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# Subcritical Water Processing of Proteins: An Alternative to Enzymatic Digestion?

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Ⓢ Supporting Information

ABSTRACT: Subcritical water is an emerging tool in the processing of bioorganic waste. Subcritical water is an environmentally benign solvent which has the potential to provide an alternative to traditional methods of protein hydrolysis without the inclusion of expensive acids or enzymes. To date, most studies on the subcritical water mediated hydrolysis of proteins have focused on the production of amino acids, rather than the intermediate peptides. Here, we investigate the specificity of subcritical water with respect to the production of peptides from three model proteins, hemoglobin, bovine serum albumin, and β-casein, and compare the results with enzymatic digestion of proteins by trypsin. In addition, the effect of subcritical water (SCW) treatment on two protein post-translational modifications, disulfide bonds and phosphorylation, was investigated. The results show that high protein sequence coverages (>80%) can be obtained following subcritical water hydrolysis. These are comparable to those obtained following treatment with tryspin. Under mild subcritical water conditions (160 °C), all proteins showed favored cleavage of the Asp-X bond. The results for β-casein revealed favored cleavage of the Glu-X bond at subcritical water temperatures of 160 and 207 °C. That was similarly observed for bovine serum albumin at a subcritical water temperature of 207 °C. Subcritical water treatment results in very limited cleavage of disulfide bonds. Reduction and alkylation of proteins either prior to or post subcritical water treatment improve reported protein sequence coverages. The results for phosphoprotein β-casein show that, under mild subcritical water conditions, phosphorylation may be retained on the peptide hydrolysis products.
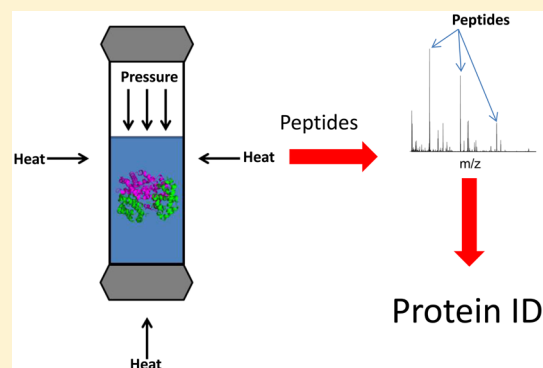
Subcritical water (SCW) is defined as water maintained at a temperature of between 100 and 374 °C and a pressure of less than 22.064 MPa, i.e., below its critical point. While in the subcritical state, water acquires unique properties, including a change in hydrogen bonding structure and an increased ionic product, K_w, which is 3 orders of magnitude greater than that of water at ambient conditions. The increase in ionic product drives the formation of hydronium (H_3O^+) and hydroxide (OH^-) ions. SCW therefore has the ability to act as either an acid or a base catalyst.[1] Consequently, SCW is regarded as a "green" solvent and is attracting interest with regards to waste and biomass conversion,[2] including hydrolysis of lignocellulose,[3−5] carbohydrates,[6−8] lipids,[6] and proteins[8−14] and extraction of bioactive compounds.[15]

The majority of studies designed to evaluate the utility of the SCW mediated hydrolysis of proteins have focused on the production of amino acids, rather than the intermediate peptides. For example, Abdelmoez and Yoshida investigated the SCW mediated hydrolysis of bovine serum albumin (BSA) for the production of amino acids in the temperature range of 200−300 °C.[13] Few amino acids were produced at temperatures of 200−225 °C and reaction times of less than 2 min and at temperatures of 275−300 °C for less than 1 min. The yield of amino acids could be increased by increasing both temperature and reaction time. Watchararuji et al. investigated the effects of temperature, time, and solid/liquid ratio parameters on the production of amino acids from agricultural byproducts.[16] Similarly, Ueno et al. considered the effects of temperature on amino acid production from SCW mediated hydrolysis of fish gelatin.[17]

In this work, we investigate the specificity of SCW in the hydrolysis of proteins with respect to the production of peptides and compare hydrolysis driven by SCW with enzymatic digestion of proteins by trypsin, the protease of choice for bottom-up proteomics.[18] SCW mediated hydrolysis has previously been used in conjunction with trypsin digestion. Jung et al. showed that pretreatment with SCW resulted in greater efficiency of trypsin digestion of porcine placenta.[19] Marcet et al. compared SCW hydrolysis and trypsin digestion for the extraction of peptides from insoluble egg yolk protein.[20] Size exclusion chromatography was used to compare peptide size distributions with trypsin resulting in peptides of ~3500 Da and SCW hydrolysis resulting in a range (from 1000 Da to 23 kDa) depending on reaction time.

Chen et al.[21] showed the direct electrospray of subcritical water solutions of ubiquitin, hemoglobin, wheatgerm agglutinin, and bradykinin by use of a high pressure electrospray source; i.e., SCW conditions were created in the electrospray source. While the peptide bradykinin remained stable, the protein samples showed evidence of unfolding and degradation. At temperatures of 160 °C, a significant decrease in intact protein ions was observed, and at 180 °C, these were virtually eliminated. High resolution mass spectrometry of superheated ubiquitin confirmed the presence of peptide hydrolysis products.

