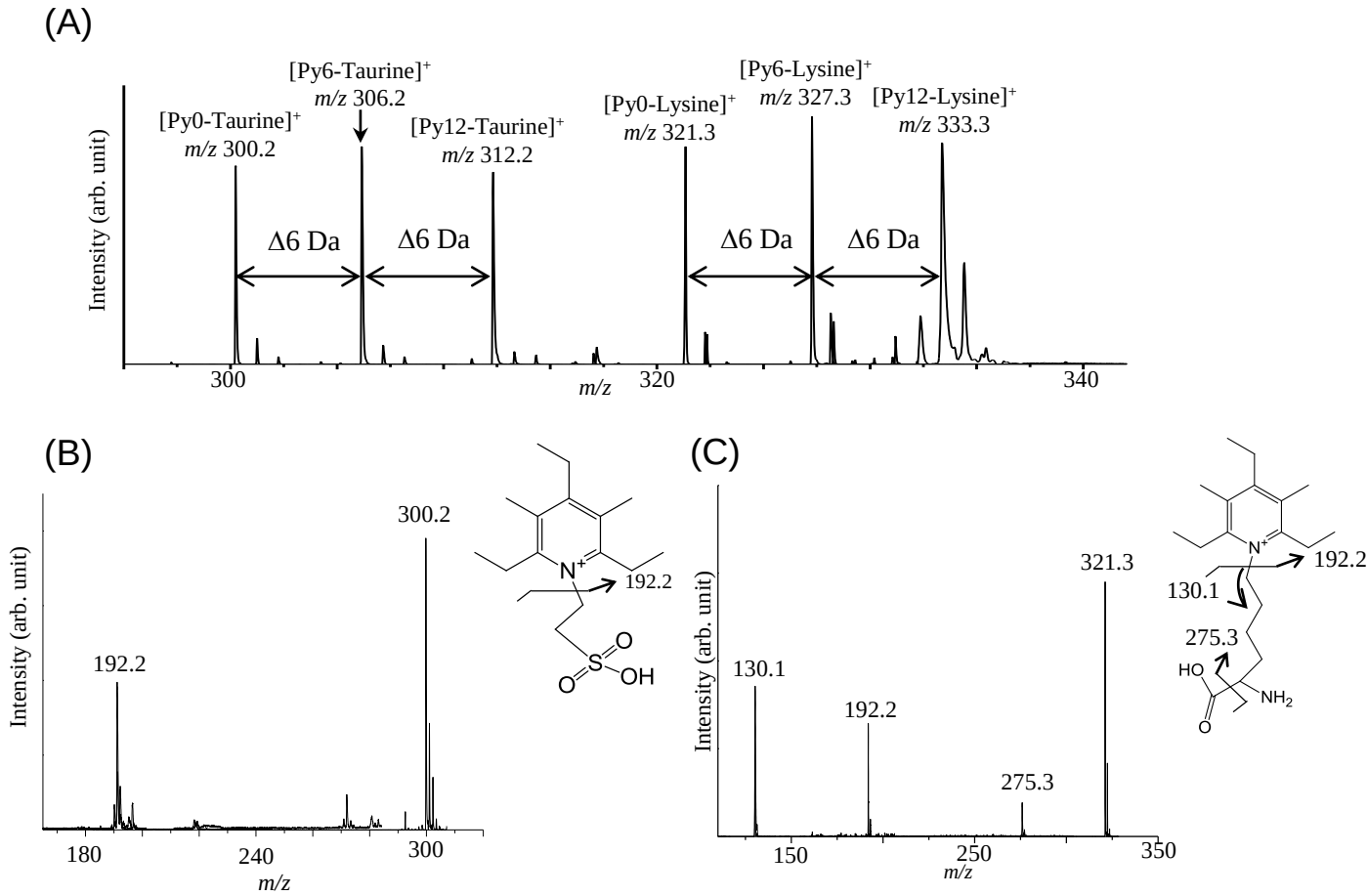


Figure 2
MS spectrum of Py-Tag labelled lysine and taurine (A). Tandem MS spectra of Py0-labeled taurine (B) and lysine (C).
MS spectrum of Py-Tag labelled peptides (D) and zoomed MS spectrum of the boxed region (E).
SP:Substance P

