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Quantitative separation of 4-hydroxyproline from skeletal muscle collagen by micellar electrokinetic capillary electrophoresis[1]

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Abstract

The phenylthiohydantoin (PTH) derivatives of 3- and 4-hydroxyproline (Hyp) were separated using micellar electrokinetic capillary electrophoresis (MEKC). The separation protocol was also used to determine Hyp content of bovine skeletal perimysial collagen preparations and whole muscle samples. Amino acids from hydrolyzed tissues were labeled using a two step procedure that involved initial reaction with *o*-phthalaldehyde (OPA) to modify primary amines followed by their precipitation under acidic conditions. In the second step, imino acids were reacted with phenyl isothiocyanate (PITC). This labeling method was rapid and the Hyp values determined in these biological samples were found to be in close agreement with conventional methods and other published reports.

Keywords: Hydroxyproline

1. Introduction

The hydroxylation of proline (Pro) to form hydroxyproline (Hyp) is a post-translational modification unique to the connective tissue proteins collagen and elastin [1]. The extent of this modification in collagen is substantial with 10–14% of the total amino acids being Hyp. The hydroxylation of Pro occurs intracellularly, shortly after synthesis while the chains are still unfolded. The hydroxylation of Pro provides additional H-bonding sites and contributes to stabilization of the triple helical conformation found in the mature molecule [2]. The analysis of Hyp has become accepted as a marker for a number of important processes including: collagen turnover [3], bone resorption [4] and metastasis in some forms of cancer [5]. In addition, Hyp determination has also been widely used to estimate the content of collagen in various tissues, especially muscle [6].

Previous methods for analysis of Hyp have predominantly used high-performance liquid chromatography (HPLC) in combination with pre-column derivativization techniques. For example, labeling with phenylisothiocyanate (PITC) followed by reversed-phase HPLC is a well established method and has advantages in increased UV absorption and stability of the PTH products [7]. However, when this method is used for Hyp or Pro analysis, the separation can suffer from co-migrating compounds (e.g., primary amino acids). To reduce these interferences, a variety of two step pre-column derivativiza-

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tion methods have been developed. Many of these procedures employed initial reaction of amino acids with OPA. OPA reacts with primary amines via Schiff base formation and leaves imino acids unmodified. Examples of the second step in these two part derivativization strategies include reaction with dabsyl chloride (dimethylaminoazobenzene-4-sulfonyl). This reagent reacts with imino acids to produce strong UV absorbing derivatives and has been useful for the HPLC determination of Hyp in urine samples [8,9]. Alternatively, a second reaction with 9-fluoromethyl chloroformate (FMOC-Cl) to produce fluorescent derivatives of imino acids has been applied to the analysis of Hyp in serum and urine [10]. Finally, a second step reaction with PITC is useful for the determination of Hyp as its stable PTH derivative in serum [11] plasma, urine and renal tissue culture media samples [12].

More recently, capillary electrophoresis has also proven to be an excellent tool for the analysis of amino acids as demonstrated by Terabe et al. [13]. Guzman et al. [14] reported that fluorescamine labeled Pro and 4-Hyp products could be separated by capillary zone electrophoresis. Most recently, FMOC-Cl labeled Pro and 4-Hyp in biological samples (serum and urine) were separated by a micellar electrokinetic chromatographic (MEKC) procedure using fluorescence detection [15].

In this paper, we describe methods for separation of Hyp isomers using micellar capillary electrophoresis. The MEKC separation utilized an amine coated column and resolved the PTH derivatives of Pro, and isomers of Hyp. These isomers included both *cis*- and *trans*-4-Hyp and 3-Hyp. In addition, a two-step labeling procedure was employed to determine the level of Hyp in bovine skeletal muscle perimysial collagen preparations and whole muscle samples.

