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Optimization and quality assessment of the post-digestion ^{18}O labeling based on urea for protein denaturation by HPLC/ESI-TOF mass spectrometry

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ABSTRACT

The post-digestion ^{18}O labeling method decouples protein digestion and peptide labeling. This method allows labeling conditions to be optimized separately and increases labeling efficiency. A common method for protein denaturation in proteomics is the use of urea. Though some previous studies have used urea-based protein denaturation before post-digestion ^{18}O labeling, the optimal ^{18}O labeling conditions in this case have not been yet reported. Present study investigated the effects of urea concentration and pH on the labeling efficiency and obtained an optimized protocol. It was demonstrated that urea inhibited ^{18}O incorporation depending on concentration. However, a urea concentration between 1 and 2 M had minimal effects on labeling. It was also demonstrated that the use of FA to quench the digestion reaction severely affected the labeling efficiency. This study revealed the reason why previous studies gave different optimal pH for labeling. They neglect the effects of different digestion conditions on the labeling conditions. Excellent labeling quality was obtained at the optimized conditions using urea 1–2 M and pH 4.5, 98.4 ± 1.9% for a standard protein mixture and 97.2 ± 6.2% for a complex biological sample. For a 1:1 mixture analysis of the ^{16}O- and ^{18}O-labeled peptides from the same protein sample, the average abundance ratios reached 1.05 ± 0.31, demonstrating a good quantitation quality at the optimized conditions. This work will benefit other researchers who pair urea-based protein denaturation with a post-digestion ^{18}O labeling method.

1. Introduction

A number of stable isotope-labeling techniques have been developed for quantitative proteomics [1–4]. Compared to other techniques, ^{18}O labeling is simple and cost-effective [5–7]. In this method, proteolytic-cleaved peptides are labeled with either $H_2{}^{18}O$ or $H_2{}^{16}O$; one sample is labeled with two ^{18}O atoms, and the other sample is labeled with two ^{16}O atoms. After labeling, the samples are mixed at a 1:1 ratio (w/w) and then analyzed by high performance liquid chromatography/mass spectrometry (HPLC/MS). Due to the mass difference of 4 Da induced by ^{18}O labeling and none difference in chromatographic retention time [8], the labeled and unlabeled peptides can be distinguished by mass Spectrometry and their relative ratios can be calculated by comparing peak intensities. The ratios of the ^{16}O- and ^{18}O-labeled peptides reflect the relative level of protein expression in the two samples.

A major problem to the ^{18}O labeling method is the variable incorporation of ^{18}O atoms, reducing the accuracy of quantitation. Differences in labeling efficiency can result from a difference in peptide sequence and structure. For example, generally peptides that have a C-terminal arginine residue are more easily labeled than peptides with a C-terminal lysine [9]. Labeling variability results in erroneous calculations of the $^{16}O/^{18}O$ ratio.

To increase the accuracy of quantitation, labeling conditions have been investigated and optimized by previous studies. In the earlier studies, proteins were digested directly in $H_2{}^{18}O$, by which the digestion and labeling are achieved simultaneously. But this method did not result in a high $^{18}O_2$-labeling efficiency. After this, it was found that the labeling efficiency is higher at a weakly acidic condition [10–12], whereas an alkaline pH is necessary for tryptic digestion. Therefore, the method decoupling peptide labeling from protein digestion was set up [9], which has been now the main method for ^{18}O labeling. In this method, protein was first digested at a pH of 8–9 in ^{16}O water followed by peptide labeling in acidic conditions using ^{18}O water. The two-step procedure has an advantage of optimizing labeling conditions without affecting digesting conditions.

However, different digestion conditions may affect following labeling conditions. In the digestion step, denaturing proteins is helpful to for digestion. Urea is a commonly used denaturation agent, and often as a chaotropic agent coupled with thiourea in

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cell lysis buffer to extract more proteins, because it can effectively disrupt protein secondary structure by destabilizing internal, non-covalent bonds like hydrogen bonds [13,14]. Previous studies optimized post-digestion labeling conditions, either using boiling for protein denaturation or digesting proteins directly in the NH_4HCO_3 buffer [10–12]. Boiling can result in more protein loss and direct digestion is not suitable for biological complex samples since the secondary protein structure remains and leads low labeling efficiency. Moreover, they drew different conclusions on the optimal labeling pH. Though some researchers have ever used urea to denature proteins for post-digestion ^{18}O labeling [15,16], the optimized conditions for ^{18}O labeling in this case have not been yet reported. In theory, use of urea can affect post-digestion labeling in two ways. One is that urea can inhibit ^{18}O incorporation directly, because ^{18}O labeling is based on an enzyme-catalyzed reaction [17]; The other way, due to the use of 8 M urea, huge amounts of NH_4HCO_3 buffer was added to dilute the urea concentration to 1 M before digestion, possibly affecting the pH of the labeling step. Hence, it is necessary to investigate and optimize a urea-based post-digestion labeling protocol.

