A New Procedure for the Specific High-Performance Liquid Chromatographic Determination of Hydroxyproline

Pier Antonio Biondi*, Luca Maria Chiesa, and **Maria Rosa Storelli**
Istituto di Fisiologia Veterinaria e Biochimica, Via Celoria 10 - 20133 Milano, Italy
Pietro Renon
Istituto di Ispezione degli Alimenti di Origine Animale, Via Grasselli 7 - 20133 Milano, Italy

Abstract

A procedure suitable for a selective high-performance liquid chromatographic (HPLC) analysis of the imino acid hydroxyproline in the presence of a large excess of amino acids is proposed. To deaminate the amino acids, the well-known reaction with nitrous acid is exploited. The *N*-nitroso derivatives of imino acids obtained are extracted in ethyl acetate, denitrosated by hydrobromic acid, and treated with dabsyl-chloride. The final HPLC separations are carried out on a reversed-phase column in a rapid isocratic run. The use of the *cis* isomer of hydroxyproline as an internal standard allows good reproducibility. As an application of the described method, the hydroxyproline content in samples containing collagen and an excess of bovine serum albumine (up to 20:1) is determined.

Introduction

trans-4-Hydroxyproline (OH-Pro) is the characteristic imino acid present in collagen. Its determination is noteworthy in clinical studies as a bone metabolism marker (1,2) and in meat science as a connective tissue marker in muscle (3,4). Among the many high-performance liquid chromatographic (HPLC) methods developed for OH-Pro measurements, the recently introduced ones exploit a previous reaction of interfering amino acids with *o*-phthaldialdehyde (OPA), which is specific for primary amino groups, and a latter treatment with a general reagent for primary and secondary amino groups such as 9-fluorenylmethyl chloroformate (FMOC-Cl) (1,4,5), phenylisothiocyanate (PITC) (6), 4-(5,6-dimethoxy-2-phthalimidinyl)phenylsulfonyl chloride (DPS-Cl) (7), and 4-dimethylaminoazobenzene-4'-sulfonyl chloride (dabsyl chloride, DABS-Cl) (2,8,9). The dabsyl derivatives have been used more than the other ones, both because of their stability and because simple ultraviolet (UV) detection at high wavelengths is allowed.

To determine OH-Pro in meat products, where a large excess of other amino acids is present, the double derivatization method using OPA and DABS-Cl has been tested, but reproducible results were not always obtained. The OPA concentration has been shown to be a critical factor, as previously reported (8).

A new procedure suitable for the elimination of amino acids containing primary amino groups is thus needed. For this purpose, the well-known reaction of primary amino moiety with nitrous acid, which is the basis of the Van Slyke procedure for protein nitrogen determination (10,11), was used. The final derivatization step was performed again using classical DABS-Cl, and HPLC analysis was carried out through an isocratic run. To increase the precision of the procedure, *cis*-4-hydroxyproline (*cis*-OH-Pro), the geometrical stereoisomer of natural OH-Pro, was introduced as an internal standard.

Experimental

Materials

DABS-Cl was purchased from Fluka (Buchs, Switzerland) and recrystallized as previously described (8). Proline (Pro), OH-Pro, *cis*-OH-Pro, collagen type I from a bovine Achilles tendon, and bovine serum albumin (BSA) were purchased from Sigma (St. Louis, MO). All other reagents and solvents were obtained from Merck (Darmstadt, Germany).

Apparatus and chromatographic conditions

A Jasco (Tokyo, Japan) HPLC system equipped with a Familic 300 S pump, a DV-312 injector, and a Uvidec 100-V detector was used. The separations were performed on a LiChrocart column (125 × 4 mm) filled with LiChrospher 100 RP-8 (5 µm) as the stationary phase (Merck). A 0.01M KH_2PO_4 solution

* Author to whom correspondence should be addressed.

