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Pressure-assisted sample preparation for proteomic analysis

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Abstract

Pressure-assisted digestion of proteins, also known as pressure cycling technology (PCT), using a Barocycler NEP 2320 was compared with the conventional method using atmospheric pressure. Our objective was to demonstrate that PCT provides more controlled enzymatic digestion of proteins than prolonged digestion at atmospheric pressure ranging from 18 to 24 h. More controlled digestion would be beneficial for studies of highly posttranslationally modified protein such as histones. For the comparison of these two techniques, recombinant and native histone H4 were used as model proteins. PCT was optimized for pressure and time, and it was found to be most effective at 15 kpsi for 120 min of incubation. In conclusion, the PCT method was found to be much faster than using atmospheric pressure. PCT was also found to allow for unambiguous control of digestion parameters and to provide a high yield of sequence coverage compared with atmospheric pressure.

Keywords

Histones; Proteomics; High-performance liquid chromatography; Barocycler NEP 2320; Sample preparation

Sample preparation for high-throughput analyses of proteins is at the center of proteomic profiling experiments [1–4]. The multi-step nature of proteomic studies makes reproducibility, standardization, and normalization key to reducing analytical variability. Other than intact protein analysis and the rarely used chemical methods for protein digestion, the majority of proteomic experiments contain a step involving the enzymatic digestion of proteins for mass spectrometry (MS)¹-based protein identification and quantitation. It is important to note that each type of proteolytic digestion can yield a different number and type of peptides, thereby limiting the scope of information related to the structure and function of the protein of interest [4]. Therefore, the concept of a universal sample preparation method has several limitations, thereby driving technological and methodological development to create new avenues for enhancing sample preparation.

Conventional enzymatic digestion protocols typically employ an overnight reaction to ensure complete digestion of peptides. Still, samples can contain cleavage sites that are missed during digestion. Namely, a reduced rate of specific peptide bond hydrolysis and/or the presence of posttranslational modifications (PTMs) contribute to this effect [5]. Several techniques, such as high-intensity focused ultrasound (HIFU) [6,7], microwave radiation

[8,9], and performing the digestion under conditions of high pressure [10,11], have been developed and modified to overcome these digestion shortcomings and to decrease the time needed for complete digestion of the protein. The technique involving high-pressure conditions applies pressure up to 40 kpsi and appears to provide improved results as compared with HIFU and microwave radiation. HIFU is based on precise modulation of ultrasound, which was a significant problem in the past; however, current ultrasound techniques have found application (e.g., electrical impedance tomography, optical tomography) [12]. On the other hand, reactions controlled by microwave radiation are so rapid that the generated reaction heat can lead to by-product formation and, in some cases, to degradation. The limitations of HIFU and microwave radiation have prompted us to test whether pressure cycling technology (PCT) can be used sufficiently for accelerated enzymatic digestion and be applicable in proteomics.

The use of PCT for enhancing enzyme activity was introduced during the early 1990s. However, the development of new instrumentation resulted in more user-friendly methods, leading to development of new applications [13,14]. Here, we evaluate the Barocycler NEP 2320 (Pressure Biosciences, South Easton, MA, USA) as a platform for analyzing the enzymatic digestion of proteins under high pressure. The Barocycler NEP 2320 applies PCT to accelerate enzymatic digestion of samples, which in turn are used for analytical purposes. In the current study, histone H4 is used as the model protein. Histones, in addition to their functions in epigenetic regulation, represent a class of proteins that can carry multiple homo- and heterogeneous PTMs, thereby altering susceptibility for enzymatic digestion, which subsequently causes additional difficulties in profiling studies. Using PCT, we were able to reduce the time of enzymatic digestion of histone H4 from 18 h to 120 min while maintaining 100% of sequence coverage identified by tandem MS (MS/MS) after digestion under atmospheric pressure.

