Songklanakarin J. Sci. Technol.
43 (5), 1443-1448, Sep. - Oct. 2021

http://www.sjst.psu.ac.th

Original Article

Separation of diethylpyrocarbonate-modified histidine-containing peptides using strong cation exchange chromatography

Pongsakorn Thawonpan[1], Supinya Thanapongpichat[2], Watthanachai Jumpathong[3], Aung Win Tun[4], Churat Weeraphan[5], Worrawit Wanitsuwan[6, 7], and Hansuk Buncherd[2, 7*]

[1] *Department of Molecular Biotechnology and Bioinformatics, Faculty of Science, Prince of Songkla University, Hat Yai, Songkhla, 90110 Thailand*

[2] *Faculty of Medical Technology, Prince of Songkla University, Hat Yai, Songkhla, 90110 Thailand*

[3] *Program on Chemical Biology, Chulabhorn Graduate Institute, Lak Si, Bangkok, 10210 Thailand*

[4] *Faculty of Graduate Studies, Mahidol University, Phutthamonthon, Nakhonpathom, 73170 Thailand*

[5] *Laboratory of Biochemistry, Chulabhorn Research Institute, Lak Si, Bangkok, 10210 Thailand*

[6] *Faculty of Medicine, Prince of Songkla University, Hat Yai, Songkhla, 90110 Thailand*

[7] *Medical Science Research and Innovation Institute, Prince of Songkla University, Hat Yai, Songkhla, 90110 Thailand*

Received: 15 July 2020; Revised: 17 November 2020; Accepted: 27 November 2020

Abstract

This work focuses on the separation of diethylpyrocarbonate-modified histidine-containing peptides by strong cation exchange (SCX) chromatography prior to mass spectrometric analysis. The decrease in positive charge of diethylpyrocarbonate-treated imidazole in histidine, masked by the incorporation of the carbethoxyl group, provides us with an opportunity to use SCX for separating diethylpyrocarbonate-modified histidine-containing peptides from the unmodified ones. In this report, the diethylpyrocarbonate-modified histidine-containing peptide is eluted faster than the untreated peptide. Furthermore, the carbethoxy group can be effectively removed to generate the unmodified imidazole in the histidine residue by neutral hydroxylamine.

Keywords: histidine modification, diethylpyrocarbonate, strong cation exchange chromatography, imidazole ring, carbethoxyimidazol

1. Introduction

Histidine is an essential amino acid containing imidazole ring, the functional group which can serve as an acid–base catalyst commonly present at the active site of several enzymes (Rebek, 1990). A number of studies on the chemical modification of the imidazole ring have suggested the importance of histidine residues in the catalytic mechanisms of enzymes (Lundblad, 2016). Diethylpyro carbonate is one of the most widely used histidine-specific reagents (Follmer & Carlini, 2005; Li, Oyala, Britt, Weintraub, & Hunsicker-Wang, 2017; Sasaki *et al.*, 2014;

*Corresponding author
Email address: hansuk.bu@psu.ac.th

Zhang *et al.*, 2016). The reaction of diethylpyrocarbonate with histidine residues results in a carbethoxylated derivative at the N-omega-2 nitrogen of the imidazole ring, N-carbethoxyimidazole, which prevents the protonation of histidine residues at a low pH (Da Poian, Carneiro, & Stauffer, 2005). The modification can be reversed by treatment with a base or a nucleophile such as hydroxylamine at neutral pH (Hunsicker-Wang & Konkle, 2018). The determination of diethylpyrocarbonate-modified histidine residues in proteins is typically achieved by mass spectrometric (MS) analysis (Barth, Bender, Kundlacz, & Schmidt, 2020; Jin, Abe, Li, & Hamasaki, 2003; Schmidt *et al.*, 2017; Willard & Kinter, 2001). However, the abundance of carbethoxy-modified peptides is relatively low compared to that of unmodified species, so that reproducible detection of the modified peptide is hampered. To be noted, the low stoichiometry of the target species is listed as one of the most common challenges related to biomolecule analysis (Ongay, Boichenko, Govorukhina, & Bischoff, 2012; Prasanna, Sidhik, Kamalanathan, Bhagavatula, & Vijayalakshmi, 2014; Thawornpan *et al.*, 2019; Thawornpan *et al.*, 2020) . To overcome the problem, isolation of diethylpyrocarbonate-modified peptides prior to MS analysis is required (Camerini & Mauri, 2015).

