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Reversed-phase high-performance liquid chromatographic analysis of hydroxyproline and proline from collagen by derivatization with dabsyl chloride

Masashi Ikeda*

Laboratory of Medical Science, Dokkyo University School of Medicine, Mibu, Tochigi 321-02 (Japan)

Kenji Sorimachi

Department of Microbiology, Dokkyo University School of Medicine, Mibu, Tochigi 321-02 (Japan)

Kazumi Akimoto

Laboratory of Recombinant DNA Research, Dokkyo University School of Medicine, Mibu, Tochigi 321-02 (Japan)

Yosihiro Yasumura

Department of Microbiology, Dokkyo University School of Medicine, Mibu, Tochigi 321-02 (Japan)

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ABSTRACT

A high-performance liquid chromatographic method for the analysis of hydroxyproline and proline has been developed. The method is based on the derivatization of the secondary amino group with dabsyl-chloride after blocking of the primary amino group with *o*-phthalaldehyde. Dabsyl-hydroxyproline and dabsyl-proline were separated from other amino acids by high-performance liquid chromatography in the gradient elution mode, and eluted at 10.27 and 16.02 min, respectively. The correlations between the peak areas of dabsyl-hydroxyproline and dabsyl-proline were linear in the range from 20–200 pmol, with equations $y = 1.10x - 0.80$ ($r = 0.999$) and $y = 1.12x - 0.52$ ($r = 0.999$), respectively. The method was applied to the analysis of rat tail collagen, and the contents of hydroxyproline and proline were 1.55 ± 0.04 and 2.03 ± 0.04 nmol/μg, respectively.

INTRODUCTION

It is well known that hydroxyproline (Hyp) is contained in collagen, and that the hydroxylation of proline is an important process in collagen biosynthesis. Therefore, the measurement of the Hyp content has previously been carried out in collagen research. Most of these methods are based on pre-column derivatization with various reagents and a high-performance chromatographic (HPLC) analysis of all the amino acids [1–4]. Recently, Paroni *et al.* [5] analysed total urinary hydroxyproline by a rapid and simple HPLC assay.

Dabsyl chloride (dabsyl-Cl) has been used as a derivatization reagent for amino acids [6,7]. This reagent was first applied to amino acid analysis by Lin and Chang [6]. Vendrell and Aviles [7]

* Corresponding author.

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reported that the dabsyl amino acids are very stable. The stability is maintained for several months when the sample is left at −20°C. In addition, detection in the visible range at 436 nm can decrease interferences caused by UV-absorbing material present in biological samples. Another advantage of this method is that the derivatization is simple and can be carried out in a relatively short time. The derivatization procedure using phenyl isothiocyanate (PITC) is more complex than that using dabsyl-Cl [8].

o-Phthalaldehyde (OPA) reacts only with primary amino groups, whereas dabsyl-Cl reacts not only with primary but also with secondary amino groups. A derivatization based on both reactions using OPA and dabsyl-Cl offers a method that selectively analyses Hyp and Pro from an amino acid mixture.

The selective derivatization of the secondary amino group, after blocking of the primary amino group with OPA, was performed in urine samples by detection of the secondary amino group with 9-fluorenylmethyl chloroformate (FMOC-Cl) [9,10] or 4-chloro-7-nitrobenzofurazan (NBD-Cl) [11]. A fluorescence photometer, however, is necessary for detecting the amino acids derivatized with these compounds, and the derivatives are unstable. Therefore, we used dabsyl chloride for detecting the secondary amino group.

As indicated above, because Hyp is an important metabolite in collagen metabolism, the present method, which uses OPA for blocking the primary amino group, and dabsyl-Cl for derivatizing the secondary amino group, has been developed to analyse both Hyp and Pro in collagen.

EXPERIMENTAL

Reagents and chemicals

Dabsyl chloride (4-dimethylaminoazobenzene-4′-sulphonyl chloride), sodium hydrogencarbonate, 2-mercaptoethanol (2-ME), a mixture of 17 standard amino acids (H-type; 2.5 μmol/ml each in 0.1 *M* HCl; Asp, Glu, Gly, Ser, His, Thr, Ala, Arg, Pro, Tyr, Val, Met, Cys, Ileu, Leu, Phe and Lys) and Hyp were purchased from Wako (Osaka, Japan). H-type means the standard amino acids for the analysis of protein hydrolysates. OPA was purchased from Kanto (Tokyo, Japan).

