Short communication

Analysis of 4-hydroxyproline using 4-chloro-7-nitrobenzo-2-oxa-1,3-diazol derivatization and micellar electrokinetic chromatography combined with laser-induced fluorescence detection

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Abstract

Micellar electrokinetic chromatography (MEKC) with laser-induced fluorescence (LIF) detection was used for internal standard (pyrrolidinol) based quantification of 4-hydroxyproline (Hyp) in muscle hydrolysates. Hydrolysates were first derivatized with *o*-phthaldialdehyde to reduce primary amine interference and then 4-chloro-7-nitrobenzo-2-oxa-1,3-diazol (NBD) to enable selective detection of secondary amines. This method allows for rapid and sensitive detection of hydroxyproline in dilute hydrolysates using commercially available capillary electrophoresis equipment. © 2000 Elsevier Science B.V. All rights reserved.

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1. Introduction

Hydroxyproline (Hyp) is a post-translational modification of proline and is found primarily in the connective tissue protein collagen [1]. Free Hyp produced during collagen degradation is not used for protein synthesis and its rate of excretion can be used as a marker for measuring collagen turnover. High levels of Hyp excretion are indicative of certain types of cancer [2] and bone diseases [3]. Hyp can also be used as a marker to estimate the collagen content of tissues and this is particularly important in animal agriculture. Meat toughness in older animals is partly related to the strengthening of connective tissue due to the formation of hydroxypyridinium cross-linkages between collagen molecules. Cross-linkages form slowly, toughen the meat and also reduce collagen solubility. The effects of collagen on meat quality can, therefore, be evaluated indirectly by measuring Hyp in soluble and insoluble fractions of meat [4].

Hydroxyproline concentrations in beef range from approximately 4 to 25 μmol per gram. After separation and hydrolysis of soluble and insoluble collagen fractions, Hyp concentrations can be as low as 20 to 30 nmol per ml. Hydroxyproline has been measured successfully in a variety of tissues using a number of means including a colorometric method

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[5], HPLC using UV absorption [6,7] and fluorescence detection [8–11] and by GC–MS [12,13]. Hyp analysis by micellar electrokinetic chromatography (MEKC) has also been recently reported [14,15] and these methods take advantage of capillary electrophoresis' (CE) high resolution, sensitivity and relatively low running costs.

We wanted to explore the possibility of developing an MEKC method for Hyp analysis using commercially available equipment. We chose 4-chloro-7-nitrobenzo-2-oxa-1,3-diazol (NBD) as our derivatizing reagent due to its compatibility with our commercial LIF detector (excitation 488 nm) and its proven use in the HPLC analysis of Hyp [8]. NBD also had a number of other advantages including its 10 fold greater reactivity with secondary versus primary amines, a short derivatization time at 60°C and derivative stability in darkened conditions [8]. For our method, we also chose to incorporate *o*-phthaldialdehyde pre-derivatization to remove primary amine interference [11,15] and the use of an internal standard (pyrrolidinol; Pyr) to optimize MEKC reproducibility.

2. Experimental

2.1. Chemicals

Mercaptoethanol, 4-hydroxyproline, *o*-phthaldialdehyde (OPA), 4-chloro-7-nitrobenzo-2-oxa-1,3-diazol (NBD), hexadecyltrimethylammonium bromide (HTAB) and the amino acid standard solution for collagen hydrolysates were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Pyrrolidinol (Pyr) was obtained from Aldrich Chemical Co. (Milwaukee, WI, USA). Boric acid, hydrochloric acid and sodium hydroxide were obtained from Fisher Scientific (Fair Lawn, NJ, USA). Methanol was obtained from EM Science (Gibbstown, NJ, USA) and water was deionized using a Milli-Q RG system (Millipore, Molsheim, France).

2.2. Equipment

Separations were carried out using a Beckman P/ACE System 5500 capillary electropherograph (Beckman Instruments, Inc. Fullerton CA) equipped with a laser induced fluorescence detector (Beckman P/ACE System Laser Module 488; 488 nm excitation, 520 emission). Samples were injected (10 s) under pressure (0.5 p.s.i.) onto a bare-fused-silica capillary column (Beckman eCAP™ Capillary tubing), 57 cm (50 cm to the detector) X 75 μm I.D. Electropherogram peak areas were integrated using Beckman P/ACE Station Version 1.0 software.