Several approaches have been developed for selective isolation of histidine-containing peptides. In general, there are two main analytical platforms for the enrichment of histidine-containing peptides. One approach involves immobilized-metal affinity chromatography (IMAC) using metal ions, e.g., nickel (II) (Kozlov *et al.*, 2007), copper (II) (Ren, Penner, Slentz, Mirzaei, & Regnier, 2003), or palladium (II) (Kikot, Polat, Achilli, Fernandez Lahore, & Grasselli, 2014). This technique is widely adopted due to its high enrichment efficiency and ease of implementation. However, analytical limitations of IMAC have been reported. Metal leaching can cause metal ion contamination in the isolated fraction (Cheung, Wong, & Ng, 2012). Also, destruction of side chain of a certain amino acids, e.g. lysine and histidine, can be observed due to metal-catalyzed oxidation reactions (Cheung *et al.*, 2012). An alternative approach relies on a selective chemical modification, i.e., sulfonation, at the N-terminal of peptides, leading to a change in the charge state of histidine-containing peptides at pH < 3.0 (Cao Dong, 2009). The resulting sulfonated histidine-containing peptides are then isolated from tryptic peptide mixture by strong cation exchange (SCX) chromatography. By using SCX chromatography, peptides are primarily separated based on the number of protonated functional groups, e.g., primary amine groups at the N-termini and imidazole group of histidine. A change in the number of functional groups can significantly affect SCX chromatographic behavior of the peptides, usually causing a retention time shift. Sulfonation coupled with SCX chromatography is a feasible approach to capturing histidine-containing peptides from complex mixtures, enabling potentially large-scale applications. However, the sulfonation at the N-terminal of peptides is irreversible, thereby limiting subsequent applications of the enriched peptides. It is noteworthy that the shift phenomenon of the peptides caused by target reaction has been applied to isolating target molecules for decades (Buncherd *et al.*, 2016; Staes *et al.*, 2017). To the best of our knowledge, a selective isolation technique for diethylpyrocarbonate-modified histidine-containing peptides is not yet available. In the present study, therefore, the advantages of diethylpyrocarbonate treatment and SCX chromatography were combined for isolation of diethylpyrocarbonate-modified histidine-containing peptides. The effectiveness of diethylpyrocarbonate modification and the chromatographic behavior of diethylpyrocarbonate-modified and unmodified histidine-containing peptides were characterized. The position of the diethylpyrocarbonate-modified residue was determined by mass spectrometric analysis. In addition, reversal of the chemical modification was studied by hydroxylamine treatment.

2. Materials and Methods

2.1 Chemicals and reagents

Diethylpyrocarbonate, hydroxylamine, and all reagents for trypsin digestion were purchased from Sigma (St. Louis, MO, USA). Trifluoroacetic acid (TFA) was from Applied Biosystems (Foster City, CA, USA), and micro C18 ZipTip pipette tips were obtained from Millipore (Bedford, MA, USA), C-18 TopTip column was purchased from Glygen (MD, USA) and a model peptide was purchased from Chempeptide (Shanghai, China).

2.2 Carbethoxylation of histidine side chain

The model peptide was incubated with diethylpyrocarbonate solution, pH 6, at molar ratio 5:1 (diethylpyrocarbonate:His), for 30 min at 25ºC. The resulting peptide was then desalted again on C18 reversed-phase TT2 top tips (Glygen), eluted with 50% acetonitrile, and dried in a vacuum centrifuge.