Here, we show the results obtained following SCW hydrolysis of three model proteins: hemoglobin (MW 15 117 Da α-globin; 15 857 Da β-globin), bovine serum albumin (BSA) (MW 66 389 Da), and β-casein (MW 23 568 Da). The aim was to determine the specificity of SCW hydrolysis of the protein backbone and to investigate the effect of two common post-translational modifications. BSA contains 17 disulfide bonds, and β-casein contains five sites of phosphorylation. A range of temperatures (160, 207, 253, and 300 °C) and reaction times (0 and 20 min) were applied. The products of SCW hydrolysis were analyzed by liquid chromatography tandem mass spectrometry (LC MS/MS). Results were compared with those obtained following trypsin digestion of the proteins and subsequent LC MS/MS analysis.

■ METHODS

Samples. Protein standards hemoglobin, bovine serum albumin (BSA), and β-casein were purchased from Sigma-Aldrich (Gillingham, UK) and used without further purification.

Trypsin Digestion. Fifty μg of standard protein was made up in 90 μL of 100 mM ammonium bicarbonate. Fifty μL of 10 mM dithiothreitol (Sigma-Aldrich, Gillingham, UK) in 100 mM ammonium bicarbonate was added and incubated for 30 min at 56 °C. Fifty μL of 55 mM iodoacetamide (Sigma-Aldrich) in 100 mM ammonium bicarbonate was added to the sample and incubated for 20 min at room temperature in the dark. Samples were digested overnight with Tryspin Gold (Promega, Madison, WI), 13 ng/μL in 50 mM ammonium bicarbonate (protein/enzyme 50:1) at 37 °C, pH 8. Proteolysis was quenched by addition of 0.5% TFA.

SCW Mediated Hydrolysis. Fifteen mg of protein was dissolved in 15 mL of deionized water (J. T. Baker, Deventer, The Netherlands) and placed inside a reaction tube consisting of stainless steel metal piping, 200 mm × 16.3 mm (internal diameter), total volume 42 cm^3, (Supplementary Figure 1), capped using SS Tube Fitting, Reducing Union, 3/4 in. × 1/4 in. (Swagelok, UK). SCW mediated hydrolysis was performed at temperatures of 160, 207, 253, and 300 °C. (Water vapor pressures at these temperatures are 613.7, 1794.8, 4216.0, and 8692.1 kPa, respectively.) Reaction tubes were placed in the oven preset to 30 °C and allowed to equilibrate for at least 10 min prior to increasing the temperature of the oven. The reaction temperature was monitored by use of a thermocouple attached to one of the reaction tubes. It took 6 min 40 s to reach 160 °C, 7 min 40 s to reach 207 °C, 10 min 30 s to reach 253 °C, and 16 min 45 s to reach 300 °C. The selected residency, i.e., 0 and 20 min, began after reaching the chosen temperature. Each experiment was performed in triplicate. The reaction was quenched by placing the reaction tubes into a bucket of ice for 5 min. One ml aliquots of the hydrolysate were then stored at −20 °C until analysis. In additional experiments,

1.5 and 0.15 mg of hemoglobin was dissolved in 15 mL of deionized water and treated as above with SCW conditions of 160 °C for 20 min.

Reduction of BSA Pre-SCW Processing. Fifteen mg of BSA was added to 8.75 mL of 100 mM ammonium bicarbonate. 3.13 mL of 48 mM DTT (Sigma-Aldrich) in 100 mM ammonium bicarbonate was added to the sample and incubated for 30 min at 56 °C. A further 3.13 mL of 264 mM iodoacetamide (Sigma-Aldrich) in 100 mM ammonium bicarbonate was added to the sample and incubated for 20 min at room temperature in the dark. Samples were introduced into the SCW reaction vessel directly.

Reduction of BSA Post-SCW Processing. Fifty μL of hydrolyzed BSA was added to 50 μL of 10 mM dithiothreitol (Sigma-Aldrich, Gillingham, UK) in 100 mM ammonium bicarbonate and incubated for 30 min at 56 °C. Fifty μL of 55 mM iodoacetamide (Sigma-Aldrich) in 100 mM ammonium bicarbonate was added to the sample and incubated for 20 min at room temperature in the dark.

Peptide Purification. Both tryptic and SCW peptides were desalted using ZipTip C18 pipet tips (Millipore, Bedford, MA, USA) according to manufacturers' instructions. The desalted samples were dried and resuspended in 10 μL of formic acid prior to MS analysis.

Liquid Chromatography. Peptides were separated using online reversed phase LC (Dionex Ultimate 3000), using a binary solvent system consisting of mobile phase A (water (J.T., Baker, Holland)/0.1% formic acid (Fisher Scientific, Loughborough, UK)) and mobile phase B (acetonitrile (J.T., Baker)/0.1% formic acid (Fisher Scientific)). Peptides were loaded onto a C18 column (LC Packings, Sunnyvale, CA), in mobile phase A and separated over a linear gradient from 3.2% to 44% mobile phase B with a flow rate of 350 nL/min. The column was then washed with 90% mobile phase B before re-equilibrating at 3.2% mobile phase B. Samples eluted directly via a Triversa Nanomate nanoelectrospray source (Advion Biosciences, Ithaca, NY) into the mass spectrometer.

Mass Spectrometry. All mass spectrometry experiments were performed on a Thermo Fisher Orbitrap Elite (Thermo Fisher, Bremen, Germany). Data acquisition was controlled by Xcalibur 2.1 (Thermo Fisher Scientific).