Materials and methods

Reagents

High-performance liquid chromatography (HPLC) gradient-grade acetonitrile (ACN) and water were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Trifluoroacetic acid (TFA, Reagent-Plus, 99%), urea, ammonium bicarbonate (ReagentPlus, 99%), iodoacetamide (IAA, Sigma Ultra), and α -cyano-4-hydroxycinnamic acid (CHCA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Molecular-grade dithiothreitol (DTT) and sequencing-grade chymotrypsin were purchased from Promega (Madison, WI, USA). Recombinant human histone H4 (P62805) was purchased from New England Biolabs (Ipswich, MA, USA). Primary human monocytes, obtained by elutriation from healthy donors as described in Refs. [15,16], were used to isolate histone fractions using a Qproteome Nuclear Protein Kit (Qiagen, Hilden, Germany). The protein fractions obtained using Qproteome were quantitated using a Pierce 660-nm protein assay and prediluted protein standards (bovine serum albumin, Thermo Scientific, Rockford, IL, USA).

Histone fractionation

The histones were isolated from the primary human monocytes using the Qproteome kit from Qiagen. The isolated histones were further fractionated using a reversed phase HPLC (RP-HPLC) system (Shimadzu Scientific Instruments, Columbia, MD, USA), which was equipped with an ultraviolet (UV) detector and Shimadzu LC Solution software for data acquisition. Chromatographic separations of 100 μ g of proteins per injection were performed using an analytical HPLC C4 column (250 $\times$ 4.6 mm, d_p = 5 μ m, 300 Å) from Advanced Chromatography Technologies (Aberdeen, Scotland). Mobile phase A was 5% ACN in water plus 0.1% TFA, and mobile phase B was 90% ACN plus 0.1% TFA.

Fractionation was initiated with 100% mobile phase A for 5 min. From 5 to 15 min, mobile phase B was ramped up to 35% and then held continuously for 10 min. From 25 to 100 min, the amount of solvent B was increased to 65%, followed by ramping up to 100% in 20 min. This condition was maintained for 5 min, and then the amount of solvent B was decreased to 0% in 5 min and held for 145 min.

Enzymatic protein digestion

Proteolytic digestion under atmospheric conditions was performed for at least 18 h but no longer than 24 h. In this article, enzymatic digestion under atmospheric pressure is referred to as the “conventional method.” In this study, we compared digestion assays of recombinant and native human histone H4 using the conventional method compared with PCT using a Barocycler NEP 2320. After completing digestions of recombinant protein, an AB SCIEX 4800 matrix-assisted laser desorption/ionization–tandem time-of-flight (MALDI–TOF/TOF) mass spectrometer (Framingham, MA, USA) was used to analyze resulting peptides. A nano liquid chromatography (nanoLC) LTQ–Orbitrap mass spectrometer was used to analyze peptides from digestions of native histone H4 isolated from human monocytes.

Conventional method of proteolytic digestion

Dried human histone H4 was dissolved in 20 μ l of 8 M urea and 0.4 M NH_4HCO_3 , and the pH was adjusted to between 7.5 and 8.5. Proteins were reduced by the addition of 5 μ l of 45 mM DTT and incubated for 15 min at 50 $^\circ\text{C}$. After cooling to room temperature, the sample was alkylated through the addition of 5 μ l of 100 mMIAA and incubated in the dark at room temperature for 15 min. Following alkylation, the digestion buffer was diluted with water to a final concentration of 2 M urea and 0.1 M NH_4HCO_3 . Chymotrypsin was added to the sample in a ratio of 1:4 (w/w) enzyme to protein. Digestion occurred at 37 $^\circ\text{C}$ for 18 h. Next, the reaction was quenched by acidifying the sample through the addition of 50 μ l of TFA.

PCT digestion

All steps of the tryptic digestion protocol preceding incubation (ambient or high pressure) were identical for the Barocycler NEP 2320 and conventional methods. However, after the sample was alkylated, in the case of using the Barocycler NEP 2320, samples were transferred to PCT MicroTubes and placed in the metal holder component of this instrument. After digestion, samples were acidified using 50 μ l of TFA to quench the reaction.