2. Experimental

2.1. Materials

Sequencing-grade modified trypsin was purchased from Promega (USA); proteinase inhibitor was purchased from Roche (Switzerland). Iodoacetamide and $H_2{}^{18}O$ (97%) were purchased from the China Isotope Company (China). HPLC-grade acetonitrile and formic acid were purchased from Fisher Scientific (USA), Ammonium bicarbonate and KH_2PO_4 were purchased from Beijing Chemical Company (China). All other chemicals and standard proteins were purchased from Sigma (USA). Water was prepared by a Milli-Q system (Millipore, USA). Reverse phase liquid chromatography column (C_{18}, 2.1 mm × 150 mm, 300 Å) was purchased from Grace Vydac (USA).

2.2. Protein digestion and Peptide ^{18}O labeling

A 500 μg protein mixture of six standard proteins (horse myoglobin, human transferrin BSA, cytochrome *c*, bovine hemoglobin and chicken ovalbumin; 100 μg each) was prepared. The mixture was denatured and reduced by a solution containing 8 M urea, 10 mM DTT and 50 mM NH_4HCO_3 at 37 °C for 4 h. Alkylation was performed in a 50 mM iodoacetamide solution at room temperature for 1 h in the dark. After alkylation, it was diluted using a 50 mM NH_4HCO_3 buffer to give a final urea concentration of 1 M. Tryptic digestion was then performed at a concentration ratio of 50:1 (total protein:trypsin, w/w). The peptide digest was divided into multiple 40 μg fractions. These fractions were lyophilized to complete dryness and then used for the labeling reaction.

For ^{18}O labeling, peptide fractions were first dissolved in a KH_2PO_4–K_2HPO_4 buffer to adjust to an acidic pH. This step was followed by a second lyophilization to complete dryness. Finally, the peptides were dissolved in ^{18}O water supplemented with 0.8 μg trypsin. After labeling at 37 °C for 20 h, residual trypsin activity was quenched by boiling for 10 min and the addition of 3% FA (v/v). The fractions were then centrifuged at 17,000 × *g* for 15 min before HPLC-ESI/TOF analysis. To investigate the effect of urea concentration on labeling quality, various urea concentrations (1, 1.5, 2, 4 and 8 M) were used in the labeling step with an acidic 100 mM KH_2PO_4 buffer. In addition, the effect of buffer pH on the labeling quality was tested with a 50, 100 and 150 mM KH_2PO_4 buffer (pH 4.45, 4.50 and 4.55, respectively) and a 100 mM KH_2PO_4–K_2HPO_4 buffer at pH 5.0, 5.5, 6.0 or 6.5. To investigate the effects of FA addition on labeling quality, 1% FA was added to one fraction to stop the digestion before ^{18}O labeling.

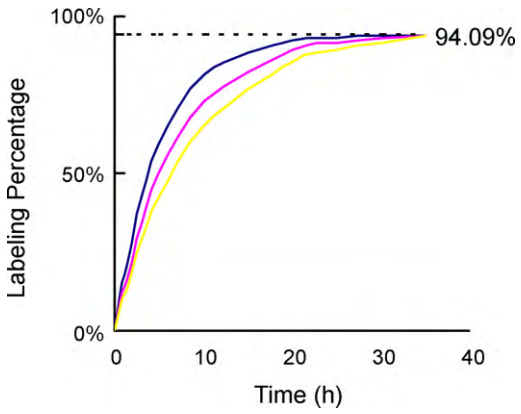

Fig. 1. The theoretical relation between the labeling percentage and the duration of the labeling reaction in the ^{18}O water (97% purity). When a labeling balance was achieved, the labeling percentage reached the theoretically largest value (94.09%).

Two fractions were labeled in ^{16}O water and ^{18}O water at the optimized conditions that urea concentration was 2 M and KH_2PO_4 concentration was 50 mM, and then mixed 1:1 (w/w) for analysis. Fifty micrograms of rat hippocampus protein extracted by a cell lysis buffer (7 M urea, 2 M thiocarbamate, 4% CHAPS, 1% DTT, 1 mM EDTA, 40 mM Tris, 40 μL/mL protease inhibitor cocktail) was digested as described above and then ^{18}O-labeled using the optimized labeling conditions.

