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Journal of Chromatography A, 1103 (2006) 368–371
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

Short communication

Capillary electrophoresis for monitoring dityrosine and 3-bromotyrosine synthesis☆

Michael Tilley[a,*], Scott R. Bean[a], Katherine A. Tilley[b]

[a] *US Department of Agriculture, Agricultural Research Service Grain Marketing and Production Research Center, 1515 College Avenue, Manhattan, KS 66502, USA*
[b] *Department of Grain Science and Industry, Kansas State University, Manhattan, KS 66506, USA*

Received 26 January 2005; received in revised form 7 December 2005; accepted 7 December 2005
Available online 27 December 2005

Abstract

Protein oxidation affects the structure of many amino acids. Variants of tyrosine are increasingly important in medical and food sciences. The synthesis of standards is essential for monitoring the disease state of patients with various illnesses and the quality of a number of food products. A method for monitoring standard synthesis of dityrosine and 3-bromotyrsoine from tyrosine using capillary electrophoresis (CE) is presented. Optimum separation was achieved using an isoelectric buffer consisting of 100 mM iminodiacetic acid (IDA) + 75 mM lauryl sulfobetaine (SB 3-12) + 0.02% hydroxypropyl methylcellulose (HPMC) in a 27 cm × 75 μm capillary at 22 kV and 45 °C. Using these conditions the tyrosine adducts could be easily separated in less than 4 min. The resolution of the CE method was similar to HPLC separations, but analysis time was distinctly shorter.
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Keywords: Capillary electrophoresis; Dityrosine; 3-Bromotyrosine

1. Introduction

Reactive oxygen species and other free radicals can result from metabolic processes as well as exposure to exogenous factors such as UV light, radiation and certain chemicals. Damage to membrane lipids, proteins, and DNA may be caused by these free radicals and is involved in various disease states. The oxidative coupling of tyrosine via free radical generating mechanisms is of general interest in several biological processes as a marker for protein oxidation/aging and as an indicator of cellular damage. Dityrosine is known to occur in amyloid fibril formation, Parkinson's disease, atherosclerotic plaque formation, brain lipofuscin granules, cataracts in eye lens, and oxidative stress. Hence, dityrosine levels in various body fluids have been posed as in vivo markers of oxidative stress and aging [1–3].

Dityrosine has various biological functions in the structure of extensible proteins such as elastin, resilin and calmodulin [4,5]. Additionally, this important structural crosslink may have a functional role in food processing [6,7].

Similarly, 3-bromotyrosine and 3,5-dibromotyrosine serve as useful indicators for identifying sites of eosinophil-mediated tissue injury in vivo. Eosinophil peroxidase has been postulated to promote oxidative tissue injury in conditions such as asthma, allergic inflammatory disorders, cancer and helminthic infections [8]. Oxidants produced in the airways of asthmatic patients determine the extent to which peroxidases contribute to the pathogenesis of the disease. Hypobromous acid (HOBr) and hypochlorous acid (HOCl), produced in these eosinophil-mediated reactions, are toxic to nearly all cell types. Both of these hypohalous acids (HOBr and HOCl) react rapidly with biological molecules causing various reactions, including crosslinking of proteins. Hypohalous acids are too reactive to measure directly, but their reactions with biomolecules leave chemical footprints of their presence. Halogenated tyrosine residues in

☆ Cooperative investigation, USDA, Agricultural Research Service and the Department of Grain Science and Industry, Kansas State University. Contribution No. 05-189-J from the Kansas Agricultural Experiment Station, Manhattan, KS 66506. Names are necessary to report factually on available data; mention of firm names or trade products does not constitute endorsement by the US Department of Agriculture over others that may also be suitable.

* Corresponding author. Tel.: +1 785 776 2759; fax: +1 785 537 5534.
E-mail address: Michael.tilley@gmprc.ksu.edu (M. Tilley).

