

Supplementary Information

Evaluation and optimization of reduction and alkylation methods to maximize peptide identification with MS-based proteomics

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A list of Supplementary Tables in Excel format:

Table S1. Peptides containing cysteine identified from triplicate experiments without the enrichment and reduction/alkylation

Table S2. Peptides containing cysteine identified from the reduction/alkylation without the enrichment (triplicate)

Table S3. Peptides containing cysteine identified from the enrichment and reduction/alkylation (triplicate)

Protocols

1. Detailed protocol for global analysis of cysteine-containing peptides from yeast whole-cell lysates

The following protocol describes global analysis of cysteine-containing peptides from yeast whole-cell lysates. The optimization steps in this study are also included in the protocol, and the steps correspond to Fig. 1.

1.1 Yeast cell culture

1) Transfer the culture media into an autoclaved Erlenmeyer flask. The size of the flask should be appropriate for the amount of yeast growing – if growing 100 mL, use 250 mL flask at least.

2) Scrape one colony of yeast from the agar plate using a sterile toothpick, swirl flask to suspend the colony in the media. Incubate at 31 °C at 250 RPM overnight.

3) Check the optical density (OD) at 600 nm the next morning. When the OD is about one, transfer media containing yeast cells into 50 mL conical tubes. Centrifuge at 800 g, 4 °C for 5 minutes.

4) Remove the media. Add 0.7 mL of water into each tube. Pipet up and down to mix the cells. Transfer to XXTuff 2 mL microvials. Centrifuge at 800 g, 4 °C for 5 minutes.

5) Remove the liquid. Put the tubes on ice until the lysis step. Otherwise, freeze at -80 °C until use.

1.2 Cell lysis

1) Prepare the lysis buffer containing 8 M urea, 1 protease inhibitor cocktail tablet per 10 mL, 75 mM NaCl, 50 mM HEPES, pH=8.2, and 0.5% sodium deoxycholate (SDC).

2) Lyse the cells

2.1) Add zirconia/silica beads and 700 uL of the lysis buffer to each XXTuff 2 mL microvials.

2.2) Lyse cells three times with Mini Beadbeater, 30 seconds each. Put on ice for 2 minutes between runs.

2.3) Transfer the supernatant to new tubes, discard the pellet.

1.3 Chloroform/methanol precipitation

- 1) Add methanol, chloroform and water to one volume of the sample at a 4:1:3 ratio. Carefully vortex. Centrifuge at 5,000 g for 10 minutes.
- 2) Remove the top layer using pipet. Do not disrupt protein pellet/film in between layers.
- 3) Add the same amount of methanol until the pellet sinks. Do not disrupt the pellet. Centrifuge at 5,000 g for 10 minutes.
- 4) Remove the liquid. Dry the pellet by air or the Speed-Vac.

1.4 Protein digestion

- 1) Prepare the digestion buffer containing 5% ACN, 1.6 M urea, and 50 mM HEPES, pH~8.2.
- 2) Add the digestion buffer to the dried protein pellet. Vortex or sonicate it.
- 3) Add Lys-C to the solution. The ratio of enzyme to proteins is 1:100 -1:200. Incubate at 31 °C with shaking overnight.
- 4) Add trypsin the next day. The ratio of enzyme to proteins is 1:100 -1:200. Incubate at 37 °C for 4 hours with shaking.
- 5) After 4 hours, quench the reaction by adding TFA until pH is less than 2.

1.5 Peptide purification

- 1) Determine the correct cartridge to use based on the amount of peptides. For each 50 mg of the packing material, load 0.9 mL of ACN. Push with a pipet bulb if necessary.
- 2) Add 0.3 mL of 50% ACN with 0.5% HOAc.
- 3) Add 0.9 mL of 0.1% TFA.
- 4) Load the peptide sample. If there is precipitate at the bottom of the tube, only add the supernatant – otherwise cartridge may be clogged.
- 5) Add 0.9 mL of 0.1% TFA.
- 6) Add 90 uL of 0.5% HOAc.
- 7) Elute with 0.35 mL of 50% ACN with 0.5% HOAc, and elute again with 0.15 mL of 75% ACN with 0.5% HOAc. Use pipet bulb to push all liquid through cartridge if necessary.
- 8) Freeze in the -80°C freezer. Dry in a Speed-Vac.