CID. The mass spectrometer performed a full MS scan (m/z 400−2000) and subsequent MS/MS (CID or ETD) scans of the seven most abundant ions that had a charge state of >1. Survey scans were acquired in the orbitrap with a resolution of 60 000 at m/z 200. Dynamic exclusion was used with a repeat count of 1 for 30 s. Automatic gain control (AGC) target for the survey scans was 10^6 charges with maximum injection time of 1 s. CID was performed in the linear ion trap using helium at normalized collision energy of 35%. Width of the precursor isolation window was 2 Th. AGC target was 5×10^4 charges with a maximimum injection time of 100 ms. ETD was performed in the linear ion trap with fluoranthene ions. Width of the precursor isolation window was 2 Th. AGC target for precursor ions was 5×10^4 with a maximum injection time of 1 s. AGC target for fluoranthene ions was 1×10^5 with maximum fill time of 100 ms. Precursor ions were activated for 100 ms (charge dependent activation time was enabled). Supplemental activation was used with normalized collision energy of 25%.

Database Search Parameters. Raw MS/MS data files from Xcalibur software (version 3.0.63; Thermo Fisher) were submitted to Proteome Discoverer software (version 1.4.1.14; Thermo Fisher Scientific) for SEQUEST searches against the
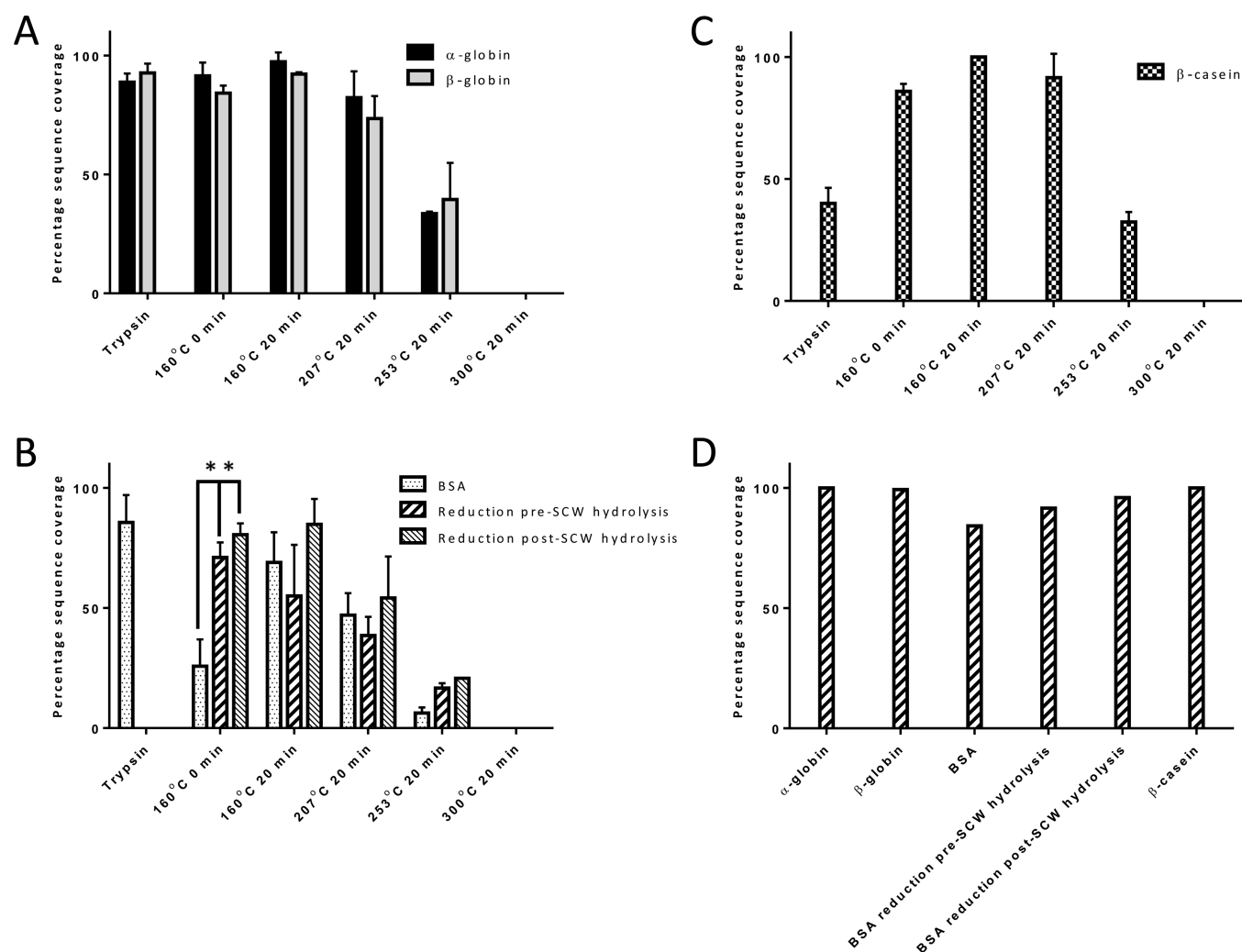
Figure 1. Mean sequence coverage obtained for trypsin digests and SCW hydrolysis at 160 °C for 0 min, 160 °C for 20 min, 207 °C for 20 min, 253 °C for 20 min, and 300 °C for 20 min for (A) α-globin and β-globin, (B) BSA, (C) β-casein, and (D) combined coverage for hemoglobin α-chain, hemoglobin β-chain, BSA, and β-casein at 160 °C for 20 min. * = $p < 0.05$ as determined by students' t test. $n = 3$. Error bars represent one standard deviation.