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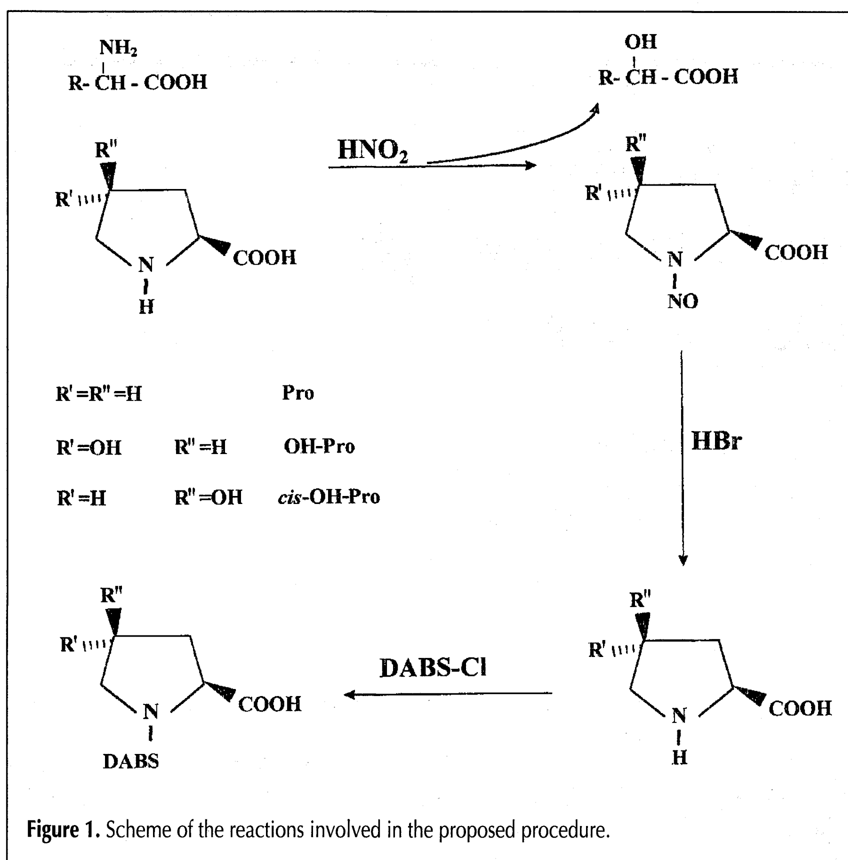

Figure 1. Scheme of the reactions involved in the proposed procedure.