2. Experimental

Capillary electrophoresis was performed with an ISCO (Lincoln, NE, USA) Model 3850 capillary electropherograph using an amine coated capillary, 70 cm (40 cm to the detector)×50 μm I.D. (Polymicro Technology, Phoenix, AZ, USA). The capillary was coated with diethanolamine essentially as described by Kuhn and Hoffstetter-Kuhn [16]. Briefly, 1 m of fused-silica capillary was treated with 1 *M* NaOH for 1 h and rinsed twice with 100 μl water. The capillary was rinsed with 100 μl each of methanol followed by acetone, to remove all moisture and then filled with 10% chloro(chloromethyl) dimethylsilane (methanol solution). The capillary was filled with silane reagent after 30 min and then incubated at 100°C for 3 h. At the end of this time, the column was flushed with methanol, followed by water (100 μl each). Three molar diethanolamine HCl (methanol solution), was introduced into the capillary, and then incubated overnight at 20°C.

Samples were loaded into the capillary by vacuum injection and detection of separated analytes was performed at 254 nm. Data was recorded and analyzed using CAESAR (Version 4.01, 1994, Roman Scientific) from Scientific Resources (Eatontown, NJ, USA). Quantitation was performed using thymine (2.0 m*M*) as an internal standard. Thymine migrated ahead of other analytes and thus created no interference.

2.1. Chemicals

Highest purity reagents were used throughout the experiments. Phenylisothiocyanate was obtained from Pierce (Rockford, IL, USA). Sodium hydroxide, hydrochloric acid, triethylamine, diethanolamine, malonic acid, chloro(chloromethyl)dimethylsilane, phthalic dicarboxaldehyde, 3- and 4-Hyp, *cis*- and *trans*-4-Hyp, methanol, sodium phosphate (monobasic), SDS and thymine were from Sigma (St Louis, MO, USA). Water used in these experiments was obtained by reverse osmosis (18 mΩ).

2.1.1. PTH labeling of Hyp standards

The preparation of PTH derivatives of Hyp isomers was performed essentially as described by Zeece and Chu [17]. Briefly, the amino acid to be labeled (e.g., 6.6 mg) was dissolved in 1.0 ml of coupling buffer containing methanol–triethylamine–water (7:1:1, v/v). The tube was flushed with nitrogen and 10 μl of PITC added per 100 μl of sample solution. The reaction was allowed to proceed for 10 min at room temp and then dried in a Centri-Vap (Fisher Scientific, Pittsburg, PA, USA).

The samples were taken up in methanol just prior to electrophoresis.

2.1.2. Preparation of muscle samples

Perimysial collagen samples were prepared from bovine skeletal muscle using the procedure of Light and Champion [18]. Muscle samples were trimmed of visible fat and external connective tissue. Twenty g of finely diced (approx. 3 mm^2) muscle was homogenized (30 s) with 100 ml of 50 m*M* $CaCl_2$ at 4°C using a Polytron. The suspension was centrifuged at 2000 *g* for 5 min. The pelleted material was washed two times with $CaCl_2$, followed by three washes with 1% SDS and three washes with water. The preparation was dialyzed vs. 40% methanol for 48 h and then collected by centrifugation (2000 *g* for 15 min). The collagen preparation was desiccated by three washes in cold (4°C) acetone, dried under a stream of nitrogen and stored at −20°C until used for analysis. Hydrolysis was performed using 50 mg of perimysial collagen with 5.0 ml of 6 *M* HCl under nitrogen for 24 h. A 100-μl aliquot (1.0 mg perimysial collagen sample) of hydrolysate was dried under vacuum in a Centri-Vap.

Whole muscle samples were also used for Hyp analysis. Finely diced bovine skeletal muscle was ground to a powder in liquid nitrogen and dried under vacuum. A 65-mg amount of muscle powder was hydrolyzed with 5 ml of 6 *M* HCl for 24 h at 110°C under vacuum and a 150-μl aliquot (2.0 mg muscle sample) was dried in a Centri-Vap.