Mass spectrometry

MALDI–TOF/TOF—In preparation for MS analysis, peptides resulting from PCT digestion of recombinant human histone H4 (1 μ g) were desalted using reversed phase ZipTip pipette tips with 0.2 μ l of C18 resin (Millipore, Billerica, MA, USA). For sample analysis, an AB SCIEX 4800 MALDI–TOF/TOF mass spectrometer was used. First, 1 μ l of digested sample was spotted onto a MALDI plate and cocrystallized with 1 μ l of CHCA matrix (5 mg/ml in 50% ACN and 0.1% TFA). The droplets were dried in a desiccator under decreased pressure. Spectra acquisition and data processing were performed using 4000 series Explorer software version 3.5.1 (AB SCIEX); the software settings were in a reflectron-positive mode at fixed laser intensity with a low-mass gate and delayed extraction. Peptide masses were acquired for m/z values ranging from 800 to 2500 Da. MS spectra were summed from 1000 laser shots by an Nd-YAG laser operating at 355 nm and 200 Hz. MS/MS spectra were acquired in 1 kV positive mode. The 1000 shots were summed in increments of 50. Database searches were performed on the SwissProt database using Mascot MS/MS Ion Search (<http://www.matrixscience.com>). The search parameters were as follows: (i) carbamidomethylation on cysteine and oxidation on methionine were variable modifications;

(ii) three missed chymotrypsin cleavage sites were permitted; (iii) the mass accuracy tolerance for the peptide (MS) was set at 15 ppm and for fragments (MS/MS) was set at 0.5 Da.

ESI-LC-MS/MS—Histone H4 isolated and fractionated from human monocytes was digested using both conventional and PCT methods. After digestion, and prior to analysis, samples were desalted using reversed phase ZipTip pipette tips with 0.2 μ l of C18 resin as per the manufacturer's protocol (Millipore). Next, samples were analyzed using a high-resolution mass spectrometry electrospray ionization (ESI)-LC-MS/MS system in a nanospray configuration (LTQOrbitrap, Thermo Scientific, West Palm Beach, FL, USA) coupled with a nanoLC system (TEMPO nano MDLC System, AB SCIEX) and using a microcapillary RP-C18 column (New Objectives, Wo-burn, MA, USA). The database search was performed using Proteome Discoverer 1.2 software (Thermo Scientific). The search parameters were as follows: (i) carbamidomethylation on cysteine and oxidation on methionine were variable modifications; (ii) three missed chymotrypsin cleavage sites were permitted; (iii) the mass accuracy tolerance for the peptide (MS) was set at 15 ppm and for fragments (MS/MS) was set at 0.5 Da.

Results and discussion

Pressure optimization

To optimize the pressure and time for the digestion for recombinant human histone H4, we performed multiple digestions at different pressures and for different time points (see Tables 1 and 2 below). Experiments were performed in triplicate. Based on manufacturer recommendations, we selected the following three pressure settings: 10, 15, and 25 kpsi. Cycling consisted of 30 cycles at 1 min each; each cycle was run for 50 s at the pressure setting of interest, followed by 10 s of stabilization at atmospheric pressure. The temperature was ambient, and chymotrypsin was used at a concentration of 0.5 μ g/ μ l. The resulting digest of the protein was analyzed using an AB SCIEX 4800 MALDI-TOF/TOF mass spectrometer for the recombinant histone H4.

The digest of recombinant histone H4 revealed seven peptides and constituted a nearly full sequence except for the first amino acid and the C-terminal fragment, a $^{100}\text{GFGG}^{103}$ (MW = 336.14) tetrapeptide. The peak list, created by 4000 series Explorer software, was used in parallel to acquire MALDI-TOF/TOF data. This data consisted of (i) the m/z ratio (including an average mass with lower and higher m/z recordings), (ii) the peak height (peak intensity), (iii) the signal/noise ratio, (iv) resolution, and (v) the area under the peak. The peak with highest intensity represents 100%.

Considering that in each experiment the only variable parameter is the applied pressure, we theorized that peak intensity would reflect the relative change in the amounts of each peptide generated by such digestion, thereby more of any given peptide in the mixture. The m/z peak of such a peptide is expected to have higher intensity. Therefore, we elected to demonstrate pressure optimization based on the relative comparison of peak intensities for each peptide generated using pressures of 10, 15, and 25 kpsi.