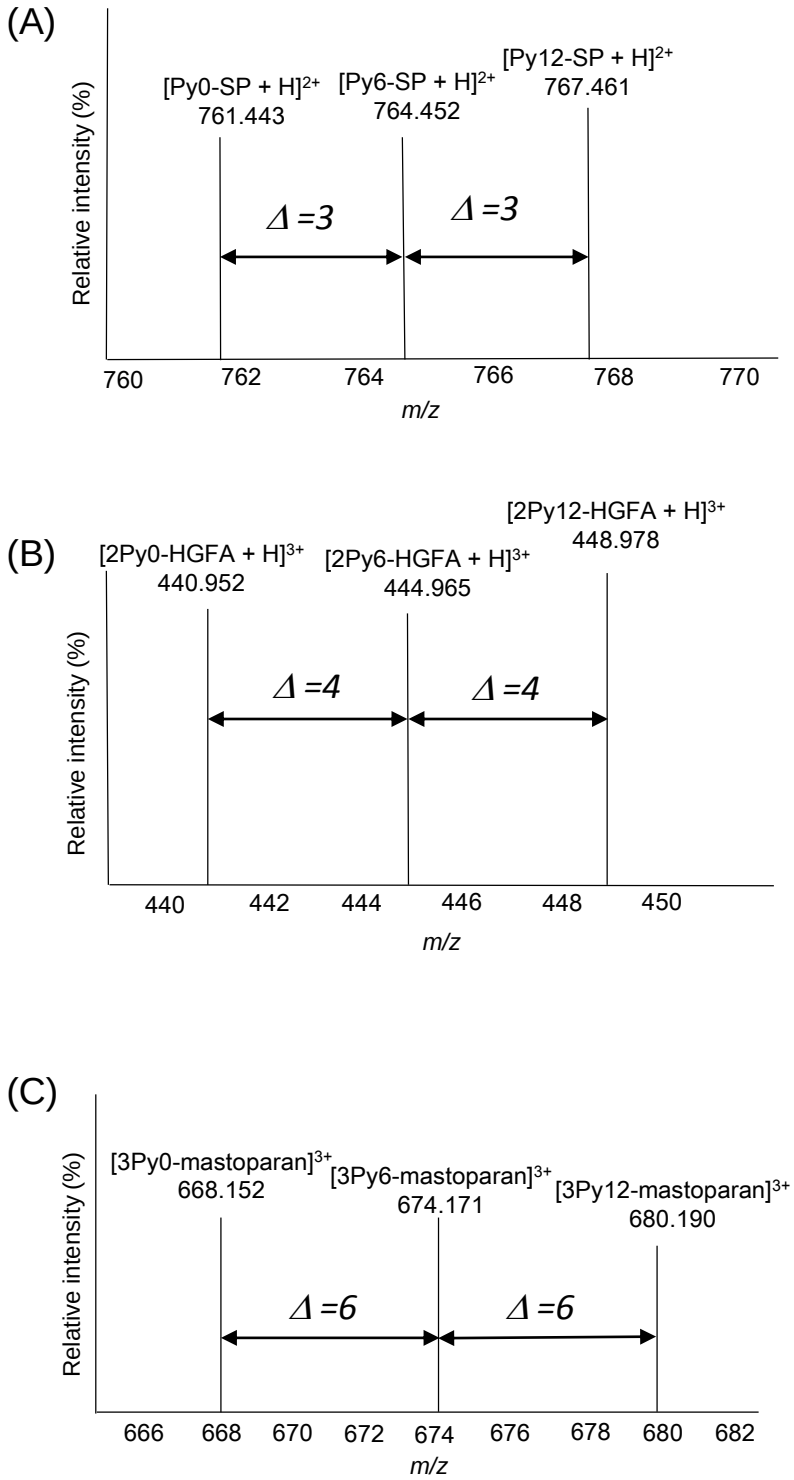


Figure 3
MS spectrum of Py-Tag labelled peptides; Substance P (A), hepatocyte growth factor activator (B) and mastoparan (C).