Reagents for both the mobile phases and the hydrolysis of the cells were obtained from Wako. Acetonitrile, methanol, N,N-dimethylformamide (DMF), distilled water, sodium acetate, acetic acid, sodium bicarbonate and sodium phosphate were either HPLC-grade or equivalent. All other chemials were of the purest grade.

Sample preparation and hydrolysis

Rat type I collagen was isolated from the rat tail tendon. The extraction of collagen and the hydrolysis were carried out according to the previously reported procedure [12]. The collagen solution was kept at −20°C until use. The protein content in the collagen solution was determined by the method of Lowry *et al.* [13], using bovine serum albumin as the standard. The mixture of standard amino acids and Hyp was dissolved in distilled water to produce a 0.1 μmol/ml solution. A 20-μl volume of the standard amino acids and the hydrolysed collagen samples were each added to plastic-stoppered tubes (50 × 6 mm I.D.) and then dried under reduced pressure.

Derivatization

Dabsyl-Cl was recrystallized as follows: 1 g of dabsyl-Cl was dissolved in acetone and then was left at −20°C overnight. Recrystallized dabsyl-Cl was completely dried *in vacuo*, 1.3 mg were dissolved in 2 ml of acetone, and 400 μl of this solution were placed in a small tube and dried again under reduced pressure.

Just before derivatization, the dried dabsyl-Cl (0.26 mg) was dissolved in 200 μl of acetonitrile, and 20-μl aliquots were added to each sample tube for derivatization.

In routine procedures, OPA was prepared for the reaction with the primary amino group by dissolving 60 mg of OPA in 2 ml of acetone and adding 30 μl of 2-ME just before use. The solution was immediately diluted 1:64 (v/v) with a 50 m*M* carbonate–bicarbonate buffer solution (pH 9.0), and 20 μl of the diluted OPA solution (3.5 m*M*) were transferred to each tube containing

amino acids and mixed well. The solution was left for 5 min at room temperature, then 20 μl of the dabsyl-Cl reagent were added and this solution was mixed. The dabsylation was carried out for 20 min at 70°C with the tubes capped or plugged. After the reaction, 60 μl of 50% acetonitrile in distilled water were added to the derivatized samples. The tubes were gently shaken, and samples of 10 μl were applied to the column.

HPLC separation of dabsyl-amino acids

The separation of amino acids was performed with the HPLC system of the Waters Chromatography Division (Millipore, Milford, MA, USA) consisting of a Model 600 solvent-delivery system, a Model 486 UV–Vis detector (fixed at 436 nm), a Model 741 data module, and a Model 3 column heater. A Wakosil $5C_{18}$ column (particle size 5 μm, 250 × 4.6 mm I.D.; Wako) was used. The gradient elution programme and mobile phases used for the selective separation of Hyp and Pro are shown in Table I.

RESULTS AND DISCUSSION

The Hyp and Pro contents of collagen are important indexes. Therefore, we focused on the development of an analytical method for the determination of both these amino acids. Several studies have reported the measurement of Hyp detected selectively from other amino acids [9,14–16].

In order to determine the suitable concentration for the OPA reaction with amino acids, we examined various concentrations of OPA made with 50 m*M* carbonate–bicarbonate buffer. When the concentration of OPA that reacted with 3.6 nmol of amino acids (200 pmol each) was increased to 7.0 m*M* from 3.5 m*M* used in the routine procedure, the peak heights of Hyp and Pro decreased. On the other hand, when the concentration of OPA was decreased to 1.8 m*M*, many unknown peaks appeared. These results suggest that a high concentration of OPA may affect the secondary amino group, and that a low concentration of OPA cannot completely block the primary amino group. Therefore, we decided that the original solution of OPA should be diluted 64-fold to obtain a suitable concentration for the reaction with amino acids; the concentration of OPA was 3.5 m*M*. Monboisse *et al.* [9] reported that the destruction of the secondary amino group was observed when a large excess of OPA was used in the absence of primary amino acids.