2.3. HPLC\ESI-TOF mass spectrometry

Samples were analyzed on an Agilent 1100 series HPLC system coupled to an Agilent ESI-TOF MS (6210) with a Vydac C_{18} column

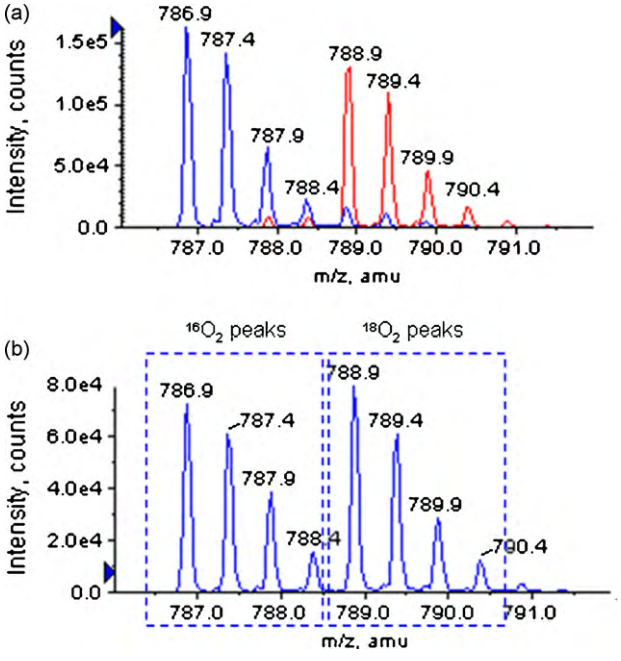

Fig. 2. An example of a peptide (*m*/*z* 786.9) spectrograph in mass spectrums. (a) The mass spectrum of the 1:1 mixture of the labeled and unlabeled peptide. (b) Overlapping mass spectrums of the labeled and unlabeled peptide. The red peaks indicate the labeled peptide and the blue peaks indicate the unlabeled peptide. At the left of the $^{18}O_2$-labeled red peaks there are two relatively small red peaks, which are $^{18}O_1$-labeled peaks.

(300 Å, 2.1 mm × 150 mm) at a flow rate of 0.2 mL/min. The injection amount was 20 μg. Gradient elution of the peptide samples was achieved with buffer A (0.1% FA in H_2O) and buffer B (0.1% FA in acetonitrile). The gradient program consisted of: 3% buffer B from 0 to 8 min, 3–40% buffer B from 8 to 68 min, 40–97% buffer B from 68 to 78 min, 97% buffer B from 78 to 80 min and 97–3% buffer B from 80 to 85 min. Post time was 10 min. ESI-TOF was performed under dry gas (10 L/min) conditions at a temperature of 350 °C. The nebulizer pressure was 35 psi, and the capillary voltage was 3500 V. The MS mass window was 300–1800 amu.

2.4. Data analysis

Molecular feature extracting software (MFE; Agilent) and the ^{18}O labeling quantitation software, Peakpair, which was developed in our lab, were used for data analysis. Due to variable incorporation of ^{18}O atoms into peptides, there were three kinds of peptides in the sample after ^{18}O labeling: $^{16}O_2$-labeled peptides, $^{18}O_1$-labeled peptides and $^{18}O_2$-labeled peptides. The peptide labeling percentage is equivalent to the percentage of the amount of $^{18}O_2$-labeled peptide divided by the total amount of peptide. The labeling percentage is