2.1.3. Labeling of amino acids from hydrolyzed samples

The OPA-PITC strategy for labeling amino acids from hydrolyzed samples was based on a modification of the procedure described by Yaegaki et al. [11]. An aliquot of hydrolyzed sample was taken up in 100 μl of 0.5 *M* Na_2CO_3, $NaHCO_3$ pH 9.5. A 2-mg amount of OPA reagent (20 mg/ml) was added per mg of sample and the mixture let stand at room temperature (20°C) for 15 min. The reaction products were precipitated by addition of 900 μl of 1.0 *M* NaH_2PO_4 pH 2.5. The precipitate was removed by centrifugation in a microfuge at 10 000 *g* for 5 min and the supernatant filtered through 0.45-μm spin-filters (Millipore, Bedford, MA, USA). A 100-μl amount of filtrate was dried in the Centri-Vap and then dissolved in 150 μl coupling buffer (described in Section 2.1.2). The vial was flushed with nitrogen and 10 μl of PITC added. The mixture was incubated at room temperature for 10 min and dried. Finally, samples were taken up in 100 μl 50% acetonitrile–water solution just prior to electrophoresis.

2.1.4. Spectrophotometric analysis of Hyp

The spectrophotometric method of Bergman and Loxley [19] was used to determine Hyp levels in muscle samples and serve as a corroborating method for capillary electrophoretic analysis. For these determinations, a 150-μl aliquot of hydrolyzed sample (perimysial collagen or whole muscle) was neutralized with NaOH and then treated with Chloramine T. The oxidized imines were then reacted with *p*-dimethylaminobenzaldehyde and the colored reaction product measured at 558 nm. This value was converted to Hyp concentration using a curve prepared from varying amounts of standard Hyp.

3. Results and discussion

3.1. Separation of Hyp isomers

Separation of Hyp isomers (PTH derivatives) was achieved in 50 m*M* Na-malonate pH 5.0 containing 75 m*M* SDS (Fig. 1) at 15 kV. The conditions used in these separations resulted from a number of experiments to optimize the resolution Hyp isomers (results not shown). These included buffers in the range of pH 2.5 to 10, SDS concentrations from 25 to 100 m*M* and a variety of coated and uncoated capillaries. The above stated conditions in combination with an amine coated capillary gave the best results. While the underlying reasons for better performance with the amine coated column were not investigated, it could be proposed that the positive charge introduced on the capillary wall may have established a dynamic equilibrium with the negatively charged SDS micelles/molecules and contributed to the separation of Hyp isomers.

The 3- and 4- positional isomers of Hyp were almost completely baseline resolved in approximately 10 min under these conditions (Fig. 1). These compounds were also well separated from Pro. Such