1.6 Disulfide bond reduction

- 1) Prepare a 1 M stock solution of DTT. Add this to the lysate until the final concentration is 5 mM.
- 2) Incubate in the dry bath at 56 °C for 25 minutes.
- 3) Remove the samples and allow them to cool to room temperature.

NOTE: Comparison of reducing reagents

- 1) For each 300 µg of the dried peptides, add 300 µL of 50 mM HEPES, pH=8.2. Vortex to dissolve the dried pellet.
- 2) Prepare 1 M stock solutions of DTT, 2-ME, TCEP, and THPP. Add this to the peptide solution to the final concentration of 5 mM. Incubate for 25 minutes in the dry bath at 56 °C.

1.7 Alkylation of sulfhydryl groups

- 1) Make sure that the lysate is at room temperature. Prepare a 0.1 mM stock solution of IAA. Add this to the peptide solution until the final concentration is 14 mM. Incubate in the dark for 30 minutes at room temperature.
- 2) Once the alkylation is done, add DTT until the final concentration is 5 mM to quench any unreacted IAA. Incubate for 15 minutes in the dark at room temperature.

NOTE: Comparison of alkylating reagents

- 1) Prepare 0.1 M stock solutions of IAA, acrylamide, N-EM, or 4-VP.
- 2) Make sure that the sample after the reduction is at room temperature. Add the stock solution of each alkylating reagent to the peptide solution to the final concentration of 14 mM. For 4-VP, also add methanol to the final concentration of 10%. Incubate in the dark at room temperature for 30 minutes.
- 3) Once the alkylation is done, add DTT until the final concentration is 5 mM to quench unreacted alkylating reagent. Incubate for 15 minutes in the dark at room temperature.

NOTE: Optimizing the alkylation reactions using iodoacetamide as the reagents

- 1) Prepare a 0.1 mM stock solution of IAA.

- a. For the concentration optimization experiment, add the stock solution to the peptide solution until the final concentration is 1, 2, 4, 8, 14, or 20 mM, respectively. Incubate in the dark at room temperature for 30 minutes.
- b. For the reaction temperature experiment, add the stock solution to the peptide solution until the final concentration is 14 mM. Incubate at room temperature, 40, 70 or 85 °C in the dry bath in the dark for 30 minutes.
- c. For the reaction time experiment, add the stock solution to until the final concentration is 14 mM in the peptide solution. Incubate in the dark at room temperature for 10, 20, or 30 minutes, respectively.

2) Once the alkylation is done, add DTT until the final concentration is 5 mM to quench unreacted IAA. Incubate 15 minutes in the dark at room temperature.

NOTE: for the reduction/alkylation at the peptide level, the samples should be desalted again after the reduction is done.

1.8 Peptide purification

Follow the steps from Part 1.5.

1.9 LC-MS/MS analysis

1) Dissolve the sample in 5% ACN 4% FA solution. Load into the autosampler of the chromatograph, and MS analysis can be found in the paper.

2. Protocol for enrichment cysteine-containing peptides

The protocol in this section was adapted from Guo et al.⁵⁴

1) After the digestion step, dissolve ~300 µg of the dried peptides in 100 µL 50 mM, HEPES (pH=8.2).

2) Prepare a 1 M DTT stock solution. Add this to the peptide solution until the final concentration is 5 mM. Incubate at 56 °C for 25 minutes. Let the solution cool down to room temperature.

3) Weigh 35 mg of Thiopropyl-Sepharose 6B resin into a microcentrifuge tube. Add 1 mL water into the tube, and keep it at room temperature for 15 minutes. The resin should absorb water and become swollen.

4) Resuspend the rehydrated resin, and keep it at room temperature for another 10 minutes.

5) Transfer the resin into a spin column. Wash with water for five times then with coupling buffer containing 50 mM HEPES, pH=8.2 and 1 mM EDTA for five times.

6) Put the plug to the bottom of the top part of the spin column after the last wash. Add the peptide mixture from step 2 into the spin column. Incubate on an end-over-end rotator at room temperature for two hours.