relevant protein sequence as obtained from uniprot. Two missed cleavages were allowed for tryptic digests. Database searches for SCW hydrolyzed samples were searched using "nonspecific enzyme". Precursor mass tolerance was 10 ppm, and MS/MS tolerance was 0.5 Da. For trypsin digests of hemoglobin and BSA, carbamidomethylation of cysteines was set as a static modification, while deamidation and oxidation were set at variable modifications. For the trypsin digest of β-casein, phosphorylation was introduced as a further variable modification. For database searches of SCW hydrolyzed hemoglobin, deamidation and oxidation were set as variable modifications. For database searches of SCW hydrolyzed β-casein: deamidation, oxidation, and phosphorylation were set as variable modifications. For SCW hydrolysis of BSA, deamidation and oxidation were set as variable modifications. When BSA was treated with DTT and iodoacetamide (both prior to and following SCW hydrolysis), carbamidomethylation was included as a static modification. In subsequent searches (see text), database searches were performed with additional variable modifications: dehydration of Asp, Glu, Thr, and Ser and pyroglutamic acid formation. All other search parameters remained the same.

Statistical Analysis. Each individual trypsin digestion/ SCW hydrolysis was completed in triplicate. Data are shown as ± SD. Significance was tested by student's t tests.

■ RESULTS AND DISCUSSION

Protein Sequence Coverage. Figure 1 shows the effect of SCW reaction temperature and time on peptide production (mean % protein sequence coverage) from hemoglobin, BSA, and β-casein, compared with enzymatic digestion of the proteins with trypsin. SCW reaction conditions were 160 °C for 0 min, 160 °C for 20 min, 207 °C for 20 min, 253 °C for 20 min, and 300 °C for 20 min. The trypsin digestion of hemoglobin (Figure 1A) gave high sequence coverage for both the α-globin (88.9 ± 3.6%) and β-globin (92.7 ± 4.0%). SCW hydrolysis at 160 °C for 0 min gave comparable protein coverage: 91.5 ± 5.6% for α-globin and 87.9 ± 3.3% for β-globin. Extending the SCW reaction time to 20 min resulted in increased sequence coverage: 97.4 ± 3.9% for α-globin and 96.2 ± 0.8% for β-globin. The sequence coverage obtained following SCW treatment at 207 °C was 82.3 ± 11.2% for α-globin and 76.7 ± 9.9% for β-globin. A sharp reduction in protein coverage was observed following SCW treatment at 253 °C (33.6 ± 0.8%

for α-globin and 41.2 ± 16.1% for β-globin), presumably due to decomposition of the peptides to component amino acids. No peptides were identified following SCW treatment at 300 °C for 20 min. The decrease in protein coverage observed between 253 and 300 °C supports previous work by Espinoza et al. which showed a decrease in the amount of peptides produced following SCW treatment of whey protein isolate above 220 °C due to conversion into amino acids.[22]

The average protein coverage obtained following SCW mediated hydrolysis (160 °C, 0 min) of BSA (Figure 1B) was 32.1 ± 3.4%, significantly lower than the coverage seen for hemoglobin under the same conditions. Maximum sequence coverage was obtained following SCW treatment at 160 °C for 20 min (69.0 ± 12.5%). Nevertheless, this maximum coverage obtained following SCW treatment falls short of that obtained following trypsin digestion of reduced and alkylated BSA (85.6 ± 11.4%). The protein coverage obtained following SCW treatment at 207 °C (20 min) and 253 °C (20 min) was 47.0 ± 9.2% and 6.3 ± 2.3%, respectively. No peptides were observed following treatment at 300 °C.

The reduced sequence coverage observed for BSA suggests that SCW treatment does not result in the cleavage of disulfide bonds. To test that hypothesis, BSA was treated with dithiothreitol and iodoacetamide both pre- and post-SCW treatment. When disulfide bonds were reduced post-SCW treatment, a significant increase ($p = 0.007$) in sequence coverage was observed for SCW conditions 160 °C for 0 min (80.5 ± 4.7%). At all other SCW conditions, the levels of sequence coverage were not significantly different to those obtained for nonreduced BSA. When disulfide bonds were reduced pre-SCW treatment, again, a significant increase ($p = 0.016$) in sequence coverage was observed for SCW conditions 160 °C for 0 min (71.1 ± 6.2%). As seen above, the coverages obtained following SCW treatment in all other conditions were not significantly different to those for nonreduced BSA.

Following SCW hydrolysis of untreated BSA at 160 °C (0 min), five cysteine-containing peptides (incorporating 4 cysteine residues) were identified of which the component cysteine residues are involved in three disulfide bonds. This observation suggests that SCW supports cleavage of disulfide bonds. Nevertheless, the cleavage is limited as demonstrated by the results following chemical reduction: reduction of BSA pre-SCW hydrolysis resulted in identification of 98 cysteine-containing peptides (incorporating 26 cysteine residues involved in 16 disulfide bonds) and reduction of BSA post-SCW hydrolysis revealed 142 cysteine-containing peptides (incorporating 32 cysteine residues involved in 17 disulfide bonds). For the peptides identified from the SCW hydrolysate of untreated BSA at 160 °C for 20 min, 35 cysteine-containing peptides were identified. Reduction of BSA pre-SCW treatment revealed 175 cysteine-containing peptides, and reduction of BSA post-SCW mediated hydrolysis revealed 232 cysteine-containing peptides. The *overall* protein sequence coverage was similar for the three conditions; therefore, the differences in observed cysteine-containing peptides suggest that only very limited disulfide bond cleavage occurs under SCW conditions of 160 °C for 20 min. These results suggest that proteins containing disulfide bonds should be reduced and alkylated either prior to or post-SCW treatment in order to maximize sequence coverage.