In order to determine the conditions under which dabsyl-Hyp and dabsyl-Pro are completely separated from the other peaks, various elution systems were examined. Fig. 1A shows the elution profile of the dabsylated mixture of 18 amino acids without the OPA treatment. The elution was carried out under the conditions described in Table I. The dabsyl-Hyp peak eluted at 10.27 min, and the dabsyl-Pro peak at 16.02 min. Fig. 1B shows the chromatogram of the dabsyl amino acid mixture treated with OPA and eluted under the same conditions. The peaks for both Pro and Hyp were completely separated from the other peaks. When the pH of solvent A was fixed at 3.5, the Hyp peak moved closer to the unknown peak b, whose elution time was 12.40 min. When the pH was increased above 3.5, the Hyp peak moved closer to the dabsyl-ONa peak or overlapped with it. The most suitable pH for solvent A was found to be 3.0, as shown in Table I.

The correlation between the peak area of Hyp or Pro and its amount was investigated in the range from 20 to 200 pmol, and linear relationships were obtained. The correlation coefficients (γ values) were 0.9998 ($y = 1.102x - 0.796$) for Hyp and 0.9997 ($y = 1.117x - 0.523$) for Pro. The relationship from 200 pmol and 1000 pmol was not linear. It appears that the amounts of dabsyl-Cl might be insufficient for dabsylation. Thus, a large excess of dabsyl-Cl is needed to react completely with a large amount of amino acids. However, when a large excess of dabsyl-Cl is used for the derivatization, several unknown peaks appear. Therefore, a large amount of amino acids should be avoided. The most suitable ratio of dabsyl-Cl to amino acids was determined to be a three- to four-fold molar excess [17].

The ε-amino group of lysine (Lys), the imidazole group of histidine (His) and the phenolic hydroxyl group of tyrosine (Tyr) all react with dab-