7) After the enrichment, remove the bottom plug and centrifuge the column at 1,500 g for 1 minute to remove unbound peptides.

8) Wash the resin five times with 0.5 mL 8 M urea. Centrifuge at 1,500 g for 1 minute after each wash.

9) Wash the resin five times with 0.5 mL 2 M NaCl. Centrifuge at 1,500 g for 1 minute after each wash.

10) Wash the resin five times with 0.5 mL 80% ACN. Centrifuge at 1,500 g for 1 minute after each wash.

11) Wash the resin five times with 0.5 mL 50 mM HEPES, pH=8.2. Centrifuge at 1,500 g for 1 minute during each wash.

12) Prepare the eluting buffer containing 20 mM DTT in 25 mM NH_4HCO_3 . Replace the bottom part of the spin column with a new and clean tube. Add 200 μL of the buffer into the resin. Incubate at room temperature for 30 minutes on an end-over-end rotator.

13) Remove the bottom plug. Elute the peptides by centrifuging at 1,500 g for 1 minute. Transfer the eluate into another microcentrifuge tube.

14) Add another 100 μL of the eluting buffer to the resin. Vortex briefly, then incubate at room temperature on an end-over-end rotator. Collect the peptides and combine the two fractions together.

15) Follow the normal alkylation step.

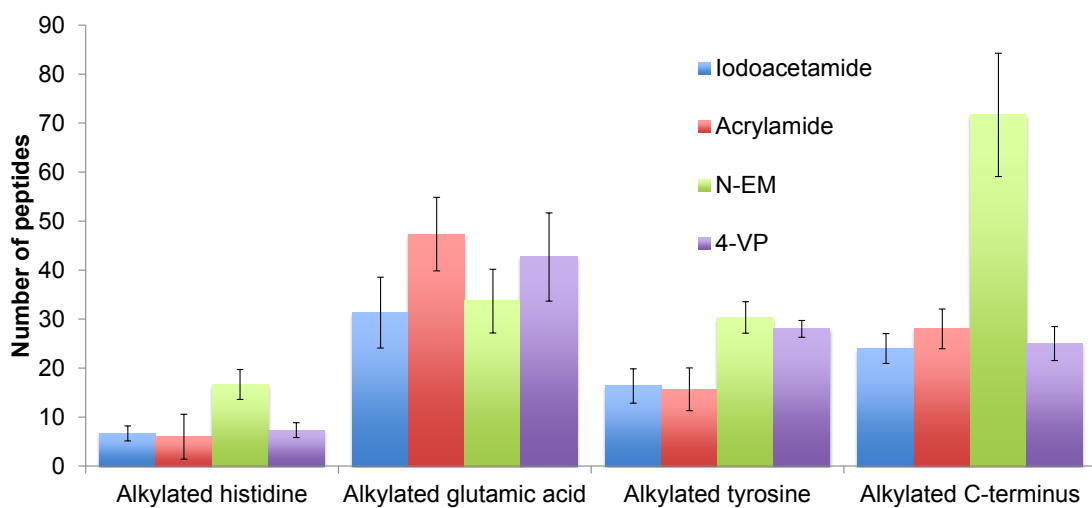


Fig. S1. Comparison of alkylating reagents: other side reactions on the side chains of histidine, glutamic acid, tyrosine, and the peptide C-terminus.