2.3 Strong cation exchange chromatography of peptides

Peptides were diluted with 50 µL of Solvent A (50 mM KCl, 10 mM potassium phosphate buffer (pH 2.9) in 20% acetonitrile). A volume of 50 µL was loaded on a PolySULFOETHYL Aspartamide column (2.1 mm ID, 10 cm length; PolyLC Inc., Columbia, USA) operated on an Ultimate HPLC system (LC Packings, Amsterdam, The Netherlands). Elution, at a flow rate of 0.1 mL/min, was performed using an isocratic pattern with 50 mM KCl, 10 mM potassium phosphate buffer (pH 2.9) in 20% acetonitrile for 20 min. After that, linear gradient from 50 to 250 mM KCl in Solvent A (Solvent B) over 20 min was followed by a gradient over 3 min to 500 mM KCl in Solvent A (Solvent B). A UV detector was used to detect absorbance at 214 nm for the effluent. After loading, effluent was manually collected in two fractions representing the following two time windows: 0–25 min and 25–35 min. Peptide fractions were desalted on C18 reversed-phase TT2 TopTip, eluted with 0.1% TFA in 50% acetonitrile, and dried in a vacuum centrifuge.

For secondary SCX runs, after diethylpyro carbonate-modification (as described below), the peptides were then dissolved in 10% SCX buffer B just before loading for the secondary SCX runs. Elution occurred under the same conditions as in the primary SCX run. Fractions were collected as described in the primary SCX. For LC-MS/MS analysis, the collected effluents of each secondary SCX run

were lyophilized, solubilized in 0.1% TFA, and desalted using C18 Ziptips (Millipore, Billerica, USA). Peptides were then eluted from the Ziptips with 0.1% TFA in 50% acetonitrile and dried in a vacuum centrifuge.

2.4 Mass spectrometric analysis

Mass spectrometric analysis of the peptide samples was performed with an amaZon Speed Iontrap having a Captive Spray ion source (Bruker) coupled with an EASY-nLC II (Proxeon, Thermo Scientific) chromatographic system. Peptides of each fraction were injected and separated with an eluent flow of 300 nL/min on an Acclaim Pepmap C18 (75µm i.d. × 25 cm, 2 cm precolumn) using a 50 min gradient of 0–50% acetonitrile and 0.1% formic acid. Some selected MS/MS instrument parameters were as follows: MS mass range, m/z 400–1500; MS/MS mass window, from m/z 100 to detected precursor ions; precursor mass window selection, m/z 4.0; number of precursor ion selections, 5; CID in Smart Frag mode with variable energy; data-dependent acquisition with active exclusion after 1 spectrum with release after 30 s.

Raw MS/MS data of the 2-D SCX peptide fractions were processed with the MASCOT DISTILLER program, version 2.4.3.1 (64 bits), MDRO 2.4.3.0 (Matrix Science, London, UK). Peak picking for MS and MS/MS spectra was optimized for a mass resolution of up to 3500 (m/Δm) and 2500, respectively. Peaks were fitted to a simulated isotope distribution with a correlation threshold of 0.7 and with a minimum signal-to-noise ratio of 2.

3. Results and Discussion

3.1 Chemical modification of histidine-containing peptide with diethylpyrocarbonate

To study the reaction of diethylpyrocarbonate with histidine-containing peptides, acetylated (ac)-VGHDYETVSR, was used as a model. The model peptide was treated with diethylpyrocarbonate using the conditions described in the method section. The efficiency of the reaction was evaluated by the conversion of ac-VGH*DYETVSR. After incubation with diethylpyrocarbonate at 25 °C for 30 min, the complete conversion of the peptide precursor was observed (data not shown), indicating the high efficiency of nucleophilic acyl substitution of histidine with the reagent. The peptide fragment fingerprinting analysis of the doubly charged material with m/z 638.82 was unequivocally identified as derived from ac-VGH*DYETVSR (Figure 1). The modification of imidazole side chain to N-carbethoxyimidazole of the histidine at y_8 and b_3 ion increased molecular mass (72.02 Da) from that of the unmodified form, with a substitution at either of the nitrogen positions (N_1 or N_3) on the imidazole ring. However, it is highly likely that N3 is chemically modified due to the available lone pair electrons that can react with the carbonyl group of diethylpyro carbonate, while the lone pair electrons of N1 are delocalizing in the imidazole ring (Christen, Griffiths, & Sheridan, 1981). In addition, bis-carboxyethylation at both N_1 and N_3 on the imidazole ring was observed but to a lesser extent. Our results are in agreement with prior similar studies (Glocker, Kalkum, Yamamoto, & Schreurs, 1996).