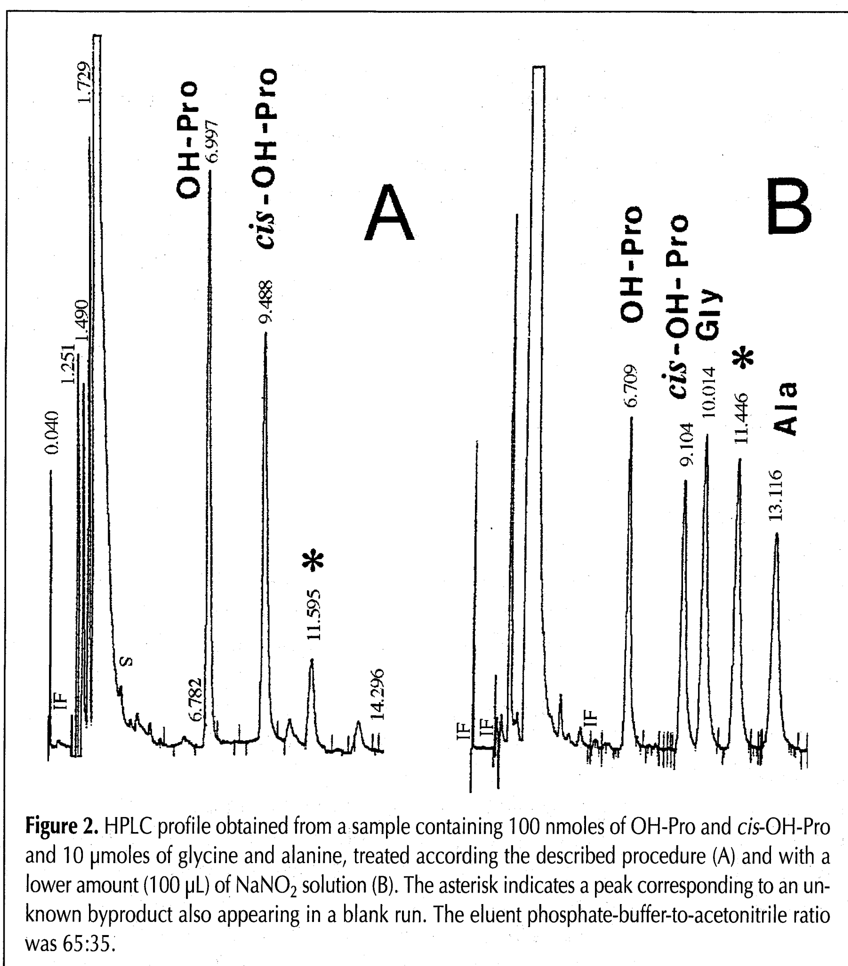

Figure 2. HPLC profile obtained from a sample containing 100 nmoles of OH-Pro and *cis*-OH-Pro and 10 µmoles of glycine and alanine, treated according the described procedure (A) and with a lower amount (100 µL) of $NaNO_2$ solution (B). The asterisk indicates a peak corresponding to an unknown byproduct also appearing in a blank run. The eluent phosphate-buffer-to-acetonitrile ratio was 65:35.

brought to pH 2.5 with H_3PO_4 and mixed with acetonitrile was used as an eluent at a flow rate of 1 mL/min; the eluate was monitored at 436 nm. The phosphate buffer-to-acetonitrile ratio was 65:35 in preliminary experiments and changed to 60:40 during final tests in the presence of Pro derivative.

Derivatization procedure

Collagen (0.5 mg/mL) and different amounts of BSA (0–10 mg/mL) were hydrolyzed for 16 h at 110°C in 6M HCl. A sample (200 µL), added with *cis*-OH-Pro (50 µL of a 1mM solution in 0.01M HCl), was kept in a mixture of ice and salt. A fresh solution of 8M $NaNO_2$ (300 µL) was slowly added under magnetic stirring, and the mixture was maintained for 5 min below 0°C and for 10 min at room temperature. Ethyl acetate (3 mL) was added, and after extraction, the organic phase was brought to dryness under a nitrogen stream. The sample was then treated with 48% HBr (50 µL) for 10 min at 70°C and again brought to dryness. Then 100 µL of 0.2M Na_2CO_3 and a solution (5 mg/mL) of DABS-Cl in acetonitrile (200 µL) were finally added, and the mixture was maintained for 10 min at 70°C. Aliquots (20 µL) were analyzed by HPLC.

Quantitative analysis

A fixed amount of *cis*-OH-Pro (50 nmoles) and different amounts (10–250 nmoles) of OH-Pro were dissolved in 6M HCl (200 µL) and treated according to the above described procedure. A calibration curve was obtained from the final ratios of the heights corresponding to OH-Pro and *cis*-OH-Pro, and from this curve, the OH-Pro contents in the analyzed samples were detected.

Results and Discussion

Figure 1 shows the scheme of the proposed procedure reactions followed by the amino acids and the imino acids Pro and OH-Pro contained in samples as well as *cis*-OH-Pro, the internal standard. The treatment with HNO_2, produced from $NaNO_2$ in acidic medium, transforms amino acids into hydroxy acids and imino acids into *N*-nitroso derivatives (12). In this step, temperature control is critical; only by keeping the samples at or below 0°C is it possible to reduce the decomposition of nitrous acid to a minimum and keep the concentration of the