0021-9673/$ – see front matter. Published by Elsevier B.V.
doi:10.1016/j.chroma.2005.12.029

proteins are specific biomarkers of hypohalous acids [9]. Recent reports in the literature have identified 3-bromotyrosine as a specific indicator of eosinophil peroxidase mediated protein oxidation [10].

Rapid and accurate methods are critical in all of the areas in which the presence of dityrosine and related molecules plays a determinant role in the course of action with either a patient monitoring a disease state or in other practical applications. For example, a recent paper by Fenaille et al. [11] demonstrated the use of dityrosine formation as an indicator of lipid oxidation in infant formula milk powder and suggested quality assurance by using tandem mass spectrometry for multiple reaction monitoring transitions to select, confirm and quantify the presence of dityrosine.

Here, we report the development of capillary electrophoresis (CE) separation as a rapid and simple method for the analysis of tyrosine, dityrosine and 3-bromotyrosine which can be used in a number of applications in diverse fields.

2. Experimental

2.1. Samples

Tyrosine crosslink standard was prepared as described in Tilley et al. [12]. Briefly, L-tyrosine was dissolved in 2 mL 0.08 M HCl, and divided into six glass 13 mm × 100 mm culture tubes. Two milliliters aqueous potassium bromate ($KBrO_3$) (0.0025–0.05%, w/v) were added to each tube, tubes were covered with aluminum foil, heated at 150 °C for 25 min in a convection oven, cooled and lyophilized to dryness if needed. The material in each tube was dissolved in 0.5 mL deionized water, centrifuged through 0.22 μm nylon centrifuge tube filters (Corning, Midland, MI, USA) at 2700 × *g* and analyzed by CE. For CE optimization, the reaction conditions were set at 10 mg tyrosine and 0.01% $KBrO_3$ and are referred to as "reaction mix" in the text.

All chemicals were obtained from Sigma (St. Louis, MO, USA) and were of the highest purity available. Commercial buffers for optimization of CE separation were obtained from MicroSolv Technology (Long Branch, NJ, USA).

Analysis of the reaction mix by HPLC revealed three prominent peaks corresponding to tyrosine, dityrosine and 3-bromotyrosine previously verified by one- (1D) and two-dimensional (2D) NMR [12]. Purified standard compounds were used to identify peaks in the CE separations.

2.2. Instrumentation

2.2.1. Capillary electrophoresis

A P/ACE 2100 instrument (Beckman Coulter, Fullerton, CA, USA) equipped with a UV detector was used for all separations. Capillaries were either 50 μm or 75 μm I.D. bare fused silica capillaries (Polymicro Technologies, Phoenix, AZ, USA) 37 cm or 27 cm in length (30 cm or 20 cm to detector, respectively). Samples were injected for 2 s at 3.45 kPa and detected by UV at 200 nm with normal polarity (inlet +, outlet −). Products were compared with individual compounds purified by HPLC. Capillaries were initially rinsed with separation buffer for 15 min followed by preconditioning at 20 kV for 5 min [13]. Prior to each separation, capillaries were rinsed with run buffer for 2 min. No post-separation rinses were made. Iminodiacetic acid (IDA) concentrations were varied from 25 to 100 mM. Separation voltages were varied from 12 to 30 kV and separation temperature was varied from 25 to 50 °C.

3. Results and discussion

3.1. CE optimization

The best separation using commercial buffers was achieved using 75 mM sodium phosphate pH 2.5 with 37 cm × 50 μm capillaries at 12 kV and 25 °C. This agrees with the findings of Lee et al [14] who identified low pH phosphate buffers as optimum for separation of aromatic amino acids. Although the separation was superior to other commercial buffers, it was not successful in separation of the tyrosine adducts and the use of dynamically coated capillaries and isoelectric buffers was investigated. The isoelectric buffer iminodiacetic acid has shown promise in the separation of peptides due to good solubility in organic solvents and a low isoelectric point [15]. Zwitterion surfactants are capable of forming a dynamic coating on the capillary surface thus minimizing analyte interactions with