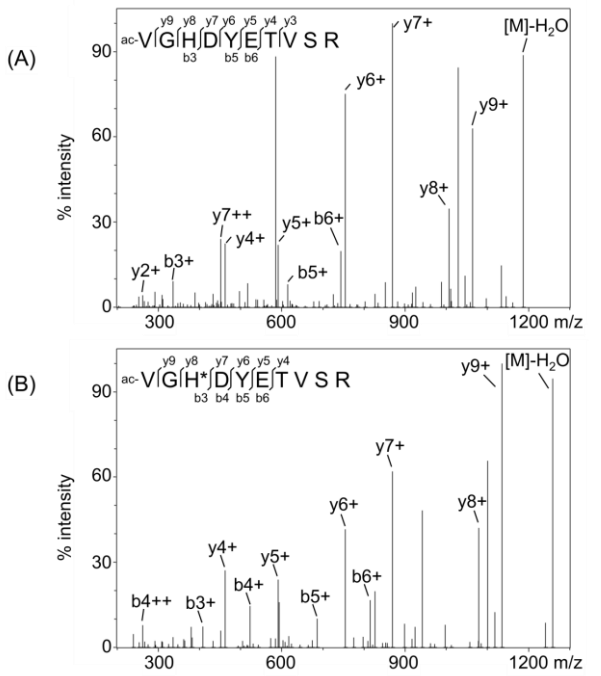

Figure 1. Mass spectra of (A) unmodified histidine-containing peptide, and (B) diethylpyrocarbonate-modified histidine (*) containing peptide

Previous studies have observed that the amino acid residues with other nucleophilic reactive side chains can also react with diethylpyrocarbonate, e.g., threonine, tyrosine, or cysteine residues at pH 7 (Mendoza & Vachet, 2008). As to our conditions set at pH 6, however, no ions with masses corresponding to a carbethoxy-modification of tyrosine or threonine were observed, indicating the specificity of the reaction towards histidyl residues under the study conditions with pH relatively acidic. To elaborate, when the -OH groups of tyrosine ($pKa_{side\ chain}$ = 10) and threonine ($pKa_{side\ chain}$ = 16, estimated from pKa isopropanol in DMSO) react with the carbonyl group of diethylpyrocarbonate, a 10-fold decrease in alkalinity of the solution results in unsuccessful deprotonation of protons in the -OH groups of tyrosine and threonine. However, because $pKa_{side\ chain}$ of histidine is 6.5 which leads to 24% of available unprotonated histidine, N3 in the imidazole ring of histidine can chemically react with diethylpyrocarbonate. Eventually, the equilibrium of the protonated histidine is shifted to the unprotonated form to further react with diethylpyrocarbonate. Although the model peptide used in the present study did not contain cysteine, the reaction with sulfhydryl group (pKa = 8.6) might be very slow according to pKa of the sulfhydryl group. In general, the sulfhydryl group can be efficiently deactivated by alkylating reagents to avoid the side reaction (Hale, Butler, Gelfanova, You, & Knierman, 2004; Sechi & Chait, 1998).

Consistent with previous studies, our results suggest that carbethoxylation of the histidine-containing peptide could be achieved by restricting pH in the range from 5.5 to 7.5 (Bhattacharyya, Bandyopadhyay, & Banerjee, 1993) as well as by slowing down kinetics using a low concentration of diethylpyrocarbonate (molar excess <50:1) (Glocker *et al.*,

1996) to obtain selective acylation (Lundblad, 2014). Based on these results, the condition of diethylpyrocarbonate modification to study SCX chromatographic behavior of the modified peptide was optimized.