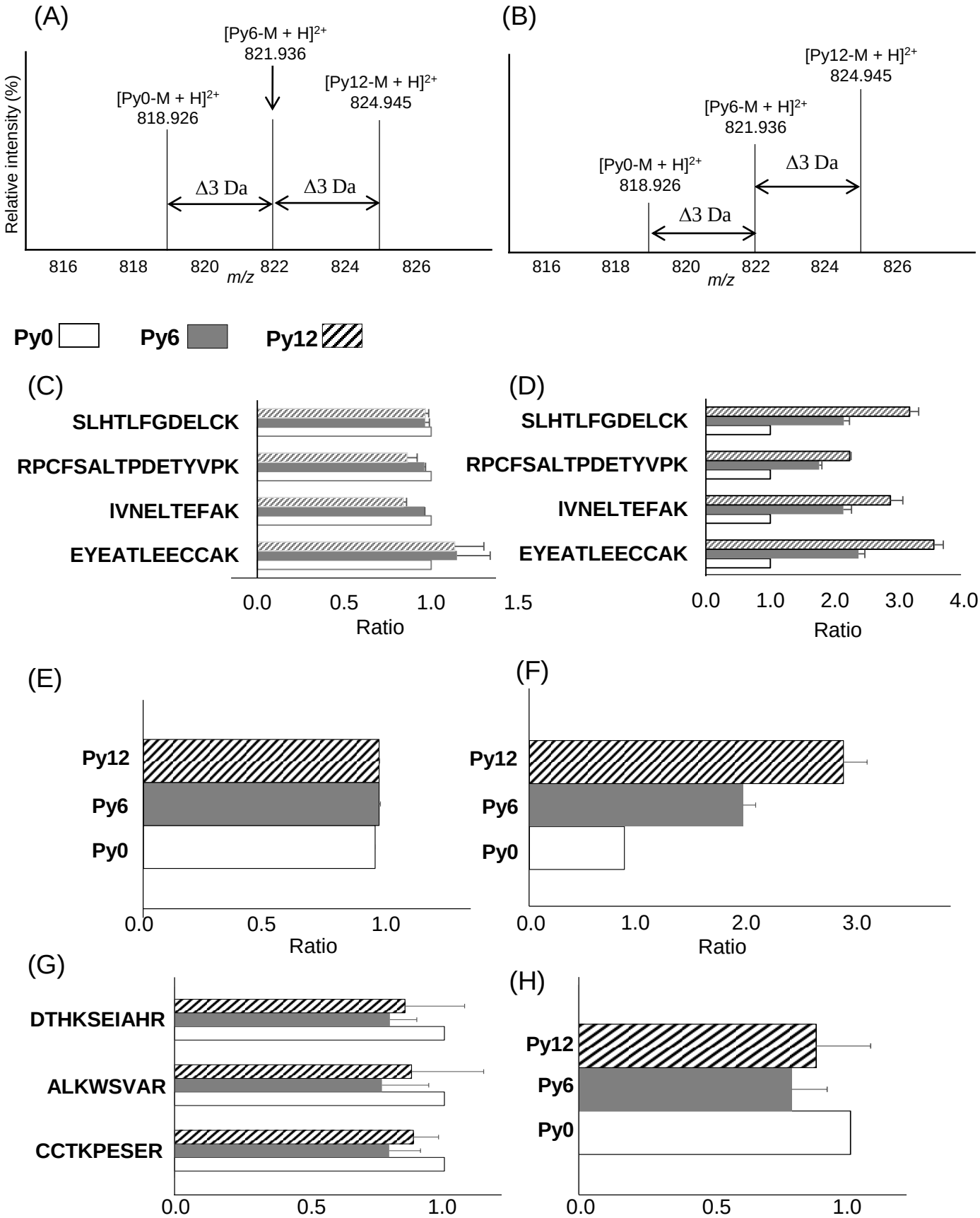


Figure 4

The Py-Tag labelled peptides from BSA; MS spectra of Py-Tag labelled SLHTLFGDELCK in a mix ratio of 1:1:1 (A) and 1:2:3 (B). Fragment peptides in a mix ratio of 1:1:1 (C) and 1:2:3 (D). The averaged value of all fragment peptides in a mix ratio of 1:1:1 (E) and 1:2:3 (F). Py-Tag labelled intact BSA in a mix ratio of 1:1:1; detected fragment peptides (G) and average value (H).