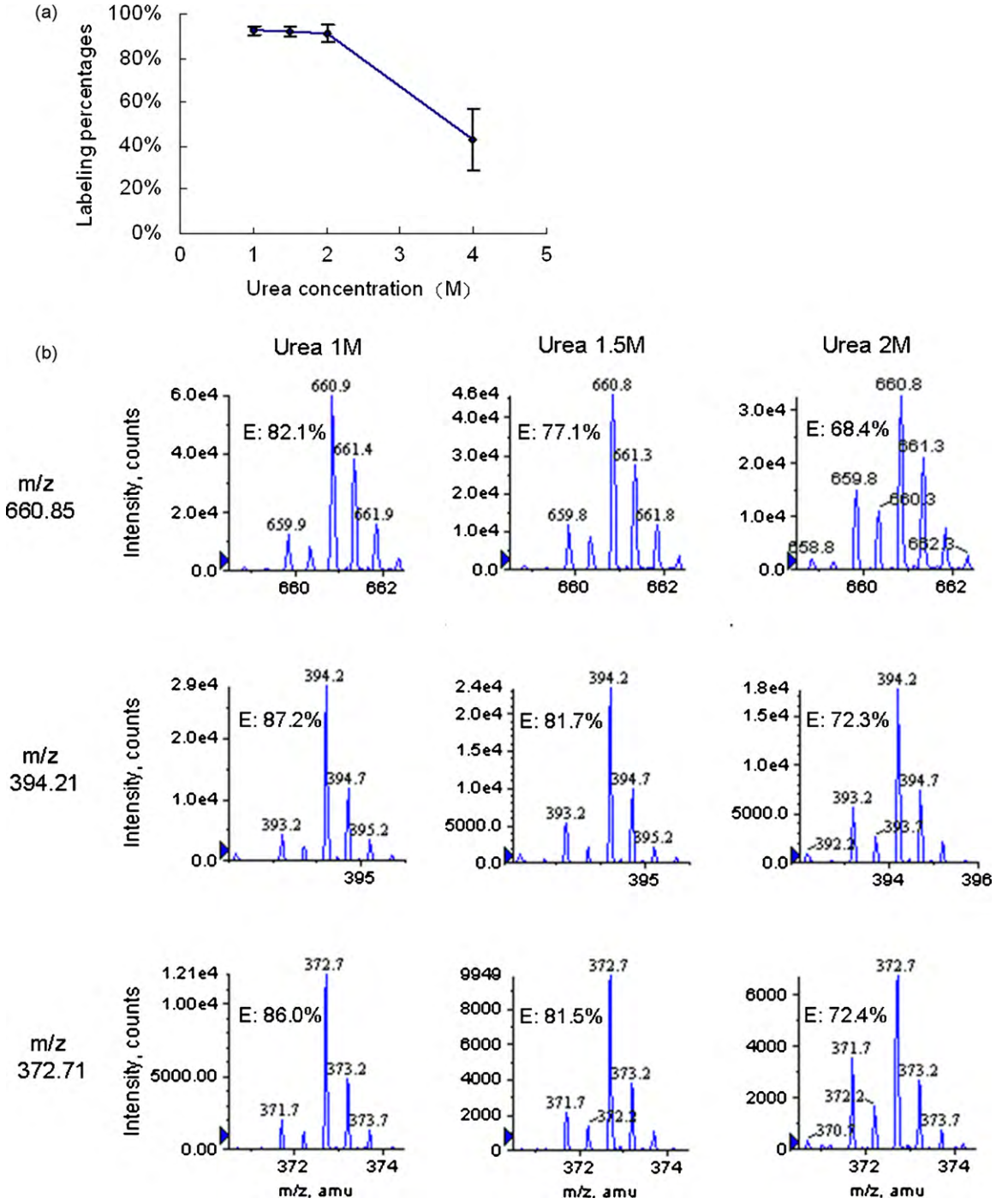

Fig. 3. The 135 most abundant peptides were chosen to investigate the labeling percentage at different urea concentration. (a) Effects of different urea concentration on the average and standard deviation of the peptide labeling percentage. The standard deviation indicates the labeling percentage variability. (b) The mass spectrums of three of the eight peptides at different urea concentration. "*E*" represents the labeling percentage.

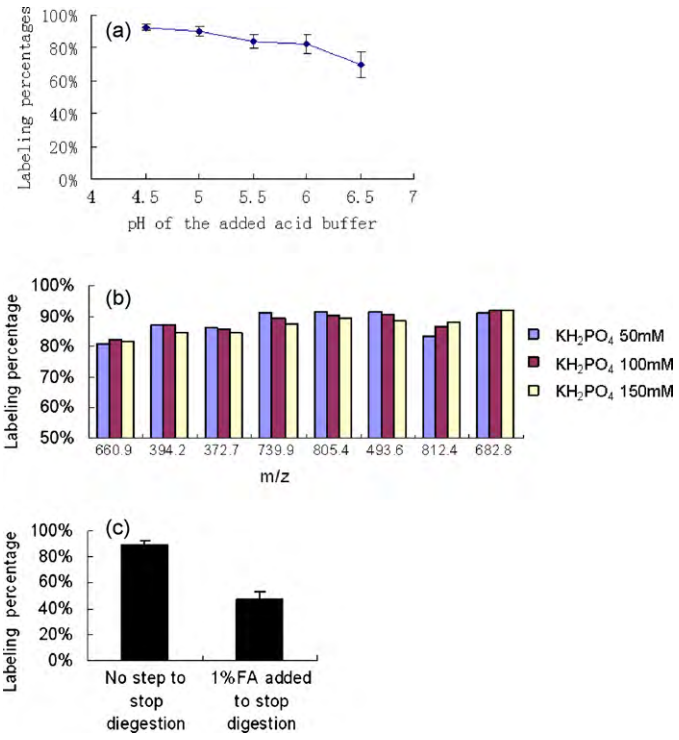

Fig. 4. The 135 most abundant peptides were chosen to investigate the effect of buffer pH on the labeling percentage. (a) Effects of buffer pH on the average labeling percentage. (b) Effects of KH_2PO_4 concentration on the labeling percentage of the eight peptides with the lowest labeling percentage. (c) FA (1%), which was added to quench the digestion reaction, reduced the labeling percentage.