2.3. Conditions

The autosampler vial locations, rinse and electrophoresis conditions are listed in Tables 1 and 2. This vial configuration is specific for users of the Beckman P/ACE System 5500 and allows for maximum unattended throughput of samples by utilizing space in the inner autosampler ring for rinse solutions. For non-Beckman users, the method can be completed by simply following the time and procedure information in Table 2. Buffers and wash solutions were filtered through 0.45 μm syringe mounted PTFE filters (Cole-Parmer, Vernon Hills, IL, USA).

2.4. Sample preparation

Four types of samples were used to develop a method for MEKC analysis of Hyp. First, standards containing Hyp and Pyr were analyzed to optimize derivatization conditions and generate a standard

Table 1
Beckman P/ACE system 5500 autosampler vial (4.5 ml) locations enabling 15 samples per batch of analyses. The autosampler has an inner ring (vials 1–10) and an outer ring (vials 11–34)

Vial	Content
1–5	Outlet Buffer[a]
6	0.1 *M* NaOH
7	Water
8–9	Rinse Buffer
10	Empty
11–15	Inlet Buffer
16–30	Sample vials
31	0.1 *M* HCl
32	Empty
33	Rinse buffer
34	Wait buffer (0 min)

[a] Buffer composition (rinse, inlet and outlet buffer): 30% methanol, 10 m*M* hexadecyltrimethylammonium bromide and 100 m*M* Sodium Borate pH 9.3 (made using boric acid made alkaline with 6 *M* NaOH).

Table 2
Beckman P/ACE system 5500 autosampler rinse and sample injection program for hydroxyproline analysis

Order	Inlet Vial	Outlet Vial	Time (min)	Procedure
1	31	10	2.5	0.1 *M* HCl rinse
2	6	32	2.5	0.1 *M* NaOH rinse
3	10	32	0.6	Air purge of column
4	7	32	2.5	Water rinse
5	8	32	2.5	Buffer rinse
6	9	32	2.5	Buffer rinse
7	33	10	2.5	Buffer rinse
8	Sample injection (vials 16–32)		10 (s)	Sample injection (0.5 p.s.i.)
9	34	8	0	0 min dip in buffer
10	11–15	1–5	7	Electrophoresis[a]

[a] Each pair of inlet and outlet buffers (ex. vials 1&11, 2&12 etc.) is used for three samples to optimize electrophoresis conditions, with a run time of 7 min at −26 kV and 20°C.

curve to establish linearity of Hyp response. Second, the Sigma amino acid standard solution for collagen was analysed to establish conditions for optimal separation of Hyp and Pyr from other NBD-amino acid derivatives. Third, standard additions of Hyp to hydrolysed bovine loin muscle were analysed to check reproducibility of analyses and recovery of Hyp, and finally, soluble and insoluble fractions of bovine loin muscle were separated, hydrolysed and recoveries of Hyp from these samples were determined.

Samples hydrolysed prior to analyses included (intact) beef loin (*longissimus lumborum*), and the soluble and insoluble collagen fractions were also from beef loin. To prepare (intact) beef hydrolysates, 1 g of loin was added to 6 ml of 7 *M* HCl in 16×150 mm screw capped pyrex tubes, purged with $N_{2(g)}$ and hydrolysed for 16 h at 110°C. Soluble and insoluble collagen fractions from three beef loins were prepared according to Hill [4]. Briefly, 4 g of each loin were homogenized with 25% strength Ringer's solution, heated for 1 h at 77°C and the soluble and insoluble fractions were separated via centrifugation. The insoluble fraction was then washed twice more with 25% strength Ringer's solution, centrifuged and the washings were combined with the original soluble fraction. Concentrated (12 *M*) HCl was added to yield final volumes of 25 ml and 54 ml for the insoluble and soluble fractions, respectively, to a final concentration of 6 *M* $HCl_{(aq)}$ for the insoluble and soluble fractions respectively. These fractions were then hydrolysed for 16 h at 110°C. After hydrolysis, samples were cooled, fil-

tered through 0.45 μm filters and internal standard (Pyr) and Hyp additions were made.