3.2 Behavior of diethylpyrocarbonate-modified histidine residue under SCX conditions

To assess the chromatographic properties of the diethylpyrocarbonate-modified peptides under SCX conditions, the retention times of the ac-VGHDYETVSR precursor and the diethylpyrocarbonate-modified peptide were compared. Under SCX conditions (pH < 3), the unmodified model peptide, ac-VGHDYETVSR, has a net charge of +2 brought about by histidine and the C-terminal arginine residue, Figure 2a. The bound peptides were eluted from the column using an increasing salt gradient (Solvent B). After treatment with diethylpyrocarbonate, the modified-imidazole ring of the histidine residue in ac-VGH*DYETVSR could no longer be protonated, which results in a net charge of +1 and thus this is eluted from the column earlier than the unmodified form, Figure 2b. The deprotonation of histidine residue after diethylpyrocarbonate-modification was also observed by Da Poian *et al.* (2005).

To further confirm that the diethylpyrocarbonate-modification of histidine residues plays an important role in the interaction between negatively charged SCX resin and positively charged amino residue, the SCX fraction of ac-VGH*DYETVSR was collected and subsequently subjected to hydroxylamine treatment. The reversal of carboxylated histidine residues to their original forms readily occurs at alkaline pH or in the presence of nucleophiles such as hydroxylamine (Miles, 1977). The resulting peptide was then desalted and analyzed again with SCX chromatography under identical conditions to the primary run. As expected, the UV profiles of the secondary SCX run of the hydroxylamine-treated fraction presented additional absorption after the start of salt gradient at the retention time corresponding to that of unmodified peptide, Figure 2C. This indicated increased net positive charge, which was attributed to the protonation of hydroxylamine-deprotected carbethoxyl imidazole ring in histidine. In addition, the absence of peak at retention time corresponding to that of diethylpyrocarbonate-treated peptide ensured the completion of the reversion. Based on these results, the diethylpyrocarbonate-modification has potential to be used as a reaction that is specific to histidine residue for separation of the modified peptide by diagonal chromatography. To be noted, application of diethylpyro carbonate-modification combined with SCX chromatography for isolation of histidine-containing peptides is useful, although care should be taken concerning side reactions. The reaction between diethylpyrocarbonate and lysine can occur to a certain extent, thus causing deprotonation of the lysine residues under SCX analysis (Dage, Sun, & Halsall, 1998).

The isolation efficiency of the technique developed in the present study was compared to isolation techniques for histidine-containing peptides. In general, the present technique showed comparable efficiency in target-peptide isolation: the recovery rate of model peptide was approximately 60%. This is comparable to the recovery rate (from protein mixture) of other well-established approaches, e.g., copper-beads (50%) (Yang *et al.*, 2016), immobilised metal affinity solid-phase extraction containing Ni(II) coupled on-line to capillary electrophoresis-mass spectrometry (60%), and capillary electrophoresis-mass spectrometry (40%) (Pero-Gascon, Giménez, Sanz-Nebot, & Benavente, 2020).

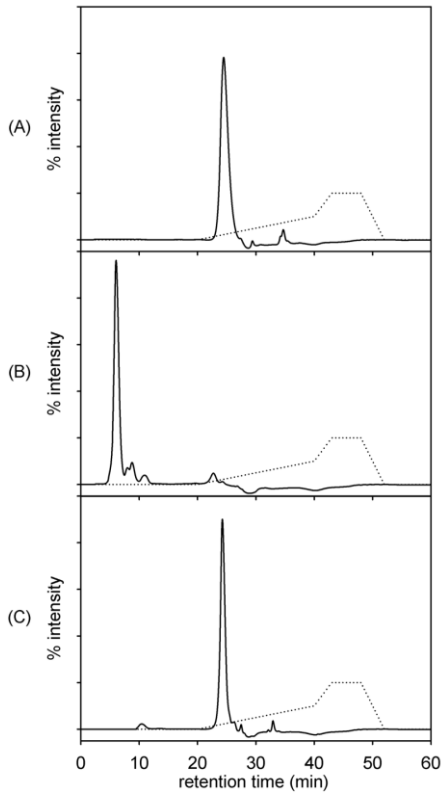

Figure 2. After injection into SCX, (A) untreated acetylated model peptides harboring one histidine (+2 charges) were eluted at retention time ~25 min. (B) The chromatographic shift observed after model peptides were modified by diethylpyrocarbonate. This step decreased net charge from +2 to +1. (C) After treatment with 0.5 M hydroxylamine, model peptides were shifted back to original retention time. Solid line represents UV absorption at 214 nm. Dash line represents salt gradient.