calculated using the following equation:

$$E = \frac{I_2}{I_0 + I_1 + I_2}$$

$$\text{Relative } E = \frac{E}{E_0}$$

E: labeling percentage; E_0: the theoretical largest labeling percentage when labeling balance is reached; I_0: the peak intensity of monoisotopic peak of $^{16}O_2$-labeled peptide; I_1: the peak intensity of monoisotopic peak of $^{18}O_1$-labeled peptide; I_2: the peak intensity of monoisotopic peak of $^{18}O_2$-labeled peptide.

The relative labeling percentage of two ^{18}O atoms was defined as labeling efficiency. To determine the accuracy of quantitation, the observed monoisotopic peak intensity of three types of labeled peptides should be corrected by eliminating the isotopic interferences derived from isotope overlap. However, in actual calculations, the complex calculation method for eliminating isotopic interferences was not used, and the peak intensity of the monoisotopic peak of $^{16}O_2$-labeled peptide was skipped, because the peak intensity of $^{16}O_2$-labeled peptides and $^{18}O_1$-labeled peptides were relatively very small compared to the peak intensity of $^{18}O_2$-labeled peptide, especially the peak intensity of the $^{16}O_2$-labeled peptide, which was not observed for most peptides.

3. Results and discussion

Enzymatic catalysis is selective and the reaction intensity depends on the structure of both the enzyme and its substrate. Urea, a denaturant, can alter protein structure so that it can inhibit the ^{18}O labeling activity of trypsin. And different kinds of peptides may have different labeling activity due to their special sequences and structures. Theoretically, the highest labeling percentage occurs

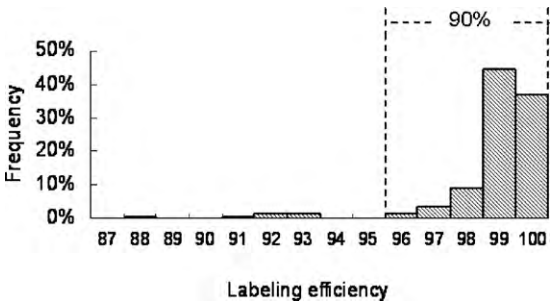

Fig. 5. The distribution of labeling efficiency obtained using 1 M urea and 100 mM KH_2PO_4 after a 20 h labeling reaction. The 135 most abundant peptides were chosen to investigate the distribution.

when the labeling balance is reached, and it depends only on the purity of the ^{18}O water. Labeling balance is a state at which the labeling reaction reaches equilibrium and obtains a highest labeling percentage. It can be illustrated in Fig. 1. For ^{18}O water with a purity of 97%, the theoretical labeling percentage is: $97\% \times 97\% = 94.09\%$. The time needed to reach the highest labeling percentage depends on the labeling efficiency. Higher labeling efficiency means less time needed to obtain a high labeling percentage. The effect of different labeling conditions on the labeling efficiency can be reflected by comparing the labeling percentage before a labeling balance is reached. When the majority of peptides do not reach the labeling balance, the labeling percentage will vary and reduce the accuracy and reliability of quantitative proteomics based on ^{18}O labeling. High quality labeling is the basis of the accurate and reliable quantitation. $^{18}O_2$ labeling percentage and its variation among different peptides are two key indexes for the assessment of labeling quality. Fig. 2 shows an example of a peptide (m/z 786.9). As shown in Fig. 2(a), the isotopic peaks of the labeled and unlabeled peptides overlapped, and the labeled peptides were not completely labeled two ^{18}O atoms, indicating that the accuracy relative quantitation will be interfered when the labeled and unlabeled peptides are 1:1 mixed for analysis. Fig. 2(b) shows the mass spectrum of this peptide from the 1:1 mixture analysis. Its expected ratio was 1, while its calculated relative ratio was 0.91.

3.1. Effects of urea concentration on labeling quality

Fig. 3(a) measures the effects of urea concentration during the labeling reaction on the average and standard deviation of the peptide labeling percentage. The standard deviation reflects the amount of variability in the labeling percentage. In the MS analysis, the peaks with highest intensity, in general, are more accurately quantified due to the relatively low interference from background and other peaks. Therefore, the 135 most abundant peptides, which were about 10% of the total, were chosen for analysis. The comparisons of labeling percentage suggested that urea dose-dependently inhibited ^{18}O labeling. The standard deviation increased with urea concentration, indicating that low labeling percentages have greater variability.