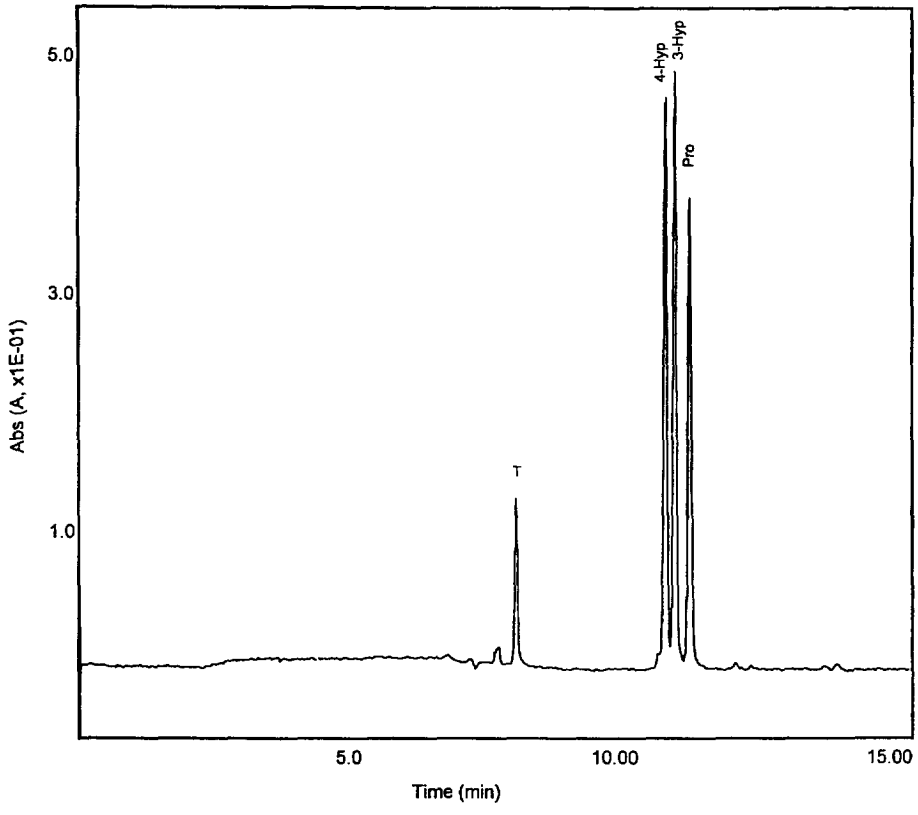

Fig. 1. Electropherogram of the PTH derivatives of 3-Hyp, 4-Hyp and Pro (5–6 pmol each). The separation was performed in 50 m*M* Na-malonate pH 5.0, 75 m*M* SDS with an amine coated column (75 cm×50 μm I.D.) at 15 kV. Thymine (T) was used as reference and internal standard.

a separation is essential for the potential application of this protocol to biological samples.

In addition, *cis*- and *trans*-4-Hyp isomers (PTH derivatives) were also resolved under these conditions (Fig. 2). The separation of these stereo isomers may have resulted from the slightly different structures of their PTH derivatives, that produced a differential interaction with SDS micelles. A mixture of all four PTH derivatized compounds (*trans*-4-Hyp, 3-Hyp, *cis*-4-Hyp and Pro) was also separated, but, without complete baseline resolution (Fig .3). The inset of Fig. 3a contains an expanded time scale representation of the original electropherogram and shows the relative separation of all four isomers. Figs. 1–3 illustrate the value of this separation procedure for the determination of 3- and 4-Hyp and its potential application to the measurement of collagen degradation from different tissues. The 3-Hyp isomer is found predominately in type IV collagen molecules that are associated with the basement membrane of cells and also in elastin containing tissues such as arterial walls [1,20].

The above separation protocol was used to determine the level of Hyp in perimysial collagen preparations and whole muscle samples from bovine skeletal muscle. The collagen in these tissues is composed predominately of type I molecules. Direct PITC labeling of hydrolyzed samples was initially tried. This approach proved problematic because a number of other peaks co-migrated with Hyp and Pro (results not shown). Therefore, a modified two step labeling procedure was employed to reduce the number of peaks as has been previously reported in HPLC methods for Hyp determination [11]. In this protocol, primary amino acids were first reacted with OPA to form Schiff bases in aqueous solution.