Results for all peptides are summarized in Table 1, and Fig. 1 is a bar graph comparing the changes in peak intensity for the two N-terminally located peptides that are postulated to be of the greatest biological importance. In all three samples (10, 15, and 25 kpsi), the peptide $^{90}\text{ALKRQGRTLY}^{99}$ showed the highest intensity, thereby setting up 100%, which were 0.24 1.2, and 1.3×10^4 , respectively. Based on these data, we postulated that we did not reach the signal saturation for this mixture of peptides under our experimental conditions, thereby allowing us to make relative comparisons. Comparisons should be

performed with caution and can be performed for only a single peptide at three digestion conditions (horizontally, e.g., for one given peptide), assuming that the rate of ionization remains constant and signal suppression from other peptides is negligible.

Based on the data presented, it was determined that a pressure of 10 kpsi was too low, as reflected by the low signal intensity overall and for each of the seven peptides. When comparing peak intensities for pressures of 15 and 25 kpsi, results indicate that 15 kpsi was beneficial for the signal intensity of peptides 1, 2, and 5 (see Table 1), which were further digested and/or degraded at 25 kpsi. If low-specificity chymotrypsin was used, we would expect that the peptide ⁷⁴TEHAKRKTVTAMDVVY⁸⁹ would be further fragmented into the following three peptides: ⁷⁴TEH⁷⁶, ⁷⁷AKRKTV-TAM⁸⁵, and ⁸⁶DVVY⁸⁹. This fragmentation is expected to result in a drop in the peak intensity for this particular intact peptide. For example, when low-specificity chymotrypsin is used, the peptide ²SGRGKGGKGLGKGGAKRHRKVL²³ could be further fragmented at 25 kpsi into the following three peptides: ²SGRGKGGKGL¹¹, ¹²GKGGAKR¹⁸, and ¹⁹HRKVL²³. Because this was not observed, we conclude that susceptibility of peptide bonds cleaved under PCT conditions is, to some extent, selective. This selective cleavage could be advantageous if one investigates proteins with point mutations, splice variants, and/or isoforms and when the aim of the experiment is not only to have maximal sequence coverage but also to have overlapping sequences. For other peptides, a pressure of 25 kpsi appears to be optimal.

It has been postulated that, from a biological point of view, the most important part for all histones is the N-terminus end (also called the “histone tail” [17]). This end is arbitrarily set to be approximately 30% of sequence from the N-terminal end of histones [18]; in histone H4, this would be the first 38 amino acids. The N-terminus portion of histone has been shown to play an important role in chromatin remodeling and transcription regulation. In summary, we conclude that 15 kpsi is the optimal pressure to digest histones for further studies.

Time optimization at 15 kpsi

Based on reasoning presented above with respect to fragmentation and pressure, we attempted to optimize digestion time at a pressure of 15 kpsi. For this part of data analysis and interpretation, we used the same criteria as we used for analysis applied to pressure optimization as described in the previous section. Results for all peptides are presented in Table 2, and Fig. 2 is a bar graph comparing the changes in peak intensity for the two N-terminally located peptides that are postulated to be of the greatest biological importance. For peptides 1, 2, 3, and 5, the optimal time appears to be 120 min; when extended, this leads to a decrease in the signal intensity. The decrease in the peak intensity for the peptides shown in Table 2 would again suggest that a longer period of time pushes the digestion reaction to yield smaller peptides.

In summary, we optimized the conditions for chymotrypsin digestion of recombinant human histone H4 as 15 kpsi for 120 cycles. Each cycle was 1 min long and consisted of 50 s of high pressure and 10 s of atmospheric pressure.

Summary of PCT using chymotrypsin

Fig. 3 shows a representative spectrum of MALDI-TOF/TOF spectrometric analysis of fragments generated from chymotrypsin digestion of recombinant histone H4 using the optimal conditions established by this study. Table 3 shows sequences of peptides, which provide nearly complete sequence coverage. It is important to note that we were able to sequence the entire N-terminal end (histone tail) of histone H4. We also performed MS/MS

analysis of five minor peaks with m/z 864.9, 1021.5, 1798.3, 2108.0, and 2331.1, but we were unable to assign them to any part of the histone H4 sequence, concluding that they are most likely part of contamination.