The efficiency of diethylpyrocarbonate modification on multiple histidine-containing peptides still remains to be studied. As the isolation of diethylpyrocarbonate-modified peptide relies on the change of SCX chromatographic behavior with a retention time shift, a single modification of histidine residues by diethylpyrocarbonate in peptide can enable the separation.

3.3 Stability of diethylpyrocarbonate-modified histidine residue under SCX conditions

The monosubstituted derivative is unstable under acidic conditions causing hydrolysis of the modification, which leads to liberation of free histidine (Lundblad, 2014). It is therefore important to examine the stability of the derivative under the acidic conditions of SCX chromatography. To do so, the diethylpyrocarbonate-modified peptide was incubated with SCX solvent A (10 mM potassium phosphate buffer (pH 2.9) in 20% acetonitrile) at 25 °C for 30, 60, 90 and 150 min.

All samples were subsequently subjected to SCX chromatography. The areas under the peaks (at 214 nm) with the retention time corresponding to the modified peptide were calculated. As shown in Figure 3, the acidic hydrolysis of the derivative gradually occurred. The half-life of the derivative was approximately 150 min, which allows for a separation based on SCX chromatography. However, further optimization of the separation conditions is essential.

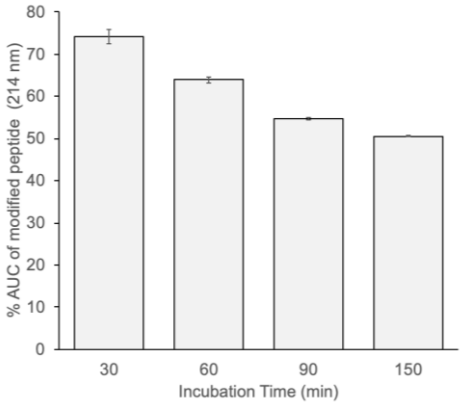

Figure 3. Hydrolysis of diethylpyrocarbonate-modified histidine under SCX conditions. The diethylpyrocarbonate-modified peptide was incubated with SCX solvent A (10 mM potassium phosphate buffer (pH 2.9) in 20% acetonitrile) at 25 °C for 30, 60, 90 and 150 min. The areas under the curve (AUC-214 nm) of the peak with the retention time corresponding to the modified peptide were calculated. Values are presented as mean ± SD (n = 3).

4. Conclusions

The modification of histidine residue by diethylpyrocarbonate can inhibit the protonation of the imidazole ring, resulting in a decrease of charge from +2 to +1 and a shift to shorter retention time compared to its unmodified form. The diethylpyrocarbonate-modified peptide can be effectively reversed to its unmodified form by treatment with neutral hydroxylamine. The chromatographic change caused by diethylpyrocarbonate modification of histidine is potentially applicable to separating histidine-containing peptides.

Acknowledgements

This work was funded by the Thailand Research Fund (grant number MRG5980075) and Prince of Songkla University (grant no. MET6302031S).

References

Barth, M., Bender, J., Kundlacz, T., & Schmidt, C. (2020). Evaluation of NHS-acetate and DEPC labelling for determination of solvent accessible amino acid residues in protein complexes. *Journal of Proteomics*, *222*, 103793.

Bhattacharyya, D. K., Bandyopadhyay, U., & Banerjee, R. K. (1993). Chemical and kinetic evidence for an essential histidine residue in the electron transfer from aromatic donor to horseradish peroxidase compound I. *The Journal of Biological Chemistry*, *268*(30), 22292-22298.

Buncherd, H., Roseboom, W., Chokchaichamnankit, D., Sawangareetrakul, P., Phongdara, A., Srisomsap, C., de Jong, L., & Svasti, J. (2016). beta-Elimination coupled with strong cation-exchange chromato graphy for phosphopeptide analysis. *Rapid Communications in Mass Spectrometry*, *30*(14), 1695-1704.

Camerini, S., & Mauri, P. (2015). The role of protein and peptide separation before mass spectrometry analysis in clinical proteomics. *Journal of Chromatography A, 1381*, 1-12.