All samples and standards, whether hydrolysed or not, were suspended in 6 *M* HCl and aliquots of these (5–40 μl) were dried in amber colored 0.6 ml polypropylene microcentrifuge tubes prior to derivatization. Samples were dried using an evaporative centrifuge (Concentrator 5301, Eppendorf, Hamburg, Germany) connected to a vacuum pump with an in-line $N_{2(l)}$ cold trap. Dried samples were then suspended in 50 μl of 0.4 *M* sodium borate (pH 9.3; sodium borate was made from boric acid made basic with 6 *M* $NaOH_{(aq)}$), mixed with 50 μl OPA (4 mg OPA and 2.08 μl mercaptoethanol per ml of 80:20, water:methanol) and allowed to react for 5 min at room temperature to derivatize and reduce primary amine interference. One hundred μl of post-OPA buffer (90:10, water:methanol containing 40 m*M* HTAB and 0.2 *M* sodium borate (pH 9.3)) was then added and mixed. To this 100 μl of NBD (5 mg per ml methanol) was added, mixed and derivatization was carried out at 60°C in a covered water bath for exactly 4 min. Samples were then cooled immediately in an ice bath in the dark (due to light sensitivity of NBD derivatives). One hundred μl of water were then added and samples were mixed to yield pH, buffer salt, and HTAB concentrations equal to the MEKC run buffer. Samples were stable for several h at room temperature enabling 15 samples to be easily analysed per sample batch.

Immediately prior to MEKC (i.e. in the 400 μl post-derivatization final volume), the concentration of Pyr was 12.5 nmol per ml for all samples and

standards. The Hyp concentrations for the standard curve and for the Hyp additions to the intact loin hydrolysates were 0, 1.25, 2.5, 5, 10 nmol per ml. The concentration of Hyp in the Sigma amino acid standard was 5 nmol per ml and 0.8 nmol per ml for the primary amino acids. The concentration of Hyp added to the soluble and insoluble fractions was 5 nmol per ml.

Samples and standards were injected for 10 s at 0.5 p.s.i., and assuming a viscosity equivalent to water, approximately 57 nl was injected per analysis containing between 0 and 713 amol (10^{-18}) of Hyp and Pyr (calculated using Beckman CE Expert software version 1.0b).

3. Results and discussion

3.1. Derivatization, MEKC conditions and linearity of Hyp response

The derivatization conditions used were similar to those reported by Ahnoff et al. [8] for the HPLC analysis of Hyp using NBD, except samples required 4 min instead of 3 min derivatization at 60°C to accommodate quantitative Pyr reaction. While looking for a compatible internal standard, it was also interesting to note that secondary amines had to be part of a ring structure for rapid NBD-derivatization to occur. The use of 10 m*M* HTAB as the micellar reagent and 30% methanol in the MEKC buffer was found to be adequate for the separation of NBD-derivatives. Increasing or decreasing the HTAB concentration effectively increased and decreased, respectively, the migration times of Hyp and other derivatives with carboxyl groups. The lack of a carboxyl group made Pyr separation from other NBD-derivatives (i.e. amino acids) fairly easy by altering buffer HTAB concentration.

A typical standard sample electropherogram is illustrated in Fig. 1A (12.5 nmol per ml Pyr and 5 nmol per ml Hyp). The Hyp standard curve was linear for all samples (analysis in triplicate, $R^2 = 0.999$) and migration times for Pyr and Hyp were very reproducible (Pyr and Hyp migration time C.V.=0.34% and 0.45%; *n*=12).
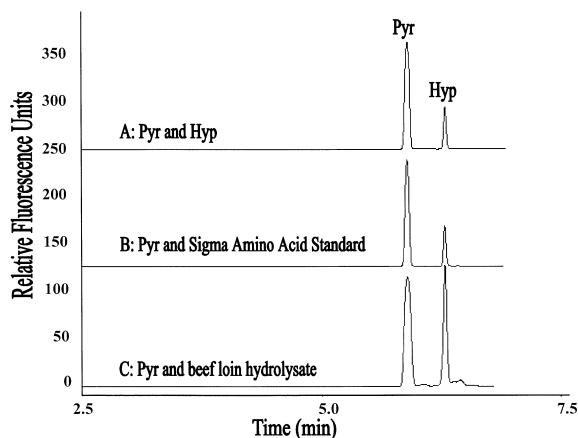