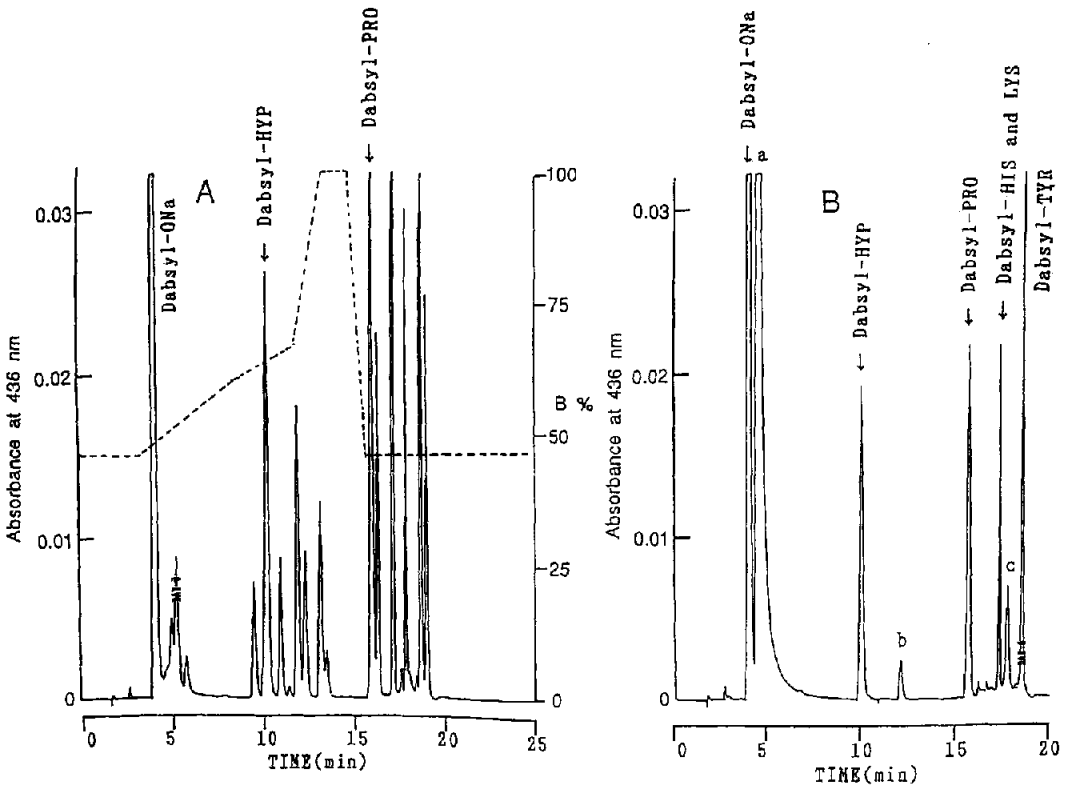

Fig. 1. Chromatograms of 18 dabsyl amino acids: (A) without the OPA reaction; (B) with the OPA reaction. The peaks a, b and c are unknowns. The amount of each amino acid was 200 pmol. The gradient elution programme is given in Table I.

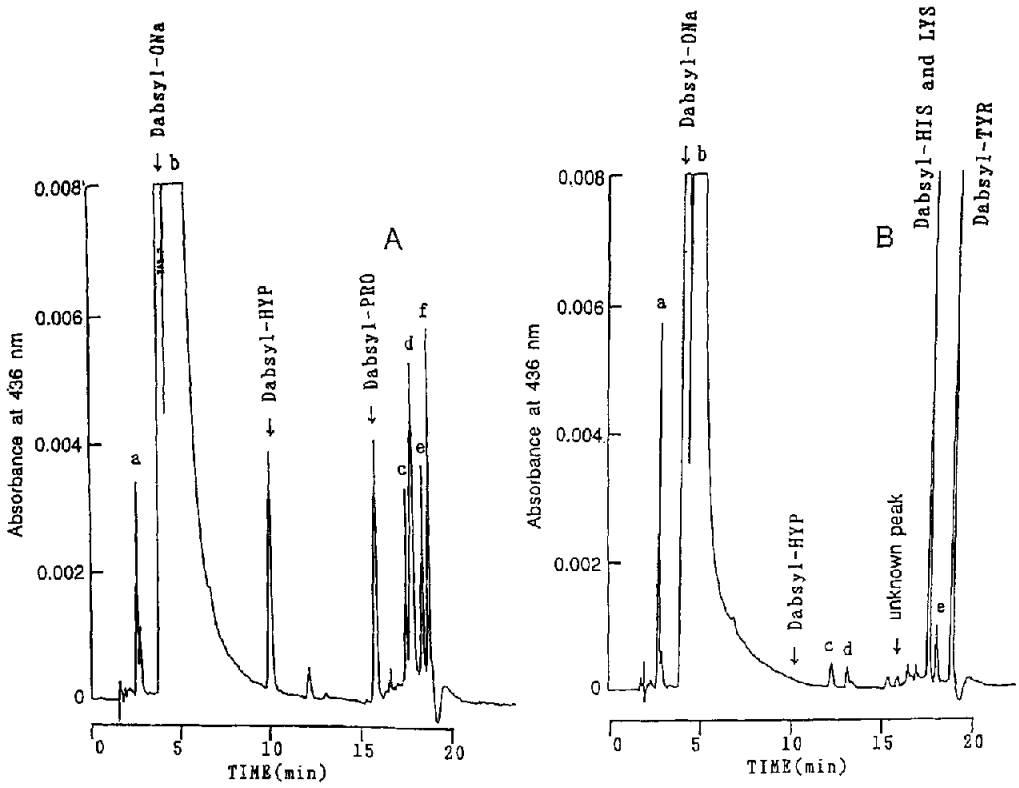

Fig. 2. Chromatograms of dabsyl amino acids: (A) the amount of each amino acid was 20 pmol; (B) the amount of His, Lys and Tyr was 1000 pmol each. The peaks a, b, c, d, e and f are unknowns. The gradient elution programme is given in Table I.