Cao Dong, Z. C., Zhang Yangjun, Han Chunguang, & Deng Yulin. (2009). Sulfonation modification-assisted enrichment and identification of histidine-containing peptides by strong cation exchange chromatography and mass spectrometry. *Chinese Journal of Chromatography*, *27*(2), 158-163.

Cheung, R. C., Wong, J. H., & Ng, T. B. (2012). Immobilized metal ion affinity chromatography: A review on its applications. *Applied Microbiology and Biotechnology*, *96*(6), 1411-1420.

Christen, D., Griffiths, J. H., & Sheridan, J. (1981). The microwave spectrum of imidazole; Complete structure and the electron distribution from nuclear quadrupole coupling tensors and dipole moment orientation. *Zeitschrift für Naturforschung A*, *36*(12), 1378.

Da Poian, A. T., Carneiro, F. A., & Stauffer, F. (2005). Viral membrane fusion: is glycoprotein G of rhabdoviruses a representative of a new class of viral fusion proteins? *Brazilian Journal of Medical and Biological Research*, *38*(6), 813-823.

Dage, J. L., Sun, H., & Halsall, H. B. (1998). Determination of diethylpyrocarbonate-modified amino acid residues in alpha 1-acid glycoprotein by high-performance liquid chromatography electrospray ionization-mass spectrometry and matrix-assisted laser desorption/ionization time-of-flight-mass spectrometry. *Analytical Biochemistry*, *257*(2), 176-185.

Follmer, C., & Carlini, C. R. (2005). Effect of chemical modification of histidines on the copper-induced oligomerization of jack bean urease (EC 3.5.1.5). *Archives of Biochemistry and Biophysics*, *435*(1), 15-20.

Glocker, M. O., Kalkum, M., Yamamoto, R., & Schreurs, J. (1996). Selective biochemical modification of functional residues in recombinant human macrophage colony-stimulating factor beta (rhM-CSF beta): identification by mass spectrometry. *Biochemistry*, *35*(46), 14625-14633.

Hale, J. E., Butler, J. P., Gelfanova, V., You, J. S., & Knierman, M. D. (2004). A simplified procedure for the reduction and alkylation of cysteine residues in proteins prior to proteolytic digestion and mass spectral analysis. *Analytical Biochemistry*, *333*(1), 174-181.

Hunsicker-Wang, L. M., & Konkle, M. E. (2018). Translation of chemical biology research into the biochemistry

laboratory: Chemical modification of proteins by diethylpyrocarbonate, *Best Practices for Supporting and Expanding Undergraduate Research in Chemistry* (Vol. 1275, pp. 165-179). Washington, DC: American Chemical Society.

Jin, X. R., Abe, Y., Li, C. Y., & Hamasaki, N. (2003). Histidine-834 of human erythrocyte band 3 has an essential role in the conformational changes that occur during the band 3-mediated anion exchange. *Biochemistry, 42*(44), 12927-12932.

Kikot, P., Polat, A., Achilli, E., Fernandez Lahore, M., & Grasselli, M. (2014). Immobilized palladium(II) ion affinity chromatography for recovery of recombinant proteins with peptide tags containing histidine and cysteine. *Journal of Molecular Recognition, 27*(11), 659-668.

Kozlov, I. A., Kermani, B. G., Melnyk, P. C., Barker, D. L., Zhao, C., Hachmann, J. P., & Lebl, M. (2007). Retention of histidine-containing peptides on a nickel affinity column. *Journal of Chromatographic Science, 45*(4), 207-211.

Li, S. Y., Oyala, P. H., Britt, R. D., Weintraub, S. T., & Hunsicker-Wang, L. M. (2017). Reactive sites and course of reduction in the Rieske protein. *Journal of Biological Inorganic Chemistry, 22*(4), 545-557.

Lundblad, R. L. (2014). *Chemical reagents for protein modification*. Boca Raton, Fl: CRC press.

Lundblad, R. L. (2016). *Chemical modification of biological polymers*. Boca Raton, Fl: CRC press.