Fig. 1. (A) Electropherogram of NBD-derivatives of Pyr (12.5 nmol per ml) and Hyp (5 nmol per ml) (B) Electropherogram of NBD-derivatives of Pyr (12.5 nmol per ml), Hyp (5 nmol per ml) and 18 other amino acids from the Sigma amino acid standard for collagen. (C) Electropherogram of NBD-derivatives of Hyp (5 nmol per ml) added to 0.95 mg per ml beef loin muscle hydrolysate. Samples were injected (10 s) under pressure (0.5 p.s.i.) onto a bare-fused-silica capillary column (Beckman eCAP™ Capillary tubing), 57 cm (50 cm to the detector;) X 75 μm I.D. The separation was performed in 30% methanol with 10 m*M* hexadecyltrimethylammonium bromide and 100 m*M* Sodium Borate (pH 9.3). Separations were completed in 7 min at 26 kV and 20°C.

3.2. Sigma amino acid standard

Fig. 1B is an electropherogram of the Sigma amino acid standard for collagen hydrolysates with added Pyr. The vial subjected to MEKC contained 12.5 nmol per ml Pyr, 5 nmol per ml Hyp and 0.8 nmol per ml of 18 primary amino acids. Hyp recovery from the Sigma amino acid standard was calculated relative to a standard sample containing only Hyp and Pyr (internal standard). Recovery of Hyp from the amino acid standard for 10 separate runs was 97.3%±3.6% C.V. These data and the eletropherogram indicate variability and interference with other amino acids were minimal when Hyp was analysed using the proposed method.

3.3. Hydrolysate standard additions

Standard additions of Hyp to loin muscle hydrolysates were analysed to ensure Hyp could be measured without interference from other derivatives in

'authentic' samples. A typical electropherogram of hydrolysed beef loin with added Pyr is illustrated in Fig. 1C. Very little interference was encountered with these analyses, and standard additions of Hyp to a muscle hydrolysate (0, 1.25, 2.5, 5 and 10 nmol Hyp per ml in a post-derivatized sample vial with approximately 0.95 mg hydrolysed muscle per ml) were linear (analysis in triplicate, $R^2 = 0.998$). Average recovery of Hyp in samples was 103.5% ± 3.5% C.V.

3.4. Hyp in soluble and insoluble fractions of meat

To ensure the method would be useful for measuring Hyp in dilute hydrolysates without extensive pre-concentration, we also analysed soluble and insoluble collagen fractions collected from three bovine loin muscles. Four g of muscle were separated into soluble and insoluble fractions according to Hill [4]. For these analyses, 2.5 and 40 μl of insoluble and soluble hydrolysates, respectively, were dried prior to derivatization. Hyp and Pyr peaks were well resolved (electropherogram not shown) and the average recovery of added Hyp was 98.3% ± 1.9 C.V. The total Hyp content for the loin muscles averaged 0.81 mg per gram of fresh muscle with an average collagen solubility of 8.3%. Total Hyp results compare favorably to previous reports [16], but collagen solubility was 3.7% higher for the present study due to the use of younger animals.

4. Conclusion

This is the first time MEKC has been used to analyse Hyp in muscle after OPA blocking of primary amine interference coupled with secondary amine derivatization with NBD. The proposed meth-od for analysis of Hyp is reproducible and sensitive. Buffer use per sample is minimal and upwards of 30 samples can be analysed per day. Further refinement of the method to reduce times for column washing and equilibration may be possible to increase the number of samples analysed per day. We feel, however, that autosampler space would still be limiting with the present system and the true economy of capillary electrophoresis may only be realized when a multi-column CE with a buffer replenishment system is used.

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