Trypsin digestion of β-casein gave an average protein sequence coverage of 40.0 ± 6.4% (Figure 1C). This low sequence coverage can be explained by the paucity of arginine and lysine residues in the protein sequence. In contrast, SCW hydrolysis at 160 °C, 0 min, gave high sequence coverage (86.0 ± 3.0%). Under SCW conditions of 160 °C for 20 min, protein sequence coverage of 100% was achieved in all replicates. A reduction in protein coverage was observed following SCW treatment at 207 °C for 20 min (91.6 ± 9.8%), and this trend continued following SCW treatment at 253 °C for 20 min (32.4 ± 4.1%). No peptides were identified at 300 °C for 20 min. The results obtained for β-casein illustrate the advantages of SCW hydrolysis over trypsin for identification of some proteins.

On the basis of the results above, the optimum SCW conditions in terms of protein sequence coverage were 160 °C for 20 min. Figure 1D summarizes the total combined coverage for the three replicates obtained at these conditions for each protein. The high protein coverages obtained suggest that these SCW hydrolysis conditions may be used as an alternative to trypsin in bottom-up proteomics.

Supplementary Figure 2 shows a plot of the percentage of peptide spectral matches versus proteolysis conditions (either trypsin digestion or SCW hydrolysis). Peptide spectral matches (PSMs) refer to MS/MS spectra which were confidently assigned to a peptide sequence in the protein database search. The percentage of PSMs for the samples treated with trypsin were consistently greater (23.2 ± 4.7% for hemoglobin, 89.8 ± 10.5% for BSA, and 31.9 ± 4.3% β-casein) than for those treated with SCW (<7% in all cases). This observation suggests that, in addition to cleavage of the peptide bond, SCW treatment results in other hydrolysis products presumably due to degradation of the amino acid side chains. To further investigate this possibility, additional protein database searches were performed in which dehydration and formation of pyroglutamic acid were set as variable modifications (in addition to deamidation and oxidation). These database searches did not result in improved sequence coverage (and in some cases sequence coverage dropped, presumably due to the challenge to the algorithm created by additional variables). Nevertheless, additional peptides were identified: On average, peptides showing water loss resulted in an additional 9% (range of 2% to 17%) of the total identifications for α-globin, β-globin, BSA, and β-casein at SCW conditions of 160 °C (0 min), 160 °C (20 min), and 207 °C (20 min). Formation of pyroglutamic acid accounted for an average of 1% (range of 0% to 3%) additional identifications. (Note that additional identifications do not correlate with increased sequence coverage as the unmodified peptides were previously identified.)