RP-HPLC fractionation of intact histones

After optimization of the PCT method using recombinant histone H4, we moved to the next step involving intact histones isolated from biological material. The source of histones used for this study was human monocytes obtained from elutriated blood of healthy donors (see Materials and methods). For preparation, histones were subjected to RP-HPLC fractionation (Fig. 4), followed by identification of collected fractions using MS. Histones H2B, H4, H2A, and H3 were eluted as separate peaks between 50 and 70 min (Fig. 4 inset). Peaks appear to be broad, which is likely due to small differences in the elution time for posttranslationally modified forms rather than due to chromatographic conditions. Histone H1 was eluted prior to the 50-min time point; however, it was not collected for the purpose of this study. A relatively broad fraction containing histone H4 was collected, as shown in the box in the Fig. 4 inset, and used for further experiments.

Chymotryptic digestion of native histone H4 using conventional and PCT methods

Equal amounts of the HPLC fractions containing histone H4 were digested using either optimized PCT or the conventional method (i.e., atmospheric pressure). Peptides resulting from these digestions were analyzed using an LTQ-Orbitrap in a nanoLC-ESI-MS/MS configuration. The reason we used MALDI-TOF/TOF for digested recombinant histone H4 and used ESI-MS/MS for histone H4 purified from monocytes was because we expected a higher complexity in histone H4 from monocytes compared with the recombinant sample. Both digestion conditions yielded 75% sequence coverage (Fig. 5), with ²SGRGKGGKGLGKGGAKRHRKVL²³ being the only missing peptide. This was expected, to some extent, because the N-terminal tail of histone H4 is extensively posttranslationally modified. Such modifications can lead to a digestion that differs from nonmodified proteins.

Conclusions

The major advantage of the PCT method using the Barocycler NEP 2320 is that it significantly reduces the time necessary to proteolytically digest proteins. For example, using the PCT method, proteins can be digested in 120 min at 15 kpsi instead of 18 h at atmospheric pressure. Other than the difference in the time for digestion, our experiments did not show significant advantages of this technology that would further enhance proteomic experiments. However, it is important to note that our experimental approach focused on only one protein, and not additional proteins that are difficult for proteolytic digestion, thereby limiting the scope of this study. For example, this technology may be beneficial for tests involving highly glycosylated proteins such as mucins. Another limitation in our study is that the only proteolytic enzyme tested was chymotrypsin. Additional proteolytic enzymes are available and should be tested in the future.

One observation from this study was that PCT using a Barocycler NEP 2320 technology platform did not show a substantial qualitative difference between 10 and 15 kpsi, and if based on peak intensity, 15 kpsi provided a more intact protein to be digested. However, there was a decrease in major peptides measured by peak intensity under 25 kpsi, which may indicate that proteolytic digestion was further forced at this pressure to yield smaller fragments. We find this phenomenon to be interesting for further explorations because it may be very useful in studies where the in-depth analysis of splice variants or isoforms is of interest. Overall, we conclude that PCT is a promising technique for more controlled sample

preparation, as compared with arbitrary overnight incubation, and clearly reduces time for this step. However, additional efforts are needed to refine the experimental protocols, and more formal studies need to be performed.

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1 Abbreviations used

MS	mass spectrometry
PTM	posttranslational modification
HIFU	high-intensity focused ultrasound
PCT	pressure cycling technology
MS/MS	tandem MS
HPLC	high-performance liquid chromatography
ACN	acetonitrile
TFA	trifluoroacetic acid
IAA	iodoacetamide
CHCA	α -cyano-4-hydroxycinnamic acid
DTT	dithiothreitol
RP-HPLC	reversed phase HPLC
UV	ultraviolet
nanoLC	nano liquid chromatography
MALDI-TOF/TOF	matrix-assisted laser desorption/ionization–tandem time-of-flight
ESI	electrospray ionization

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Fig.1. Pressure optimization at 30 min for 10, 15, and 25 kpsi for two peptides from the N-terminal tail portion of recombinant human histone H4.

Fig.2. Time optimization at 15 kpsi for 30, 120, and 200 min for two peptides from the N-terminal tail portion of recombinant human histone H4.