A urea concentration of 4 M had obvious effects on the average and standard deviation of the labeling percentage; however, at a urea concentration of 1–2 M, a minimal effect was observed, indicating that most of the peptides reached a labeling balance at a urea concentration below 2 M. The peptides reaching a labeling balance cannot be used to reflect the effects of urea concentration on the labeling quality, but the peptides that did not reach a labeling balance can. The average labeling percentages of the eight peptides that had the lowest labeling percentage in the 135 most abundant peptides were $88.1 \pm 3.2\%$, $84 \pm 4\%$, $77.5 \pm 6\%$ at the urea concentration of 1, 1.5 and 2 M, respectively. All the eight pep-

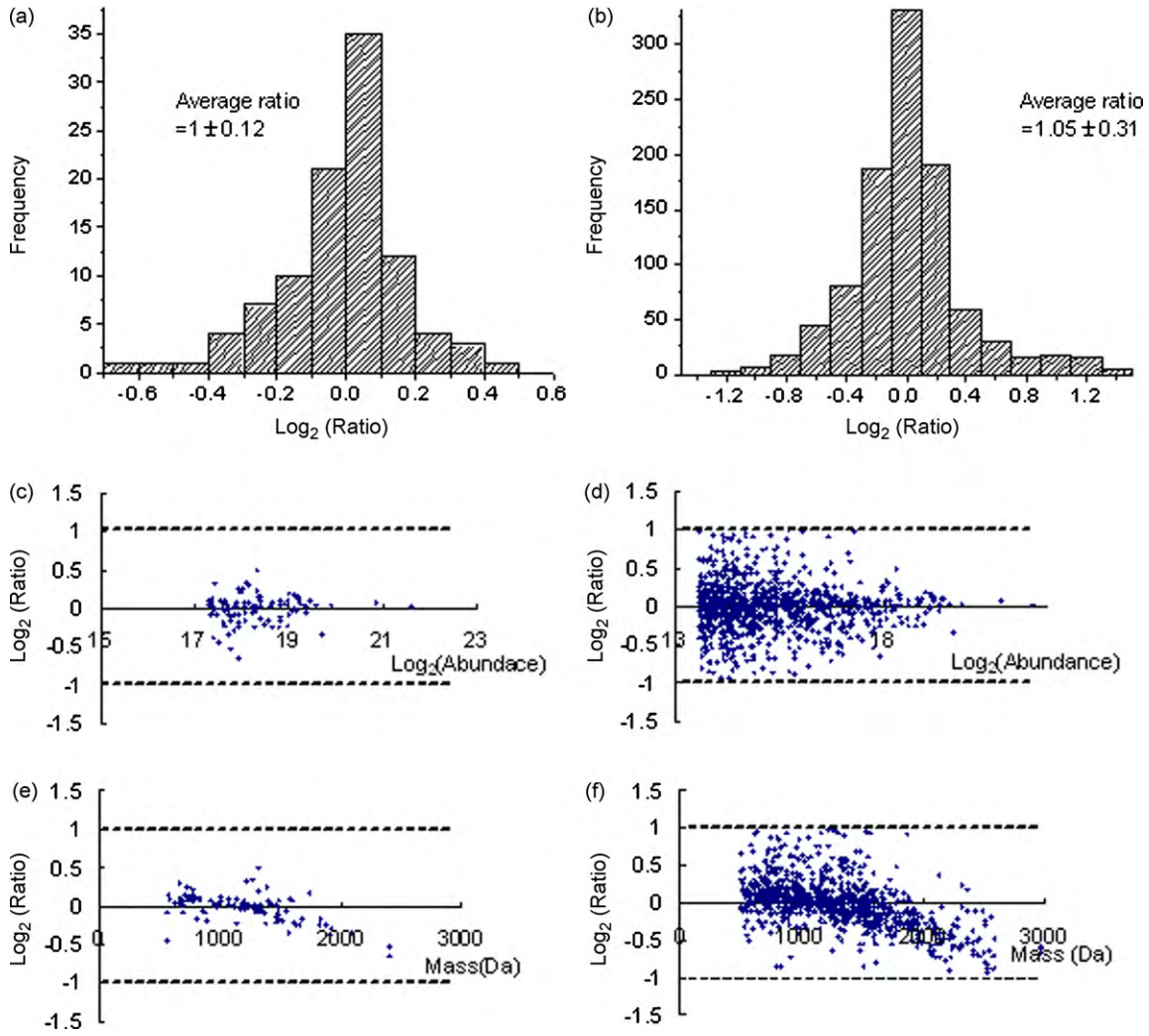

Fig. 6. The distribution of the peptide Log_2 (Ratio) values ($^{16}O/^{18}O$) in the $^{16}O/^{18}O$ 1:1 mixture analysis of two same samples. (a), (c) and (e) use the 100 most abundant peptides for analysis, while (b), (d) and (f) use the 1000 most abundant peptides for analysis.