Supporting information

Concurrent mass spectrometric analysis of multiple samples using Py-Tag reagents

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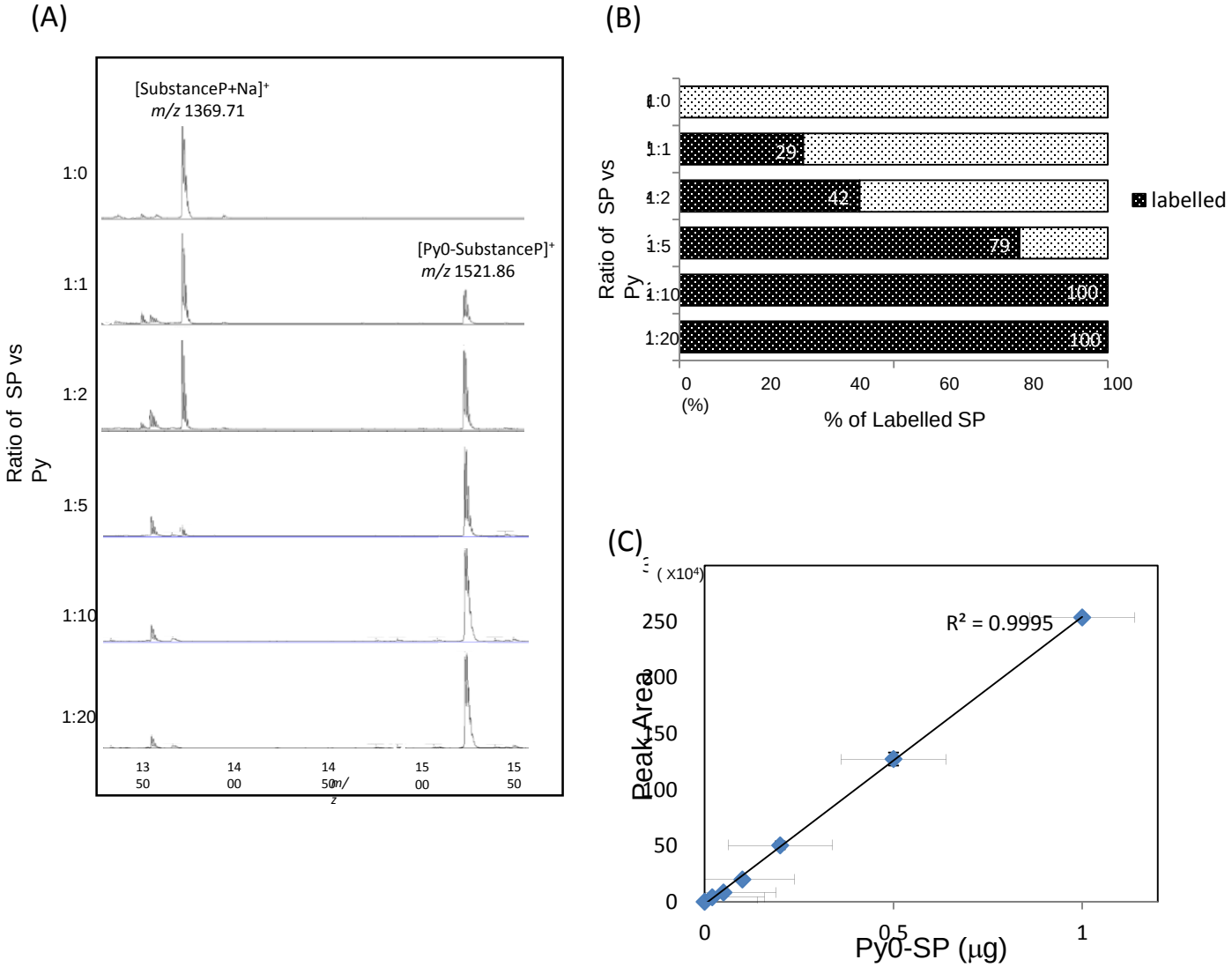