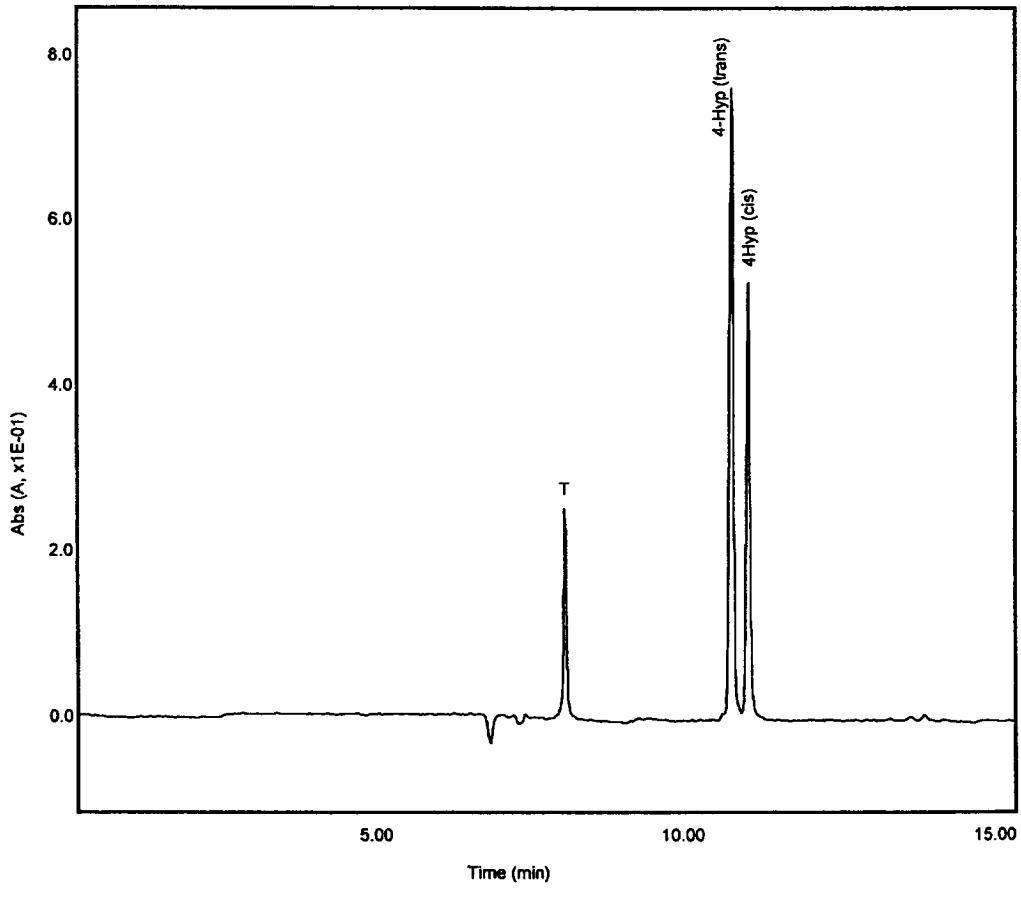

Fig. 2. Electropherogram of PTH derivatives of *cis*-and *trans*-4-Hyp (7 and 10 pmol, respectively). The separation conditions were identical to those listed in Fig. 1.

However, column separation of OPA products prior to PITC labeling was omitted in favor of precipitation. The products of OPA reaction were soluble, but could be effectively precipitated by a combination of increased ionic strength and lowered pH (2.5). It is important to note that OPA labeling should be performed in aqueous solution as it was found that inclusion of organic solvent such as methanol or acetonitrile greatly reduced recovery. This may have resulted from OPAs reaction with imino acids under nonpolar conditions. It was also found that inclusion of mercaptoethanol in the OPA reaction to produce stable isoindol derivatives of primary amines [21] was not necessary. In addition, lowering the pH of isoindol derivatives, resulted in precipitates from which, imino acids were more difficult to extract. The recovery of Hyp using the labeling procedure described here was routinely found to be 94±5%. This value represents the mean and S.D. of eighteen determinations.

Bovine perimysial collagen fractions and whole muscle samples were prepared, hydrolyzed and labeled using the two step procedure described in Sections 2.1.2 and 2.1.3. The capillary electrophoretic separation of these samples was essentially free of interfering analytes with 4-Hyp (trans) migrating in about 11 min (Figs. 4 and 5). Pro consistently migrated 0.5 min later than Hyp. The identity of these peaks was established from separate runs with co-injected standards. As expected, no 3-Hyp was detected in either of the bovine samples that contained predominately type I collagen. Comparison of