tides displayed a tendency that the labeling percentages decreased as the urea concentration increased from 1 to 2 M, indicating an obvious dose-dependent inhibition of urea on labeling percentage. The mass spectrums of three of the eight peptides are shown in Fig. 3(b). For all the three peptides, the relative intensity of the single ^{18}O atom-labeled peaks to the double ^{18}O atom-labeled peaks was increased with the urea concentration from 1 to 2 M, indicating the labeling efficiency was affected by urea. However, it was just a small amount of the 135 peptides that were affected by a urea concentration of 2 M. These data suggest that the optimal urea concentration for labeling is below 2 M for a 20-h reaction. Even for a urea concentration of 2 M, the standard deviation of the labeling percentage was only 4%, suggesting minimal viability among different peptides. When labeling at 2 M urea, the amount of ^{18}O water can be reduced by one-half, reducing the cost.

3.2. The optimal pH of the acidic buffer for labeling

Fig. 4(a) shows the effects of the pH value of the acidic buffer on the labeling quality. The labeling quality gradually decreased with an increase in pH of the 0.1 M KH_2PO_4–K_2HPO_4 buffers from 4.5 to 6.5. And a range of KH_2PO_4 concentrations from 50 to 150 mM had no obvious effect on the labeling quality, even for the eight peptides with the lowest labeling percentage (Fig. 4(b)). These data suggest that the optimal pH for labeling was maintained using a KH_2PO_4 buffer of 50–150 mM at pH 4.5. Fig. 4(c) showed that the use of the FA to quench the digestion reaction severely affected the labeling efficiency, suggesting that the FA was not removed completely during lyophilization and thereby inhibited the labeling reaction by decreasing the pH. Hajkova et al. [12] stopped the digestion reaction by adding formic acid (FA) to a final concentration of 1% and found an optimal pH of 6.0 for trypsin-based ^{18}O labeling. Hence, this optimal pH achieved in their study was not the actual pH of the labeling reaction, but just the pH of the acidic buffer used to adjust pH for labeling. Lyophilization does not remove the NH_4HCO_3 that was added during digestion, which can also affect the pH of the labeling reaction, depending on the amount of the addition. These should be the reasons why previous studies obtained different optimal pH for the labeling reaction. Changing the conditions of the digestion reaction can affect the labeling efficiency, so the optimal labeling conditions reported in previous studies may not suit for the case when digestion conditions were changed.

3.3. Quality assessment of the post-^{18}O labeling at the optimized labeling conditions

When the labeling reaction was performed at 1 M urea and 100 mM KH_2PO_4 for 20 h, the mean labeling efficiency of the 135 most abundant peptides reached 98.4 ± 1.9%. As shown in Fig. 5, the labeling efficiency varied from 88 to 100%, but most of them

distributed larger than 98% and up to 90% of the peptides obtained a labeling efficiency larger than 95%, demonstrating better labeling quality was achieved compared with previous studies that used urea for protein denaturation [15,16]. Variable ^{18}O atom incorporation decreased the accuracy of relative quantitation since the $^{16}O/^{18}O$ ratio is calculated based on the double ^{18}O labeling. At the optimal conditions the labeling efficiency almost reached the ceiling and differed slightly from one another, indicating that an average labeling efficiency can be used for the correction of the incomplete labeling.

Qian et al. [18] analyzed the 1:1 mixture of ^{16}O- and ^{18}O-labeled peptides from a blood plasma protein sample without use of urea for protein denaturation, and obtained a mean ratio of 891 peptides 1.02 ± 0.23. Fig. 6 shows the analysis results of the 1:1 mixture of the ^{16}O- and ^{18}O-labeled peptides from the same protein sample in this study. The $^{16}O/^{18}O$ ratios of the 100 and 1000 most abundant peptides were analyzed individually. The mean ratio for the 100 most abundant peptides was only 0.996 ± 0.11, and 1.05 ± 0.31 for up to 1000 peptides. The mean ratio is closely around 1, indicating that the accuracy satisfies the requirements of the relative quantitation, and the optimized protocol is reliable for comparative proteomics.