active reagent nitrosonium ion high. Two advantages were achieved by the extraction of *N*-nitroso derivatives of imino acids in ethyl acetate: a higher handling speed due to the higher volatility of the organic solvent with respect to the aqueous mixture in the subsequent evaporation step, and the elimination of the high NaCl amount, which would interfere with the final DABS-Cl treatment. In preliminary experiments, the initial aqueous sample was directly brought to dryness before the HBr treatment, but the yields of the final DABS-Cl reaction were markedly lower. According to a described procedure (13), the dabsyl derivatives were obtained in acceptable yields only when the salt content was eliminated before the HBr treatment by extraction of organic solutes with acetone from a dried sample. However, the extraction with ethyl acetate was finally adopted because of both rapidity and recovery yields.

The treatment with HBr performs the denitrosation of secondary amino groups as often described in *N*-nitroso amine analytical studies (14–16). Preliminary tests attempted to exploit the presence of HCl in the initial reaction mixture to perform the denitrosation step immediately after the nitrosation step by bringing the samples to dryness at a high temperature; however, the reaction yields appeared lower in comparison with HBr treatment. After the subsequent evaporation step, the addition of Na_2CO_3 solution assured that the neutralization of the residual traces of HBr and the alkalinization of the medium were suitable for the final derivatization step.

Because the retention times of glycine and alanine dabsyl derivatives were similar to that of OH-Pro, they were used in preliminary tests as representative interfering amino acids, which allowed the analysis of all the analytes by an isocratic HPLC run. In this way, the optimum $NaNO_2$ amount, reaction time, and temperature of the deamination step were studied using up to a 200:1 excess of primary amino acids in order to point out the conditions that were finally proposed and applied to the protein hydrolysate samples.

In Figure 2, typical chromatograms corresponding to samples formed as described and subjected to the proposed procedure using different nitrite amounts are shown. The efficiency of the deamination performed by HNO_2 and the need for an adequate amount of this reagent to exaustively eliminate a large excess of amino acids are evident. In the HPLC profile, only an unknown peak with a retention time between those corresponding to glycine and alanine and produced by the reagents appears in addition to the amino acid derivative peaks without interfering with the analyte ones.

As for the chromatographic conditions, an acidic elution buffer was preferred to neutral ones that are often used in other DABS-amino acid determinations in order to separate the major reagent peak in the void volume, which would otherwise interfere with the analyte peaks. When samples also containing the more hydrophobic Pro derivative were to be analyzed, the organic modifier was increased in the eluent mixture in order to make the HPLC run more rapid. The retention times of OH-Pro derivatives decreased but still remained well-separated.

Collagen from a bovine Achylles' tendon was used as an example of protein containing OH-Pro. BSA was chosen as a protein model without OH-Pro and was added to collagen in increasing amounts to represent specimens containing traces of OH-Pro in the presence of an excess of other amino acids. A typical chromatogram obtained from a sample containing collagen and BSA in a weight-to-weight ratio of 1:10, hydrolyzed and treated according the proposed method, is given in Figure 3. With respect to the above reported standard chromatograms, in addition to the expected Pro derivative peak and apart from the above mentioned "reagent" peak, only a small background resulted from the large amount of amino acids present in the hydrolyzed sample, thus demonstrating again the selectivity of the overall procedure.