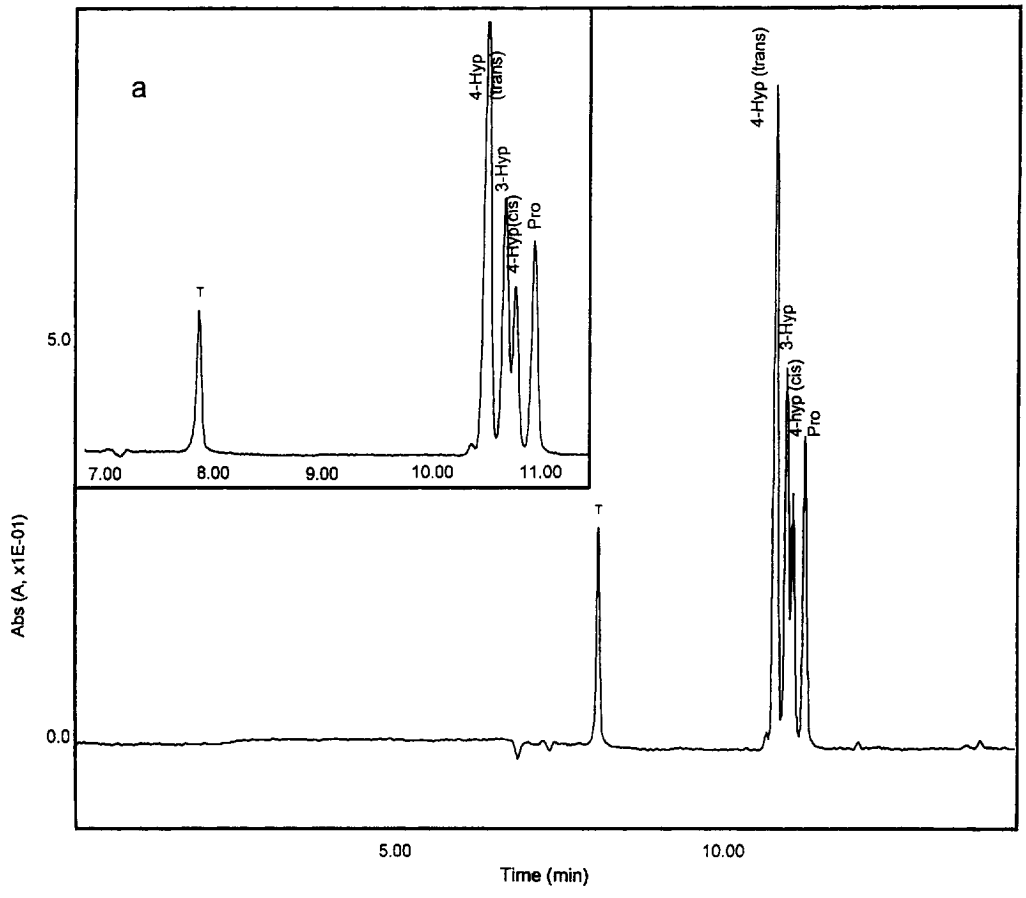

Fig. 3. Electropherogram of a mixture of PTH Hyp isomers and Pro. The separation contains 3-Hyp (6 pmol), *cis*- and *trans*-4-Hyp (4 and 10 pmol, respecitvely) and Pro (5 pmol). Separation conditions were identical to those listed in Fig. 1. The inset (a) in this figure represents a computer program derived (Caesar 4.01) time scale expansion of the electropherogram.

these separations (c.f., Figs. 4 and 5) revealed that the ratio of Hyp to Pro was greater in the perimysial fraction than in whole muscle. The perimysial sample was composed mostly of collagen, whereas the whole muscle sample contained Pro from noncollagen proteins as well.

The amount of 4-Hyp in these samples was calculated from a standard curve (not shown) with quantitation performed using an internal standard (thymine). The standard curve of the ratio of peak area (4-Hyp/thymine) was linear in the range of 1.0 to 16 pmol (r^2=0.998) with a lower detection limit of 1.0 pmol.

The CE method for 4-Hyp determination described here found 76.8±3.7 and 21.1±0.8 mg Hyp/g of dry sample (mean and S.D. of nine determinations) for perimysial collagen fraction and whole muscle, respectively. This latter value for sternomandibularis muscle is in close agreement with Nguyen and Zarkadas [22] who reported 15.2 mg Hyp/g sternomandibularis muscle on a dry weight basis. The values determined for the bovine samples were also in close agreement with those obtained by the spectrophotometric method on these same samples. Perimysial collagen and whole muscle samples were found to contain 86.0±1.2 and 22.8±0.1 mg Hyp/g dry sample, respectively, using the method of Bergman and Loxley [19]. The CE method values for Hyp were slightly lower than those determined by the spectrophotometric method, but, corresponded to 92.5% and 89% for perimysial and whole muscle samples, respectively.