In the SCW experiments described above, 15 mg/15 mL of protein was used in each reaction tube. Twenty μL of hydrolysis product (equivalent to 20 μg of starting protein) was desalted using ZipTip C18 pipet tips and loaded onto the LC column. In the tryptic digestion experiments, 50 μg of protein was digested and analyzed. Although comparable amounts of starting protein are ultimately analyzed by LC MS/MS in the two experiments, the amount of protein in the SCW reaction is much higher. The suitability of the SCW setup for lower protein concentration was therefore investigated for hemoglobin solutions of concentration 0.1 and 0.01 mg/mL (160 °C for 20 min). SCW hydrolysis using 0.1 mg/mL of hemoglobin gave comparable sequence coverage to that described above for both α-globin (97.2 ± 2.6%) and β-globin (94.3 ± 1.6%). Sequence coverage remained high when using 0.01 mg/mL of hemoglobin (i.e., 150 μg of starting product) for both α-globin (99.5 ± 0.4%) and β-globin (91.7 ± 3.4%). Supplementary Figure 3 shows the summed survey scan mass
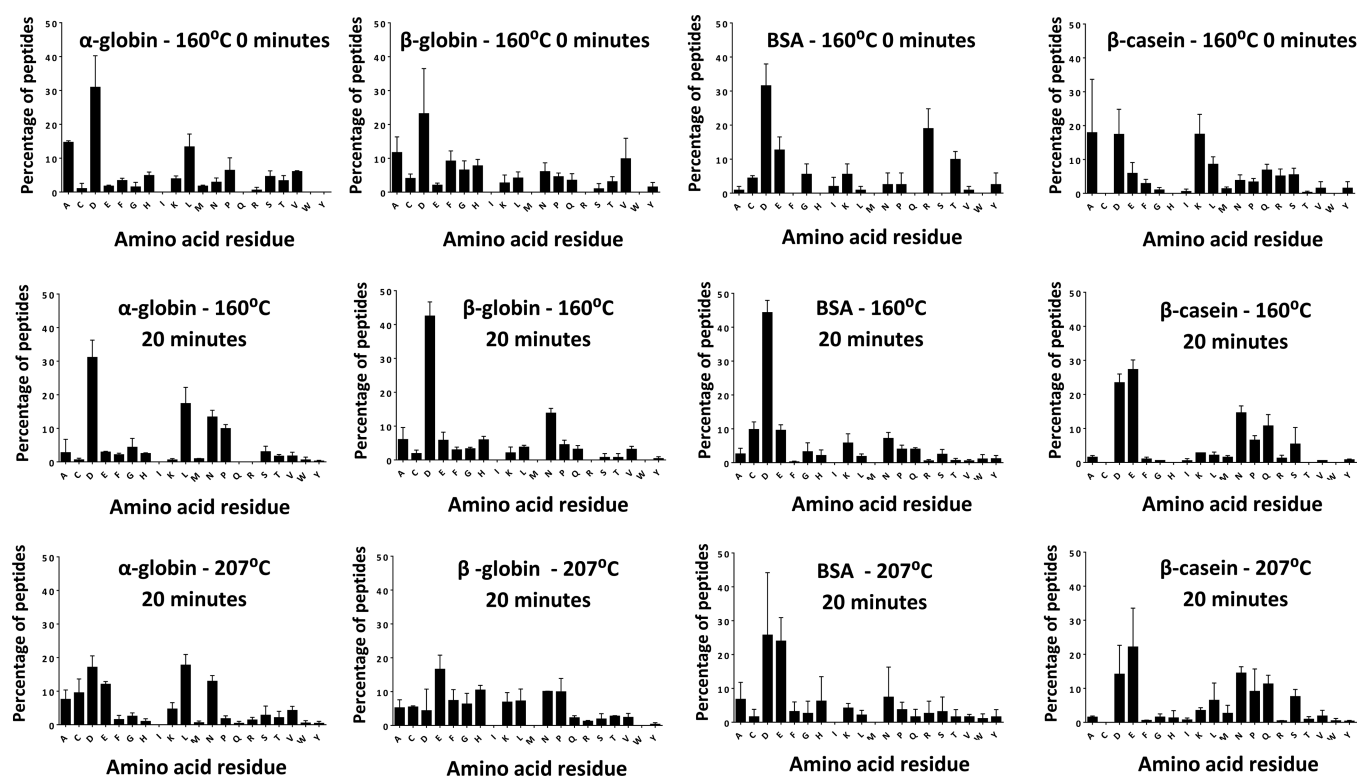
Figure 2. Specificity of SCW hydrolysis: Plots of the % of peptides identified against the amino acid residue immediately preceding the peptide N-terminus for proteins hemoglobin, BSA, and β-casein under SCW treatment at 160 °C (0 min), 160 °C (20 min), and 207 °C (20 min). $n = 3$. Error bars represent one standard deviation.

spectra at each of the three concentrations. The base peak in these mass spectra corresponds to 3+ ions of [VHLTPEEKS-AVTALWGKVNVD-H_2O]. The signal intensities are 8.5×10^6, 1.3×10^6, and 2.6×10^6, i.e., within an order of magnitude, across the concentration range. (Note that the automatic gain control was used in each experiment to control the number of ions entering the orbitrap.) These results suggest that the SCW hydrolysis would be suitable as a proteomics sample preparation method.

Specificity of SCW Mediated Hydrolysis of Model Proteins. Figure 2 shows plots of the percentage of peptides resulting from SCW treatment at 160 and 207 °C versus the amino acid residue in the protein sequence immediately preceding the N-terminus of the resulting peptide, i.e., the amino acid residue adjacent to the peptide's N-terminus. (A similar analysis of the amino acid at the peptide's C-terminus is given in Supplementary Figure 4). Peptide summary maps are shown in Supplementary Figure 5. SCW treatment at 160 °C for 0 min showed preferential cleavage toward the N-terminus of aspartic acid in α- and β-globin. The percentage of identified peptides which follow an aspartic acid residue was 30.9 ± 9.37% in the α-globin and 23.1 ± 13.39% in the β-globin, several fold higher than the natural abundance of aspartic acid residues in both α- and β-globins (5.7% in both cases). Similar results were seen for (nonreduced) BSA where 31.5 ± 6.5% of peptides was preceded by an aspartic acid residue, significantly higher than the natural abundance of aspartic acid in the BSA sequence (6.9%). The percentage of SCW peptides which follow an aspartic acid residue in β-casein was 17.4 ± 7.5%, again several fold greater than the natural abundance of the residue in the protein (1.9%). No favored cleavage adjacent to the structurally similar glutamic acid residue was observed. At

this temperature, we also observed favored cleavage toward arginine in BSA (19.4 ± 5.9%) and lysine in β-casein (17.39 ± 5.94%).

Similar results were seen following treatment at 160 °C for 20 min. The percentage of peptides with N-terminus adjacent to an aspartic acid residue was 31.1 ± 5.0% (α-globin), 42.4 ± 4.2% (β-globin), 44.3 ± 3.7% (BSA), and 23.4 ± 2.6% (β-casein). For β-casein, there appears to be some preferential cleavage adjacent to glutamic acid residue (27.2 ± 2.9%), much higher than the natural abundance of glutamic acid (9.1%). At this temperature, we do not observe favored cleavage toward arginine in BSA (0.5 ± 0.5%) or lysine in β-casein (2.8 ± 0.0).