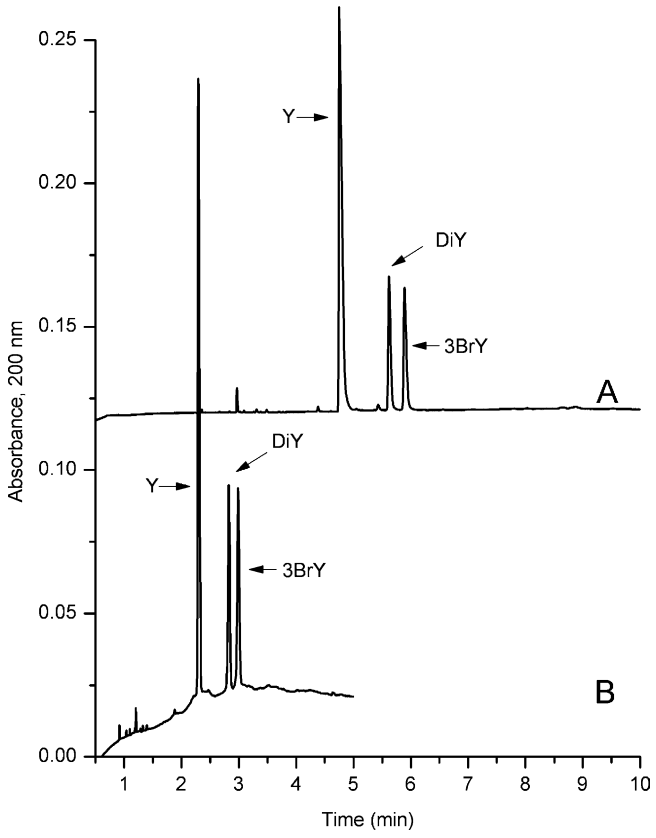

Fig. 1. Capillary zone electrophoresis separation in isoelectric buffer: (A) 100 mM iminodiacetic acid (IDA) with 75 mM SB 3-12 at 22 kV and 45 °C using capillary 37 cm × 50 μm I.D.; (B) addition of 0.02% HPMC to the buffer and capillary length 27 cm × 75 μm I.D. Peaks: Y, tyrosine; DiY, dityrosine; 3BrY, 3-bromotyrosine.

the capillary wall [16]. The use of surfactants also provides increased hydrophobic selectivity through interaction between the analytes and the surfactant micelles which may facilitate the separation of structurally related molecules. This can occur through interaction of the analytes with detergent micelles in a MEKC separation. We found IDA buffer with the zwitterionic detergent lauryl sulfobetaine (SB 3-12) at 75 mM to be optimal for the separation of tyrosine adducts within 6 min using 37 cm × 50 μm capillaries at 22 kV and 45 °C (Fig. 1A). As this concentration of SB 3-12 is above the critical micelle concentration, micelles would be present for the analytes to interact with. Thus this method would be considered an MEKC separation.

Separations were further enhanced by the addition of hydroxypropyl methylcellulose (HPMC) to the buffer and shortening the total capillary length to 27 cm and internal diameter of 75 μm. Under these conditions, all three tyrosine compounds were separated in less than 3 min. The optimized separation conditions are shown in Fig. 1B and were 100 mM IDA buffer containing 75 mM SB 3-12 and 0.02% HPMC used at 45 °C and 22 kV. It was interesting that adding HPMC reduced separation times considerably. Although the exact reason for this is unknown, it was suggested by a reviewer that the SB 3-12 may have formed a positively charged layer on the inner capillary wall at the acidic pH used in the separations. This in turn, would induce a reversed EOF, opposite to the migration of the positively charged analytes, which would slow the migration of the analytes. Adding HPMC to the separation buffer may have reduced the reversed EOF caused the by the SB 3-12, which once reduced, would allow the analytes to move through the capillary faster.