Mendoza, V. L., & Vachet, R. W. (2008). Protein surface mapping using diethylpyrocarbonate with mass spectrometric detection. *Analytical Chemistry, 80*(8), 2895-2904.

Miles, E. W. (1977). Modification of histidyl residues in proteins by diethylpyrocarbonate. *Methods in Enzymology, 47*, 431-442.

Ongay, S., Boichenko, A., Govorukhina, N., & Bischoff, R. (2012). Glycopeptide enrichment and separation for protein glycosylation analysis. *Journal of Separation Science, 35*(18), 2341-2372.

Pero-Gascon, R., Giménez, E., Sanz-Nebot, V., & Benavente, F. (2020). Enrichment of histidine containing peptides by on-line immobilised metal affinity solid-phase extraction capillary electrophoresis-mass spectrometry. *Microchemical Journal, 157*, 105013.

Prasanna, R. R., Sidhik, S., Kamalanathan, A. S., Bhagavatula, K., & Vijayalakshmi, M. A. (2014). Affinity selection of histidine-containing peptides using metal chelate methacrylate monolithic disk for targeted LC-MS/MS approach in high-throughput proteomics. *Journal of Chromatography. B, Analytical Technologies in the Biomedical and Life Sciences, 955-956*, 42-49.

Rebek, J. (1990). On the structure of histidine and its role in enzyme active sites. *Structural Chemistry, 1*(1), 129-131.

Ren, D., Penner, N. A., Slentz, B. E., Mirzaei, H., & Regnier, F. (2003). Evaluating immobilized metal affinity chromatography for the selection of histidine-containing peptides in comparative proteomics. *Journal of Proteome Research, 2*(3), 321-329.

Sasaki, S., Kobayashi, M., Futagi, Y., Ogura, J., Yamaguchi, H., & Iseki, K. (2014). Involvement of histidine residue His382 in pH regulation of MCT4 activity. *PLoS One, 10*(4), e0122738.

Schmidt, C., Macpherson, J. A., Lau, A. M., Tan, K. W., Fraternali, F., & Politis, A. (2017). Surface accessibility and dynamics of macromolecular assemblies probed by covalent labeling mass spectrometry and integrative modeling. *Analytical Chemistry, 89*(3), 1459-1468.

Sechi, S., & Chait, B. T. (1998). Modification of cysteine residues by alkylation. A tool in peptide mapping and protein identification. *Analytical Chemistry, 70*(24), 5150-5158.

Staes, A., Van Damme, P., Timmerman, E., Ruttens, B., Stes, E., Gevaert, K., & Impens, F. (2017). Protease substrate profiling by N-terminal COFRADIC. *Methods in Molecular Biology, 1574*, 51-76.

Thawornpan, P., Jumpathong, W., Thanapongpichat, S., de Jong, L., Phongdara, A., & Buncherd, H. (2019). Mechanistic investigation of phosphoprotein enrichment by fly ash-based chromatography. *Journal of Chromatography. B, Analytical Technologies in the Biomedical and Life Sciences, 1118-1119*, 1-6.

Thawornpan, P., Thanapongpichat, S., Tun, A. W., Jumpathong, W., Jong, L. d., & Buncherd, H. (2020). Isolation of nucleic acids using fly ash as a low-cost adsorbent. *Analytical Letters*, 1-14.

Willard, B. B., & Kinter, M. (2001). Effects of the position of internal histidine residues on the collision-induced fragmentation of triply protonated tryptic peptides. *Journal of the American Society for Mass Spectrometry, 12*(12), 1262-1271.

Yang, J., Tian, F., Zhang, M., Zhao, Y., Qian, X., Cai, Y., & Ying, W. (2016). Targeted histidine-peptide enrichment improved the accuracy of isobaric-based quantitative proteomics. *Analytical Methods, 8*(26), 5255-5261.

Zhang, X., Liu, J., Huang, L., Yang, X., Petersen, R. B., Sun, Y., Gong, H., Zheng, L., & Huang, K. (2016). How the imidazole ring modulates amyloid formation of islet amyloid polypeptide: A chemical modification study. *Biochimica et Biophysica Acta, 1860*(4), 719-726.