At 207 °C, there appears to be a reduction in specificity toward the C-terminus of aspartic acid residues, and a more uniform distribution among the amino acid residues is observed. The percentages of peptides whose N-terminus was adjacent to aspartic acid in the full length protein are 17.1 ± 3.4% (α-globin), 4.3 ± 6.3% (β-globin), 25.7 ± 19.1% (BSA), and 14.1 ± 8.6% (β-casein). (The large standard deviation seen in BSA is due to an outlier in the data: in one replicate, 7.2% of peptides was preceded by an aspartic acid.) There also appears to be some specificity toward glutamic acid residues in β-globin (16.5 ± 4.2), BSA (23.9 ± 7.0%), and β-casein (22.1 ± 11.5%), higher than the natural abundance of glutamic acid residues in β globin (5.5%), BSA (10.1%), and β-casein (9.1%).

These results both support and contrast work by Kang and Chun which demonstrated that SCW treatment of fish waste at 200 °C results in liberation of aspartic acid and serine in high quantities relative to the other amino acids, indicating that these residues are more susceptible to hydrolysis by SCW.[23] Our results show favored cleavage at aspartic acid residues but not serine residues.

Scheme 1. Proposed Mechanism for Weak Acid Hydrolysis of the Asp-X Bond[26,27]
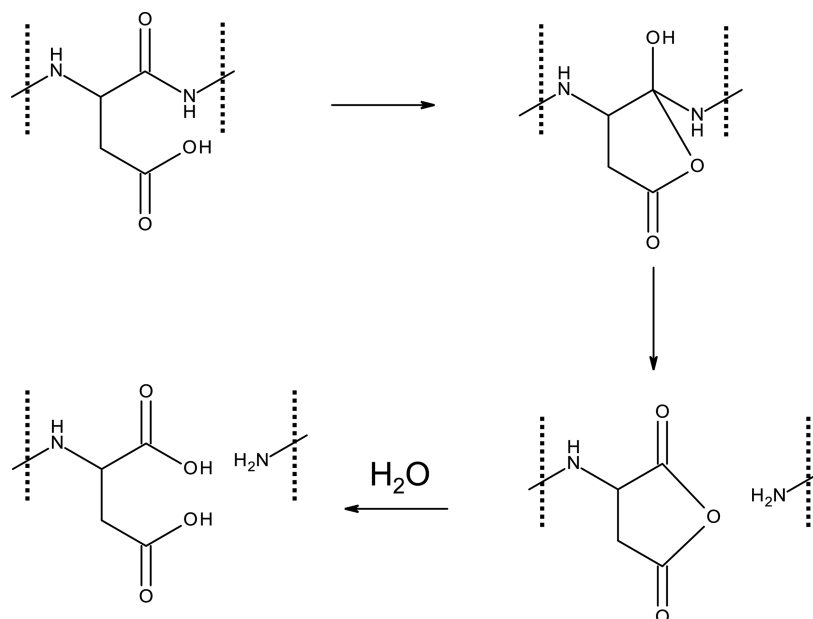
Table 1. Summary of Phosphopeptides and Unmodified Peptides Containing Known Sites of Phosphorylation Observed Following SCW Hydrolysis or Trypsin Digestion of β-Casein[a]

peptide sequence	theoretical mass	calculated mass	ΔPPM
Phosphopeptides Identified Following SCW Hydrolysis (160 °C, 0 min) of β-Casein			
IEKFQsEEQQQTED	1817.7356	1817.7423	3.6474
KIEKFQsEEQQQTED	1945.8306	1945.8395	4.5379
RELEELNVPGEIVEs	1791.8292	1791.8347	3.0304
RELEELNVPGEIVEsL	1904.9132	1904.9211	4.1104
RELEELNVPGEIVESLs	1991.9452	1991.9551	4.9349
RELEELNVPGEIVEsLss	2238.9099	2238.9137	1.6660
RELEELNVPGEIVESLSssEESITRINK or RELEELNVPGEIVESLsSsEESITRINK or RELEELNVPGEIVESLssSEESITRINK	3316.5477	3316.5649	5.1732
Unmodified Peptides Containing Known Sites of Phosphorylation Identified Following SCW Hydrolysis (160 °C, 0 min) of β-Casein			
RELEELNVPGEIVES	1712.8701	1712.8694	0.4087
RELEELNVPGEIVESL	1825.9542	1825.9602	3.2859
RELEELNVPGEIVESLS	1912.9862	1912.9902	2.0910
Phosphopeptides Identified Following Trypsin Digestion of β-Casein			
FQsEEQQQTEDELQDK	2061.8285	2061.82197	3.1671

[a]s is phosphoserine.

Partridge and Davis showed that weak acid hydrolysis of proteins results in preferential release of aspartic acid.[24] In the presence of weak acids, neutral carboxyl groups in the asparagine side chain act as proton donors in the hydrolysis of the adjacent peptide bonds. The production of aspartic acid rather than glutamic acid was attributed to the lower pK_a of aspartate. Similarly, Fisher and co-workers showed that chemical treatment of proteins with formic acid results in specific cleavage at aspartic acid residues.[25] The results described above suggest that weak acid hydrolysis of proteins occurs under SCW conditions. Scheme 1 shows the proposed mechanism for cleavage of the Asp-X bond.[26,27] Weak acid hydrolysis of proteins has also been observed in studies employing microwave-assisted nonenzymatic digestion. Microwave assisted acid hydrolysis of proteins was first demonstrated by Li and co-workers using 6 M HCl[28] and subsequently trifluoroacetic acid.[29] Hua et al.[30] showed that use of dilute formic acid resulted in specific cleavage C-terminal of aspartic

acid residues, a finding echoed by Swatkoski et al.[31] in their work using acetic acid.