Fig.3. MALDI-TOF mass spectrum of recombinant human histone H4 digested using the PCT method. The numbered peaks match those generated by conventional chymotrypsin digestion, providing 95% sequence coverage, and are listed in Table 3.

Fig.4. RP-HPLC chromatogram showing fractionation of histones using UV detector set at 214 nm. The inset shows an enlarged range of the peptides containing peaks assigned to respective histones.

Fig.5.
Comparison of peptide identification using digestion on high (Barocycler NEP 2320) pressure and atmospheric (conventional) pressure: (A) conventional digestion; (B) histone H4 sequence (P62805 H4_HUMAN); (C) PCT digestion.

Table 1
Optimization of PCT pressure based on relative intensity of MALDI-TOF/TOF peptide peaks.

Number	Molecular mass (Da)	Peptide and its position in protein sequence	Peak height (peak intensity)		
			10 kpsi	15 kpsi	25 kpsi
1	2203.08	² SGRGKGGKGLGKGAKRRHKVL ²³	129 ± 3.88	120 ± 6.21	23 ± 1.53
2	1750.86	²⁴ RDNIQGITKPAIRRL ³⁸	637 ± 17.53	1749 ± 172.21	918 ± 47.33
3	1268.78	³⁹ ARRGGVKRISGL ⁵⁰	13 ± 0.67	26 ± 1.07	47 ± 1.51
4	1078.56	⁵¹ IYEETRGVL ⁵⁹	19 ± 1.60	23 ± 1.45	39 ± 2.46
5	1666.74	⁶⁰ KVFLENVIRDAV ⁷³	131 ± 8.99	1275 ± 118.96	553 ± 32.57
6	1848.77	⁷⁴ TEHAKRKTVTAMDVVY ⁸⁹	230 ± 14.28	1458 ± 119.70	1549 ± 148.86
7	1205.57	⁹⁰ ALKRQGR ⁹⁹	2320 ± 277.70	11139 ± 1474.80	12241 ± 1221.65

Table 2
Optimization of PCT time based on relative intensity of MALDI-TOF/TOF peptide peaks.

Number	Molecular mass (Da)	Peptide and its position in sequence	Peak height (peak intensity)		
			30 min	120 min	200 min
1	2203.08	² SGRGKGKGLGKGAKRRHKVL ²³	120 ± 6.21	454 ± 22.84	23 ± 2.00
2	1750.86	²⁴ RDNIQGITKPAIRRL ³⁸	1749 ± 172.21	5126 ± 472.10	2606 ± 144.99
3	1268.78	³⁹ ARRGGVKRISGL ⁵⁰	26 ± 1.07	93 ± 2.04	44 ± 1.42
4	1078.56	⁵¹ IYEETRGVL ⁵⁹	23 ± 1.45	29 ± 1.53	41 ± 2.60
5	1666.74	⁶⁰ KVFLENVIRDAVTY ⁷³	1275 ± 118.96	2612 ± 111.01	1996 ± 148.70
6	1848.77	⁷⁴ TEHAKRKTVTAMDVVY ⁸⁹	1458 ± 119.70	2590 ± 212.12	5276 ± 508.08
7	1205.57	⁹⁰ ALKRQGRITLY ⁹⁹	11139 ± 1474.80	9013 ± 1080.66	13520 ± 1781.94

Table 3

Peptides generated using conventional chymotrypsin digestion of recombinant human histone H4.

Number	Peptide sequence	Position	Molecular mass (Da)
1	SGRGKGGKGLGKGGAKRHRKVL	2–23	2203.08
2	RDNIQGITKPAIRRL	24–38	1750.86
3	ARRGGVKRISGL	39–50	1268.78
4	IYEETRGVL	51–59	1078.56
5	KVFLENVIRDAVTY	60–73	1666.74
6	TEHAKRKTVTAMDVVY	74–89	1848.77
7	ALKRQGRTLY	90–99	1205.57
8 [*]	RDNIQGITKPAIR	24–36	1481.80
9 [*]	ARRGGVKRISGLIY	39–52	1523.66
10 [*]	LENVIRDAVTY	63–73	1292.53

^{*} Overlapping peptides.