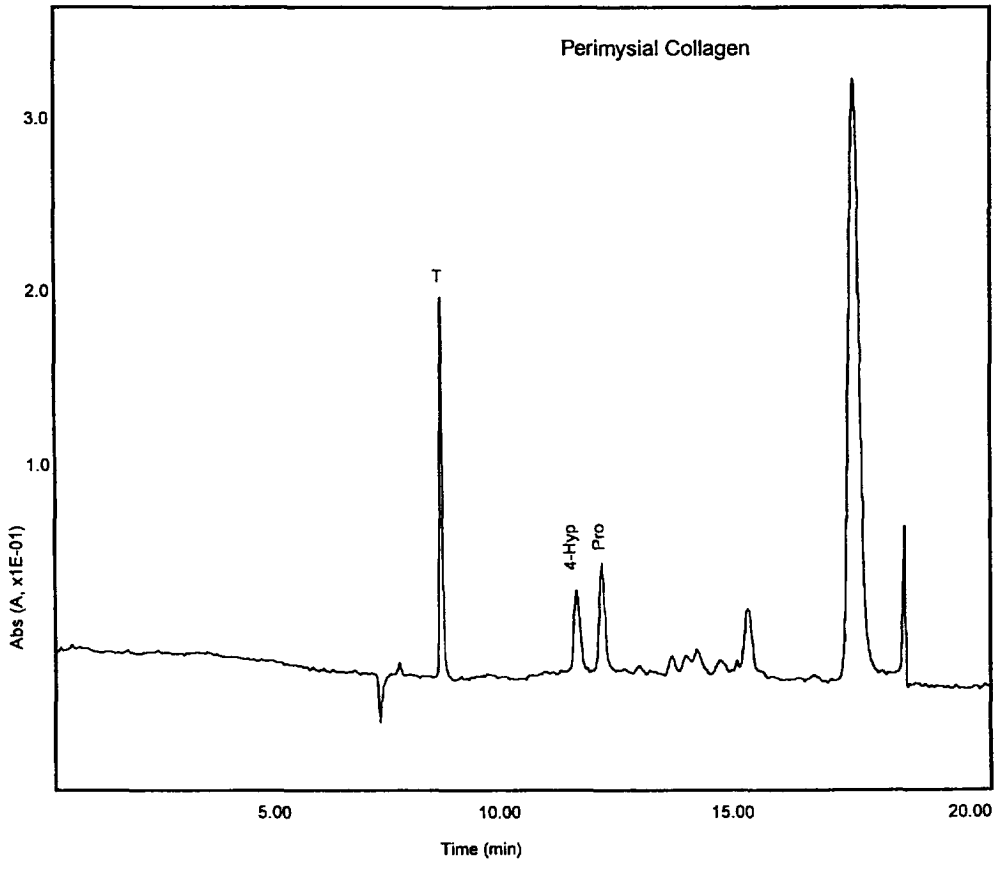

Fig. 4. Electropherogram of hydrolyzed bovine skeletal perimysial collagen. The hydrolyzed sample was reacted with OPA followed by precipitation of the products under acidic conditions, and labeling of imino amino acids with PITC. The separation conditions were as described in Fig. 1. This separation contained 2.4 pmol Hyp.

Using the CE method described here, the lowest reliable limit of detection of PTH-Hyp standards was approximately 1 pmol. The determination of Hyp level in sternomandibularis muscle was typically performed on aliquots of hydrolysates corresponding to 1–2 mg of sample. This level of routine analysis could be lowered to the μg range through combined use of microwave hydrolysis [23], OPA-FMOC derivatization [10] and CE separation of fluorescent derivatives [15].

CE has unique advantages over HPLC methods for Hyp determination. Some of the previous HPLC methods for Hyp determination in biological samples such as serum and urine, have relied upon two step derivatization protocols that also required two separations [11,12]. This approach increases analysis time and the amount of sample required. HPLC methods also showed some difficulty in resolution of PTH 3- and 4-Hyp [12] but were successful for the FMOC derivatives [10]. In contrast, the CE procedure described here used a two step derivatization procedure with a simple precipitation step to remove the OPA reaction products without affecting Hyp and Pro recovery. The amount of sample required (1–2 mg) was small and the separation protocol was able to resolve 3- and 4-Hyp as well as *cis*- and *trans*-isomers. Additionally, the ability of CE to readily resolve various forms of Hyp in extremely small samples makes it a viable alternative to HPLC. For example, CE would be well suited to studies of collagen turnover in muscle and fibroblast cell culture systems or in biopsy samples.