The optimum pH for weak acid hydrolysis of Asp-X is ~2.[26,31] The pH of subcritical water is between 5.5 and 5.7 in the temperature range used in this work.[15] It may be possible therefore to mediate the reaction by addition of a weak acid to the protein solution prior to SCW treatment; however, that was not attempted here.

It is worth noting that the SCW peptide summary maps shown in Supplementary Figure 5 suggest that, following initial cleavage of the peptide bond, secondary cleavage of the peptide products occurs. That occurrence would explain the "block-like" appearance of the summary map (which is not evident in the results obtained for trypsin).

SCW Mediated Hydrolysis and Phosphorylation. β-casein is phosphorylated at serine residues at positions 15, 17, 18, 19, and 35; see Supplementary Figure 6. SCW treated samples of β-casein were analyzed by liquid chromatography

electron transfer dissociation (ETD) tandem mass spectrometry (LC ETD MS/MS). ETD retains labile post-translational modifications on peptide backbone fragments, thereby enabling localization of sites of modification.[32] The results were compared with those obtained following trypsin digestion of β-casein and subsequent LC ETD MS/MS. Observed phosphopeptides (and observed unmodified counterparts) are summarized in Table 1, with annotated mass spectra of the phosphopeptides shown in Supplementary Figure 7. No phosphopeptides were identified following SCW treatment at 160 °C for 20 min or at temperatures above 160 °C.

In the trypsin-digested β-casein sample, one phosphosite (Ser35) was identified, belonging to the peptide FQsEEQ-QQTEDELQDK (s is phosphoserine). This phosphopeptide was observed in each of three repeats. The remaining phosphosites fall within the tryptic peptide ELEELNVP-GEIVEsLsssEESITR; however, that peptide was not detected in any of the repeats. The peptide comprises a high number of glutamic acid residues, in addition to four acidic phosphate groups, and does not favor formation of positive ions.

In contrast, phosphopeptides containing all of the phosphosites (Ser15, Ser17, Ser18, Ser19, and Ser35) were identified following SCW treatment at 160 °C for 0 min. Seven phosphopeptides were identified across three repeats. Two peptides contained pSer35, and a further two contained pSer15. One peptide contained pSer15, pSer17, and pSer18. One peptide containing Ser 15 and Ser17 was identified but was observed to be modified at Ser17 only. Finally, a doubly phosphorylated peptide was observed which contained four potential modification sites (Ser15, Ser17, Ser18, and Ser19). The two phosphosites could be localized to Ser17, Ser18, and Ser19 but could not be unambiguously assigned due to lack of cleavage between the serine residues. The observation of phosphopeptides with under-occupancy of known modification sites, together with the observation of unmodified peptides, suggests that SCW treatment can result in removal of phosphorylation. This process is exacerbated under harsher SCW conditions: No phosphopeptides were identified from samples treated for 20 min at 160 , 207, 253, or 300 °C. Nevertheless, under milder conditions, sufficient phosphorylation is retained to identify all modification sites.

CONCLUSION

We have shown that SCW hydrolysis of proteins followed by LC-MS/MS of the peptide products results in high protein sequence coverages. Sequence coverages obtained were comparable to those obtained with trypsin. The majority of the experiments described here used 15 mg of starting protein (1 mg/mL); however, the results also showed that SCW treatment of 150 μg of protein (0.01 mg/mL) gave very high protein sequence coverage (>90%). Interestingly, despite the high sequence coverage, the percentage of peptide spectral matches, i.e., MS/MS spectra that were confidently assigned to a peptide sequence, was low (<7%). That suggests that in addition to hydrolysis of the peptide bond cleavage is occurring elsewhere in the protein, presumably in the amino acid side chains. Further work to investigate this hypothesis is ongoing.

SCW treatment under mild conditions (160 °C; 0 or 20 min) shows favored cleavage of the Asp-X bond. That observation can be explained by a weak acid hydrolysis mechanism. Under slightly harsher conditions, favored cleavage of the Glu-X bond is observed in some cases (β-casein 160 °C for 20 min and 207 °C for 20 min; BSA 207 °C for 20 min).

This observation may be due to the increased ion product at higher temperature compensating for the higher pK_a of the glutamate.

SCW treatment resulted in very limited cleavage of disulfide bonds. To achieve high protein sequence coverages, it was necessary to reduce the disulfide bonds and alkylate the cysteine residues either prior to or post-SCW treatment. Under mild SCW conditions, phosphorylation generally remains on the peptide hydrolysis products, and all known phosphorylation sites were identified in β-casein; however, there was some evidence for dephosphorylation. In summary, subcritical water hydrolysis has the potential to be an efficient, cheap, and fast alternative to enzymatic digestion of proteins.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.anal-chem.6b01013.

> Photograph of SCW reaction tube; plot of % peptide spectral matches following protein database search versus treatment conditions; summed survey scan mass spectra from LC MS/MS analysis of SCW treated hemoglobin; plots of % of peptides identified versus C-terminal amino acid residue; peptide summary maps; amino acid sequence of β-casein; ETD MS/MS spectra of phosphopeptides from β-casein (PDF)

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Notes
The authors declare no competing financial interest.

ACKNOWLEDGMENTS

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