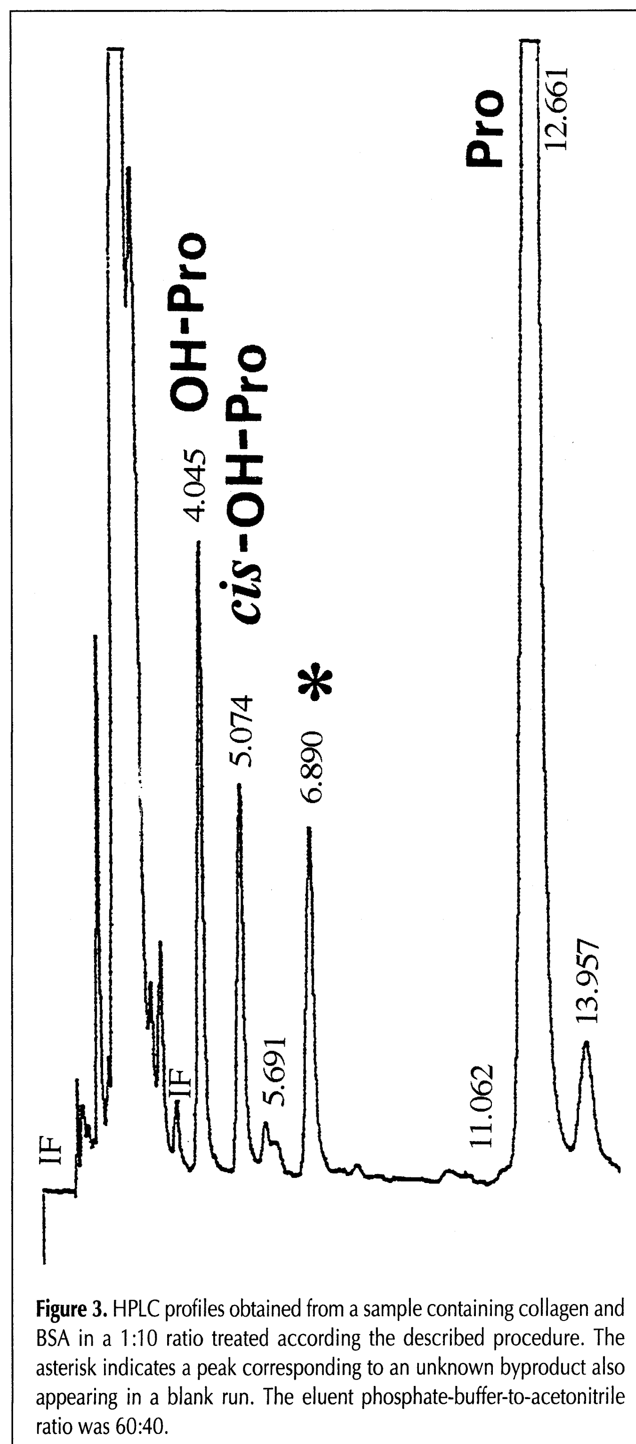

Figure 3. HPLC profiles obtained from a sample containing collagen and BSA in a 1:10 ratio treated according the described procedure. The asterisk indicates a peak corresponding to an unknown byproduct also appearing in a blank run. The eluent phosphate-buffer-to-acetonitrile ratio was 60:40.

In the range of 10–250 nmoles of OH-Pro and in the presence of 50 nmoles of *cis*-OH-Pro, the relationship between the height ratios (R_h) of OH-Pro to *cis*-OH-Pro and the OH-Pro amounts resulted as linear according the following equation:

$$R_h = 0.023 \times \text{OH-Pro (nmoles)} - 0.022$$

The correlation coefficient was 0.996. The precision of the method was tested on samples containing collagen (50 µg) and BSA in a 1:10 ratio. The within-day (5 replicates) and the between-day (5 replicates) mean contents of OH-Pro in collagen (nmoles/mg) were 926 ± 42 (coefficient of variation: 4.5 %) and 907 ± 57 (coefficient of variation: 6.3 %), respectively. No significant differences appeared in the collagen content when it was analyzed either alone or with BSA in 1:10 or 1:20 weight-to-weight ratios. Despite the various steps involved in the described procedure, the use of an internal standard as similar to OH-Pro as its geometric stereoisomer allows very acceptable reproducibility and affordable quantitative data.

Conclusion

The proposed method appears suitable for the determination of small amounts of OH-Pro in the presence of a large excess of other amino acids. On the contrary, when large amounts of amino acids do not interfere with the OH-Pro analysis, the use of OPA for their elimination can be considered the method of choice because it is simpler and more rapid in comparison with the HNO_2 treatment. Works are in progress to apply the described procedure to OH-Pro determination in meat products.

Acknowledgments

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