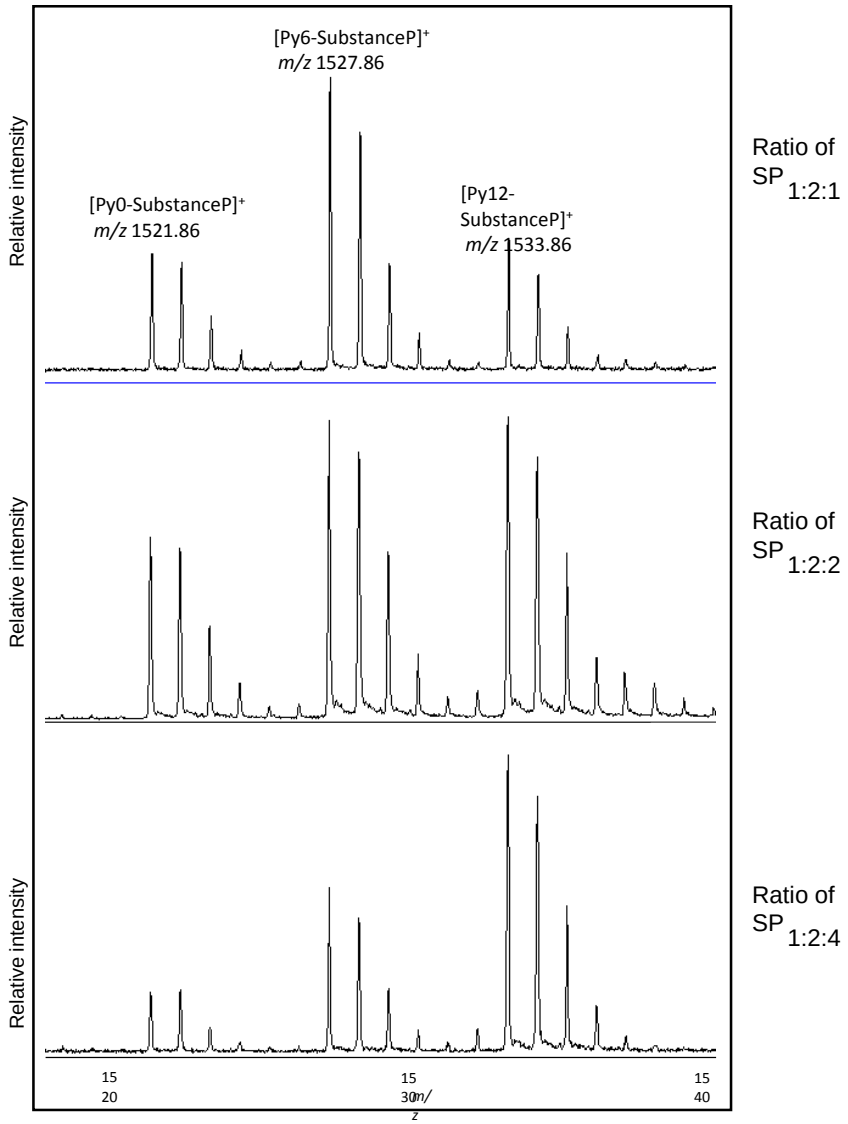
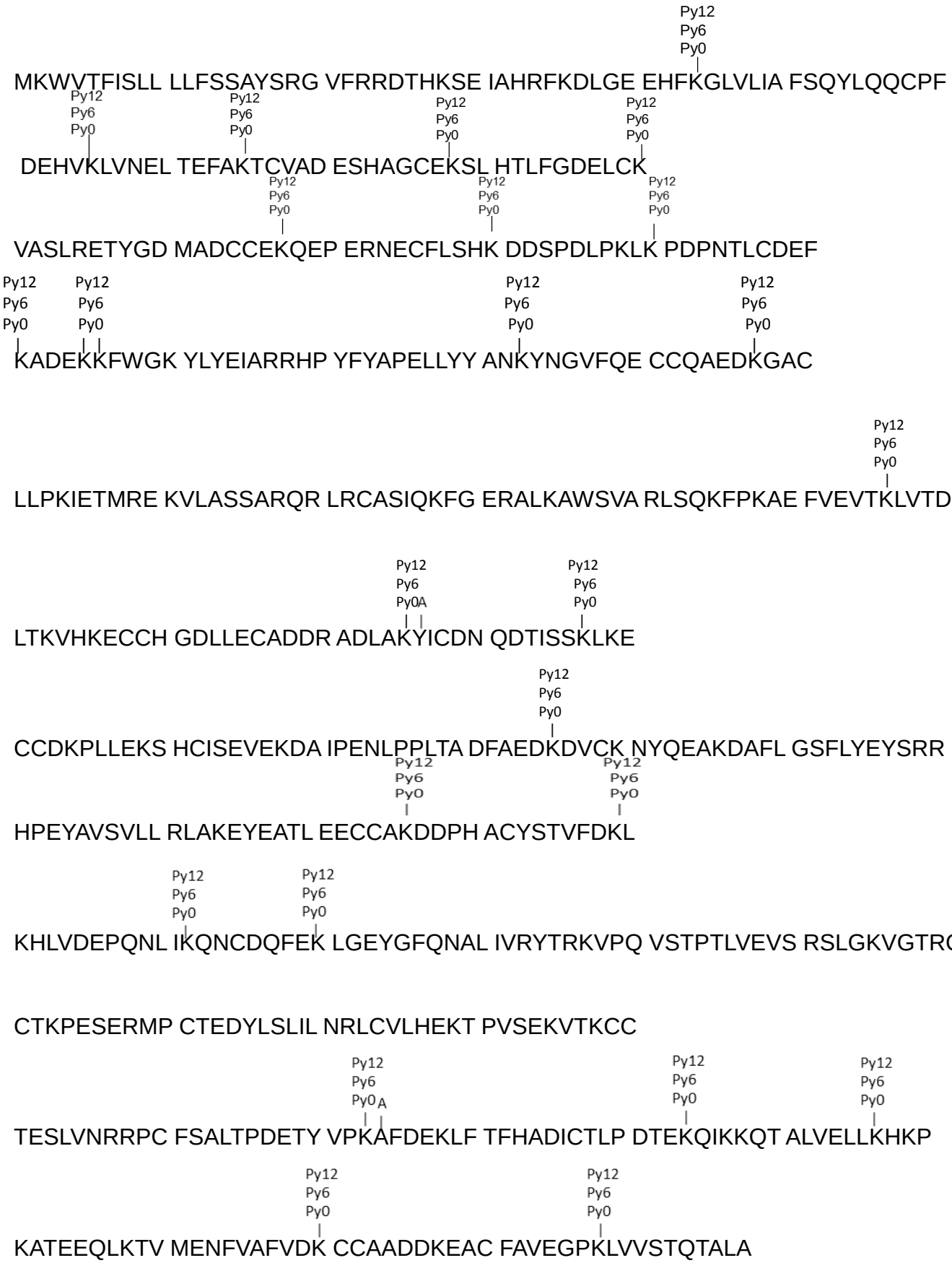