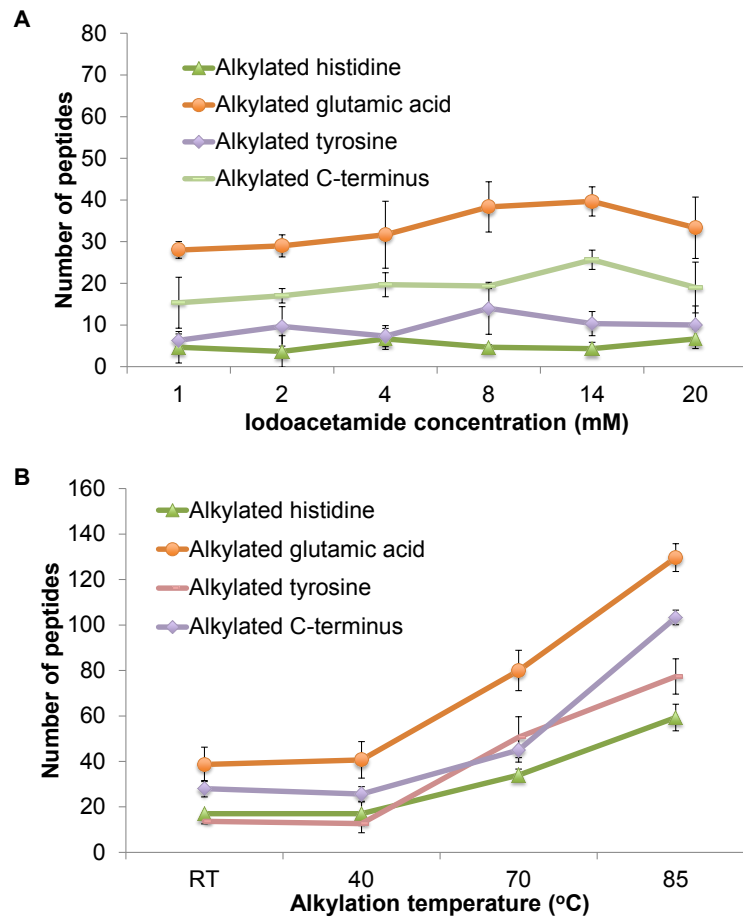


Fig. S2. Other side reactions from the alkylation with iodoacetamide at different concentrations (A) and temperatures (B).