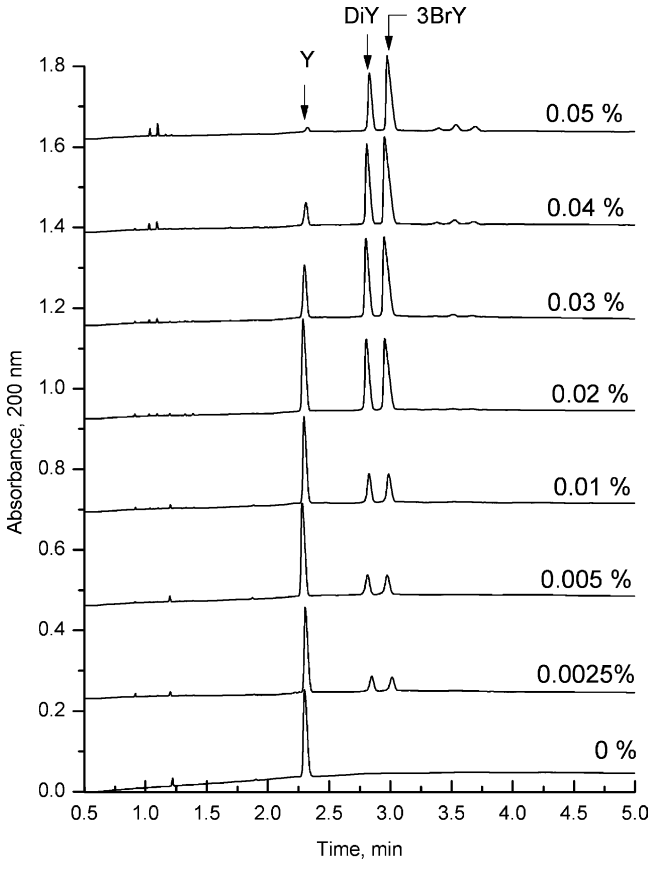

Fig. 2. Capillary zone electrophoresis separation of reaction mix resulting from increasing concentrations of potassium bromate using the optimized method—100 mM iminodiacetic acid + 75 mM SB 3-12 + 0.02% HPMC in a 27 cm × 75 μm capillary at 22 kV and 45 °C. Peaks: Y, tyrosine; DiY, dityrosine; 3BrY, 3-bromotyrosine.

3.2. Use of optimum separation conditions

The optimized CE method was used to rapidly evaluate products formed during synthetic reaction conditions. For synthesis of the tyrosine products, concentrations of tyrosine and potassium bromate as well as reaction time and reaction temperature were varied. Fig. 2 shows the separations and product yields found with increasing potassium bromate concentrations. Separations were completed in about 3 min. This methodology allows rapid analysis of a large number of samples and is particularly useful in experiments where optimization of product synthesis is critical. It also has potential in evaluation of synthesis of these compounds.

4. Conclusion

This study presents a CE method for rapid evaluation of synthetic conditions for tyrosine adducts; dityrosine and 3-bromotyrosine. This optimized method may also be useful in separation of tyrosine and other aromatic amino acids and adducts. The procedure described herein was used to rapidly evaluate reaction conditions. For this work, reaction time and temperature as well as levels of tyrosine and potassium bromate were optimized. Rather than lengthy HPLC analyses, this method can be used to provide a rapid and accurate analysis of tyrosine, dityrosine and 3-bromtyrosine. Using the CE method we found the limit of detection to be less than 25 nmol compared to the HPLC-fluorescence detection of below 1 nmol. The concentration of dityrosine and 3-bromotyrosine varies in biological samples. CE method lacks the sensitivity of the HPLC method but it could be increased by sample preparation such as solid phase extraction and detection using laser induced fluorescence.

Acknowledgements

We thank Sushma Prakash (USDA-ARS GMPRC) for technical assistance. This work was supported by the Kansas Agricultural Experiment Station, Kansas State University, and USDA-NRI grant 2002-01752.

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