syl-Cl and form didabsylated derivatives [7,17,18]. In the present study, the primary amino group was blocked by OPA. Thus, only the ε-amino, imidazole, and phenolic hydroxyl groups reacted with dabsyl-Cl. These compounds were examined under the conditions described in Table I to check the effects of these compounds on the analysis of Hyp and Pro. One of these dabsyl peaks overlapped with the Pro peak. As shown in Fig. 2B, an unknown peak appeared at the same elution time as that of Pro. On the other hand, the dabsyl-Hyp peak did not overlap with other dabsylated amino acid peaks. The correlation coefficient was checked between the unknown peak area, which appeared at the position of dabsyl-Pro, and the amount of Lys, His and Tyr (100–1000 pmol each). These responses were linear (γ value 0.9925). Judging from the calculation of the data shown in Figs. 2A and B in the range of 20 pmol, the peak area of Pro was estimated to be *ca.* 7.2% of the peak area of dabsyl-Pro, owing to the dabsyl amino acids, such as Lys, His and Tyr. Thus, when small amounts of Pro are analysed in a sample containing large amounts of Lys, His and Tyr, the data related to Pro may be inaccurate. The unknown peak was confirmed to be the peak derived from His.

In order to determine whether or not this method is applicable to the analysis of Hyp and Pro, rat tail tendon collagen was used (Fig. 3). The contents of Hyp and Pro were 1.55 ± 0.04 and 2.03 ± 0.04 nmol/μg, respectively. Furthermore, to check the recovery of the present method, the contents of Hyp and Pro in the same collagen sample were analysed by the PITC method [12]. The contents of Hyp and Pro were 1.70 ± 0.17 and 1.98 ± 0.21 nmol/μg, respectively. The data were consistent between the two methods.

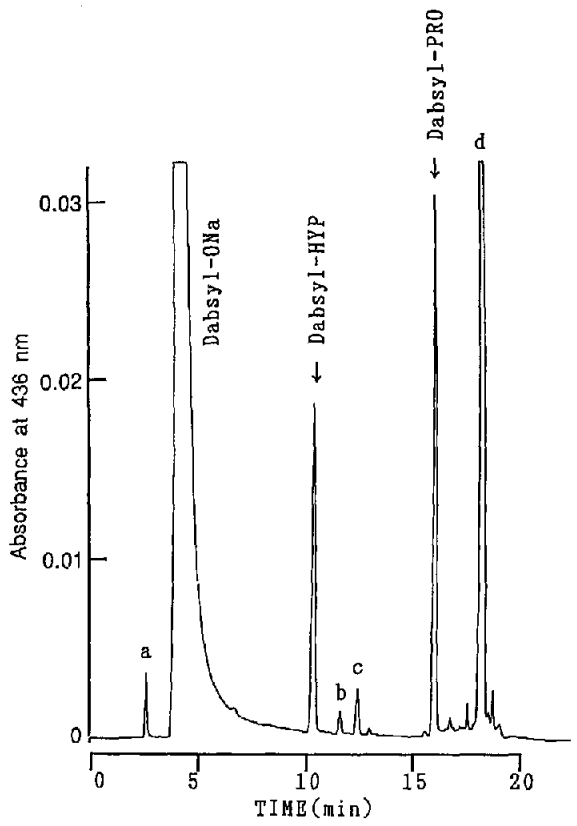

Fig. 3. Chromatogram of digested rat tail collagen treated with OPA and then with dabsyl-Cl. The elution was carried out by gradient elution (Table I). The amount of collagen applied to the column was 0.105 μg. The peaks a, b, c and d are unknowns. The gradient elution programme is given in Table I.

TABLE I

ELUTION SOLVENTS AND PROGRAMME

Th pH of solvent A (40 m*M* NaH_2PO_4) was adjusted to 3.0 with H_3PO_4. The UV–Vis detector was set at 436 nm. The column was maintained at 50°C. Solvent B, a mixture of acetonitrile and distilled water (4:1), was used with 4% DMF (v/v).

Time (min)	Flow (ml/min)	Solvent		Curve
		A (%)	B (%)	
Initial	1.5	55	45	Isocratic
3.0	1.5	55	45	6
8.0	1.5	40	60	6
12.0	1.5	33	67	6
13.0	1.5	0	100	6
15.0	1.5	0	100	6
15.5	1.5	55	45	6

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