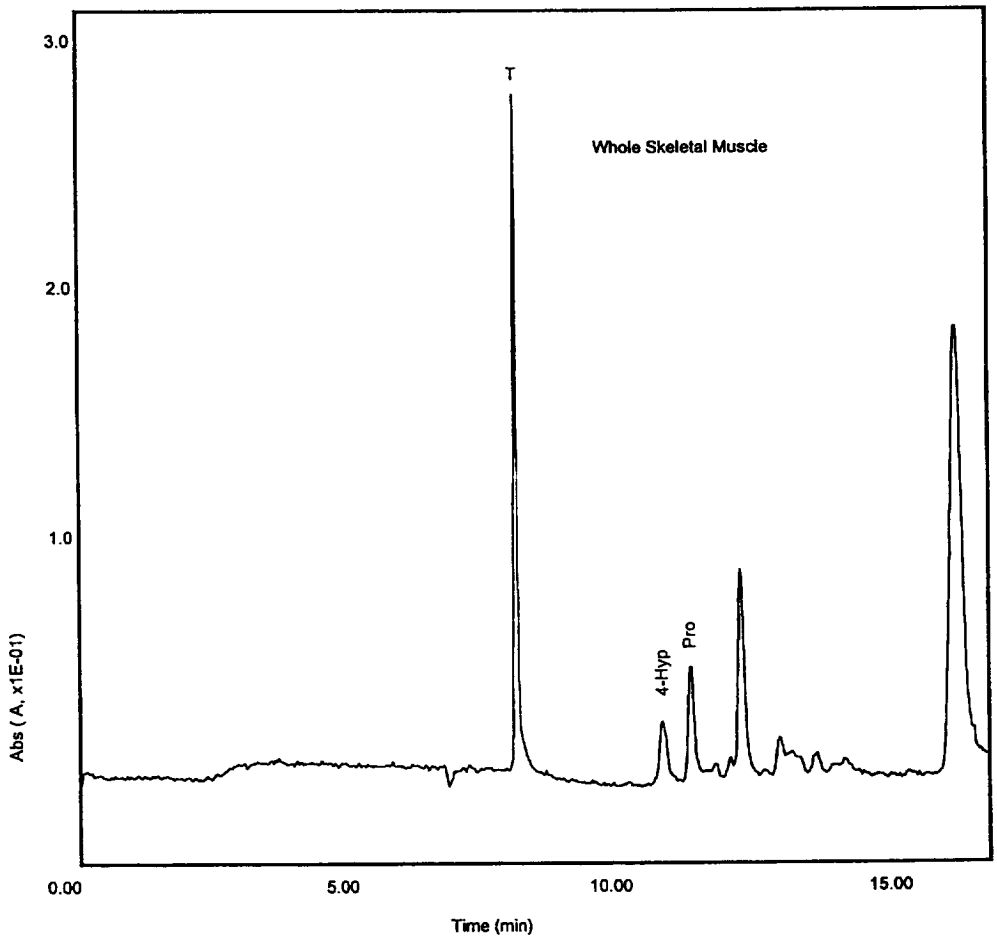

Fig. 5. Electropherogram of hydrolyzed bovine sternomandibularis muscle. The separation conditions were as described in Fig. 1. The hydrolyzed sample was labeled in a two step procedure as described in Fig. 4 and the separation contained 1.0 pmol Hyp.

4. Conclusion

An MEKC procedure is described here for the separation of the PTH derivatives of 3- and 4-Hyp at the picomole level using UV detection. This separation protocol was combined with a two step labeling procedure involving initial reaction with OPA to remove primary amino acids, followed by precipitation. The imino acids were then reacted with PITC. The combined labeling and separation protocols facilitated rapid determination of Hyp in skeletal muscle samples. Sensitivity of the procedure required only mg levels of sample. A reduction in sample size reduces analysis time and contributes to the goal of performing Hyp determinations with very small samples, such as those encountered in tissue culture or biopsy samples.

Acknowledgments

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