Supporting information 1

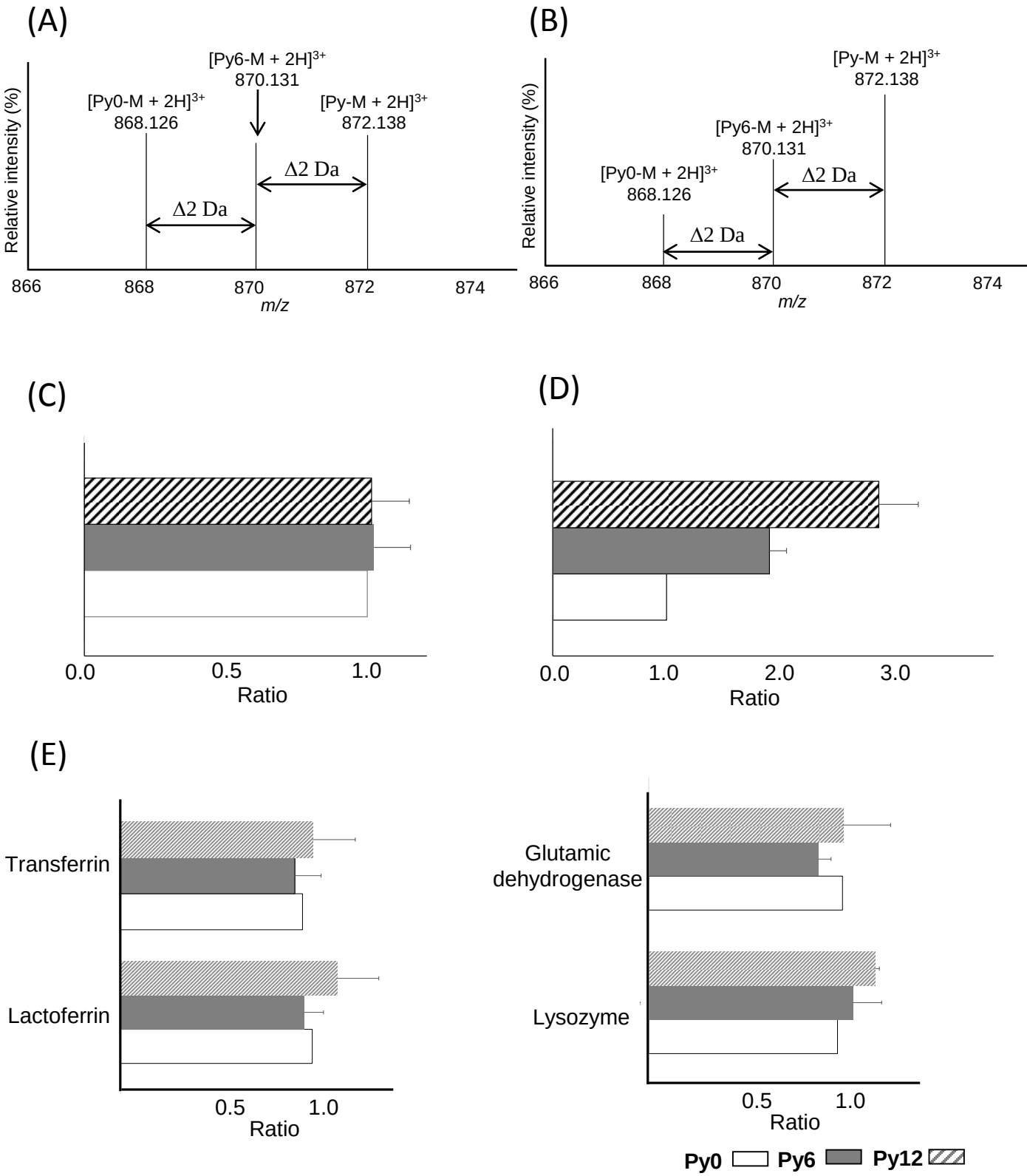
The labelling efficiency of Py-Tag and the linearity of the labelling for peptide. (A) MALDI-MS spectrum of Py0-labelled Substance P(SP) with indicated ratio. (B) Percentage of Py0-labelled SP with indicated ratio. (C) Quantitation of Py0-labelled SP by HPLC analysis.



Supporting information 2
Detection of various ratios of peptides.
The SP of abundance ratios 1:2:1, 1:2:2, 1:2:4 were labelled with Py0, 6 and 12, respectively, and then mixed, MALDI-MS analysis was performed.



Supporting information 3
The amino acid sequence of BSA and a Py-Tag labelled peptide.



Supporting information 4

The Py-Tag labelled peptides from GAPDH; MS spectra of Py-Tag labelled RVIISAPSADAPMFVMGVNHEK in a mix ratio of 1:1:1 (A) and 1:2:3 (B). The averaged value of all Py-Tag labelled fragment peptides in a mix ratio of 1:1:1 (C) and 1:2:3 (D).

Comparative analysis of various proteins by the averaged value of all Py-Tag labelled fragment peptides in a mix ratio of 1:1:1 (E).