The Log_2(ratio) values of the 100 most abundant peptides in Fig. 6(a) and 1000 in Fig. 6(b) are all around 0, but the latter distributed more widely than the former. The ratios of 90% of the 100 most abundant peptides vary from 0.8 to 1.2. No peptides have a ratio value out of the range of 0.5–2. In addition, 90% of the ratios of the 1000 most abundant peptides vary from 0.6 to 1.4. Only 0.3% of these peptides have a ratio value out of the range of 0.5–2. If ratios out of the range of 0.5–2 are judged as differential peptides, only a very small portion of the peptides will be falsely quantified, indicating that a very good quantitation quality was achieved at the optimized labeling conditions.

Fig. 6(c) and (d) shows that the distribution of the Log_2(ratio) values fluctuated more with the decrease in peptide intensity, which illustrates, to some degree, that the peak intensity has an influence on the accuracy of quantitation. As mentioned above, the peaks with larger intensities are more easily and accurately quantified in the MS analysis due to a relatively small interference from background peaks. Manual analysis found that the ratios whose Log_2 value was out of the range of -1 to 1 did not reflect the actual values, and the errors were mainly induced by the software processing. Therefore, in order to improve peak extraction and quantification in the data analysis, the software should be further improved.

Fig. 6(e) and (f) shows that the distribution of the peptide Log_2(ratio) values displayed a profile that they trended to decrease with the increase in molecular mass, indicating that the interferences of the isotopic peaks from the $^{16}O_2$- and $^{18}O_1$-labeled peptides on the isotopic peaks from the $^{18}O_2$-labeled peptides increased with the increase in molecular mass. Therefore, it is necessary to eliminate isotopic interferences by calculation, especially for the peptides with a large mass, to increase the accuracy of quantification. Some previous studies have made useful work in this regard [16,19,20].

3.4. Labeling quality of the complex biological sample at the optimized labeling conditions

We tested the optimized ^{18}O labeling method on highly complex biological samples. The peptide digest from rat hippocampi proteins were labeled for 20 h at the conditions that urea concentration was 2 M and KH_2PO_4 concentration was 100 mM. The average labeling efficiency of the 100 most abundant peptides reached $97.2 \pm 6.2\%$. As can be seen in Fig. 7, the labeling efficiency of most of them distributed in the range of 99–101% and up to 84% of the peptides obtained a labeling efficiency larger than 95%, suggesting that a high level of labeling quantity was achieved for highly complex biological samples. For a minimal part of peptides, their labeling efficiency was measured larger than 100%, due to errors introduced by the software analysis.

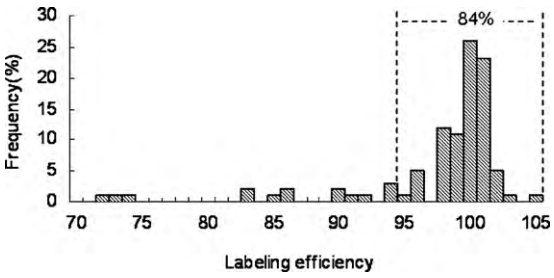

Fig. 7. The labeling efficiency distribution of 100 most abundant peptides in the analysis of a rat hippocampus protein sample.

4. Conclusions

Urea is extensively used in proteomics to denature proteins for proteolytic digestion. Our study demonstrated that the use of urea in the digestion adversely affected post-digesting ^{18}O labeling depending on concentration. However, a urea concentration between 1 and 2 M had minimal effects on labeling, suggesting that the optimal urea concentration for ^{18}O labeling was below 2 M. An acidic buffer of pH 4.5 was optimal for adjusting the pH of the labeling reaction, and the KH_2PO_4 concentration in a range of 50–150 mM did not have significant difference in labeling quality. It was also demonstrated that the use of the FA to quench the digestion reaction was not suitable for a post-digestion labeling method. Present study revealed the reason why previous studies drew different conclusions on the optimal pH for post-digestion labeling. It was that they used different digestion conditions. For a digestion and labeling decoupled approach, in spite of optimizing labeling conditions without affecting digestion conditions, different digestion conditions can affect following labeling conditions.

The labeling percentage and its viability among different peptides are two key indexes for the assessment of labeling quality. Excellent labeling efficiency $98.4 \pm 1.9\%$ for a standard protein mixture was achieved at the optimized conditions, and $97.2 \pm 6.2\%$ for a complex biological sample, demonstrating the optimized protocol for post-digestion ^{18}O labeling. Due to high labeling efficiency, a good quantitation quality was also proved through a 1:1 mixture analysis of the ^{16}O- and ^{18}O-labeled peptides from the same protein sample. The average abundance ratios reached 1.05 ± 0.31, ensuring the reliability in determining peptide relative changes for comparative proteomics. This work will benefit other researchers who pair urea-based protein denaturation with a post-digestion ^{18}O labeling method.

Acknowledgements

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