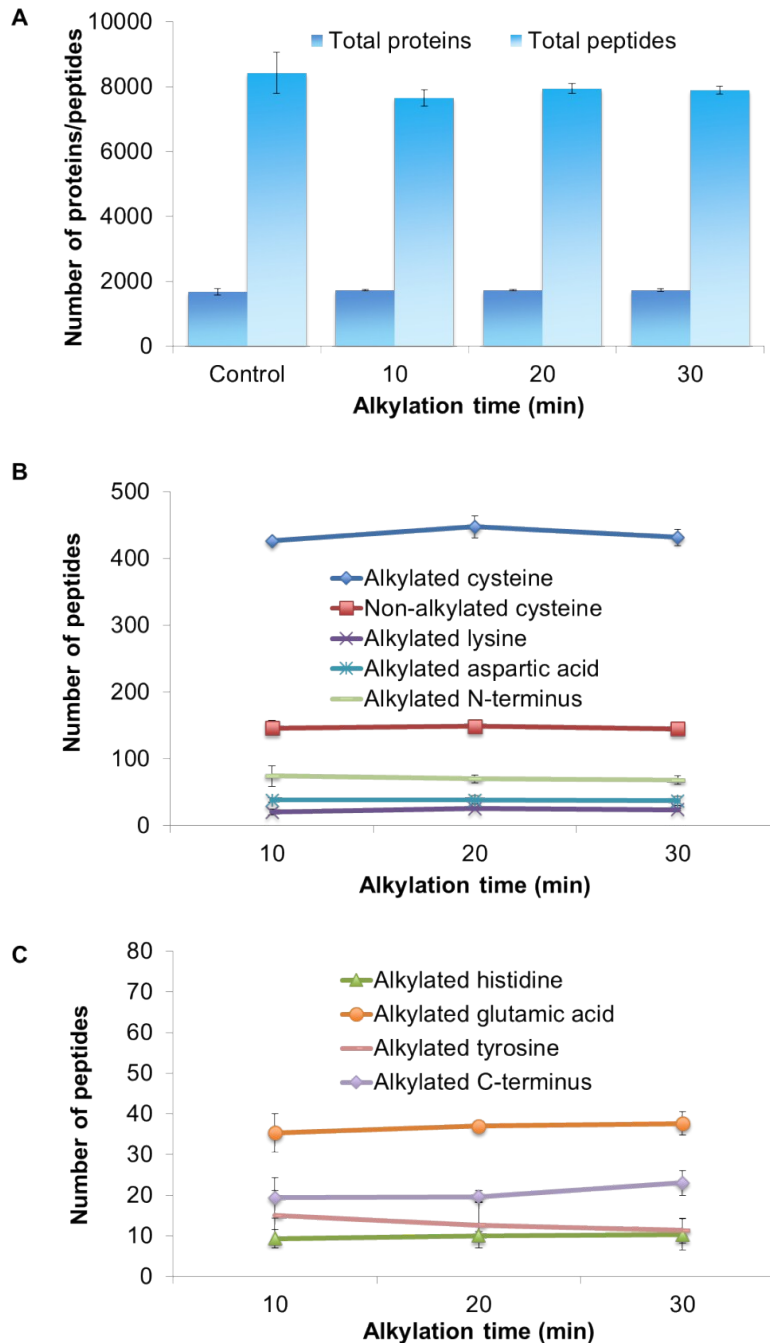


Fig. S3. Optimization of alkylation conditions. (A) Effects of alkylation time on the identification of proteins and peptides. (B) The number of identified peptides with alkylated cysteine, free cysteine (due to incomplete reaction), or the side reactions on the N-terminus and the side chains of lysine and aspartic acid from different alkylation times. (C) Other side reactions from the alkylation with iodoacetamide at different reaction times.

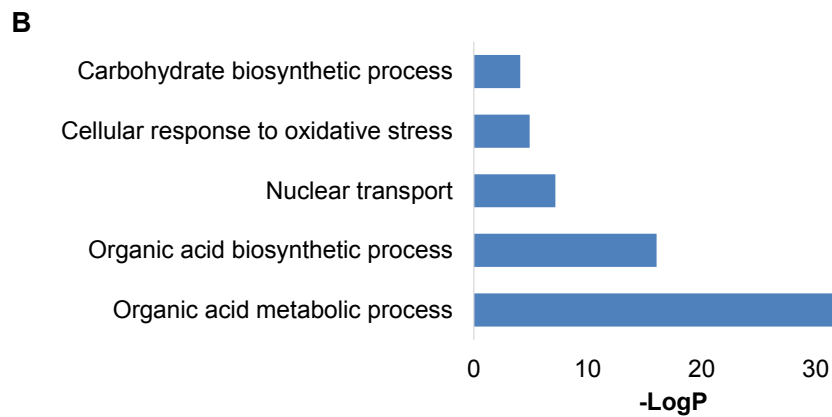
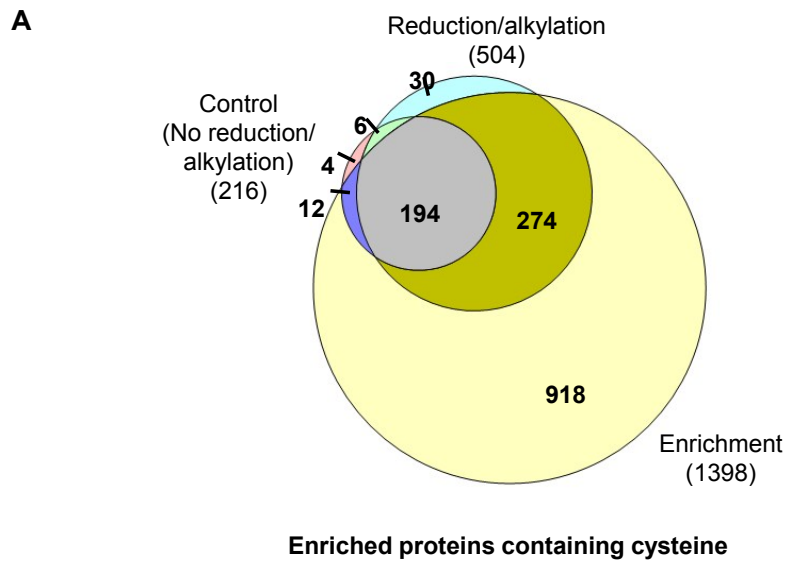


Fig. S4. (A) Overlap of identified proteins containing cysteine from the experiments without the enrichment nor reduction/alkylation (No reduction/alkylation), the experiments with only the reduction/alkylation (Reduction/alkylation), and the experiments with both the enrichment and reduction/alkylation (Enrichment). (B) Clustering of proteins with cysteine from the enrichment experiment according to the biological process.