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Short communication

Rapid determination of dabsylated hydroxyproline from cultured cells by reversed-phase high-performance liquid chromatography

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Abstract

A high-performance liquid chromatographic method was modified for the determination of hydroxyproline in cultured cells derived from rat liver. First, the primary amino group in the cell hydrolysate was blocked with *o*-phthalaldehyde, then the secondary amino group was derivatized with 4-dimethylaminoazobenzene-4′-sulfonyl chloride (dabsyl chloride). The dabsylated sample was treated with ethyl acetate to obtain a simple chromatographic elution profile of the cell hydrolysate. Dabsylhydroxyproline and proline were separated from other compounds by high-performance liquid chromatography in the gradient elution mode, and eluted at 4.71 and 8.00 min, respectively. The method was applied to the determination of hydroxyproline contained in cultured cells, the result being 25.4 ± 3.6 pmol/μg.

1. Introduction

Collagen is one of components that consist of extracellular matrices. Therefore, collagen research contributes to the understanding of the cell to cell interaction both in vivo and in vitro. In addition, a cell culture system is a useful tool for the investigation of the metabolism of certain compounds in vitro, and it has been reported that fibroblasts [1–5] and condrocytes [6] produce collagen in vitro. In order to investigate collagen fibre formation in liver cirrhosis, and to contribute to the development of the drug for this lethal disease, we used M cells [7], derived from rat liver, which formed a collagen fibre network, and found that dextran sulfate inhibited collagen fiber formation [8].

In the metabolism of collagen, hydroxylation of proline (Pro) is an important process in collagen biosynthesis. Recently, we modified the method to one that simultaneously determined seventeen amino acids and hydroxyproline (Hyp) derivatized with phenyl isothiocyanate by high-performance liquid chromatography (HPLC) [9], and more recently developed a method for the rapid selective determination of dabsyl-Hyp and -Pro contained in rat tail collagen [10]. However, our previous method was

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not applicable to the determination of Hyp in cells, because the sample contained many unknown compounds that interfered with the HPLC determination of small amounts of Hyp. In this study, we modified the method for the rapid (10 min) determination of Hyp; *o*-phthalaldehyde (OPA) was used for blocking the primary amino group and dabsyl-Cl for derivatizing the secondary amino group, then ethyl acetate extraction was carried out to obtain a simple elution profile before the application of HPLC. The simple elution profile is useful for the rapid determination of Hyp contained in cultured cells.

2. Experimental

2.1. Reagents and chemicals

Dabsyl chloride (4-dimethylaminoazobenzene-4′-sulfonyl chloride), sodium hydrogencarbonate, 2-mercaptoehtanol, acetone, ethyl acetate, a mixture of seventeen standard amino acids, hydroxyproline and reagents (either of HPLC grade or equivalent) for both the mobile phases and hydrolysis (acetonitrile, methanol, N,N′-dimethylformamide, distilled water, sodium acetate, acetic acid and sodium hydrogencarbonate) were purchased from Wako (Osaka, Japan). *o*-Phthalaldehyde was purchased from Kanto Chemical (Tokyo, Japan) and 3,4-dehydro-D,L-proline from Sigma (St. Louis, MO, USA).

2.2. Sample preparation and hydrolysis

Rat liver cells (M) [9] were cultured in DM-160 medium [11] supplemented with 10% foetal bovine serum. Cells were harvested after 10–14 days of culture, and the cell pellets were hydrolysed with 6 *M* HCl at 110°C for 24 h. The protein content in the cells was determined by the method of Lowry et al. [12], using bovine serum albumin as the standard.

2.3. Derivatization

The method was described in detail in a previous paper [10]. For the derivatization, 20 μl of the cell hydrolysate, 20 μl of triethylamine and 20 μl of 3,4-dehydro-DL-proline at 0.1 μmol/ml were mixed in a glass tube, then the mixture was dried under reduced pressure. To block the primary amino acid, 20 μl of OPA solution (14.9 m*M*) were added to the dried samples (cell hydrolysates or standard amino acids), and mixed well. The solution was left for 5 min at room temperature, then 20 μl of the dabsyl-Cl reagent in acetonitrile (0.52 mg per 100 μl) were added to the sample solutions and mixed well. Dabsylation was carried out for 20 min at 70°C in capped tubes, then 70 μl of 50% acetonitrile in distilled water and 50 μl of distilled water were added to the samples.

2.4. Ethyl acetate extraction

In order to shorten the analysis time, it is important to eliminate extra peaks in HPLC. Monboisse et al. [13] carried out diethyl ether extraction to remove the compounds that interfered in Hyp determination by HPLC. In this study, ethyl acetate was used instead of diethyl ether. After the derivatization, 500 μl of ethyl acetate and 50 μl of distilled water were added to the sample solution and mixed well. The solutions were centrifuged at 8060 *g* (12 000 rpm) for 5 min at room temperature to separate the aqueous phase and the organic solvent phase. The volume of the aqueous phase decreased to almost 100 μl, as acetonitrile moved into the organic phase during ethyl acetate extraction. Aliquots of the aqueous phase were applied to the column.

2.5. HPLC separation of hydroxyproline

The separation of amino acids was performed with a Waters HPLC system (Millipore, Milford, MA, USA). The detection of dabsylamino acids was carried out at 436 nm. The column was Puresil C_{18} (particle size 5 μm, 150 × 4.6 mm I.D.) (Waters), maintained at 50°C. The gradient elution programme and mobile phases used for the separation of Hyp and Pro are given in Table 1.

Table 1
Elution programme for separation of dabsyl-HYP

Time (min)	Solvent A (%)	Solvent B (%)	Curve[a]
0	43	57	
0.5	50	50	6
1.6	60	40	6
2.0	0	100	6
3.5	0	100	6
4.0	43	57	6

The pH of solvent A (150 m*M*, pH 3.0) was adjusted with H_3PO_4. Solvent B, acetonitrile–distilled water (4:1), was used to make 4% (v/v) DMF. The column was maintained at 50°C and the eluent flow-rate at 1.0 ml/min.
[a] The elution program 6 recommended by Waters means a linear gradient.

3. Results

In the previous study [10], dabsyl-Hyp and -Pro from collagen were determined within 20 min by HPLC. In this study, when the elution programme shown in Table 1 was used, the determination of Hyp and Pro in the presence of sixteen other amino acids was completed within 10 min. Under these conditions, dabsyl-Hyp and -Pro eluted at 4.71 and 8.00 min, respectively. The internal standard, 3,4-dehydroproline, eluted at 7.6 min, and was separated completely not only from dabsyl-Hyp but also from dabsyl-Pro.

The relationship between the amount of Hyp and the peak area was investigated in the range 0–300 pmol. The vertical axis (y) represented the ratio of the peak area of Hyp to that of the internal standard (3,4-dehydroproline) and the horizontal axis (x) the amount of Hyp. The relationship was linear and expressed by the equation $y = 0.0049x = 0.0016$, $r = 0.9996$.

The method was applied to the determination of Hyp in cell hydrolysates (Fig. 1). Fig. 1A represents the cell hydrolysate treated with OPA to block the primary amino group and without ethyl acetate extraction. A small dabsyl-Hyp peak and a large dabsyl-Pro peak were identified, but many unknown peaks were observed. When the OPA reaction was omitted but ethyl acetate extraction was carried out, the separation of the dabsyl-Hyp peak from other peaks was incomplete, as shown in Fig. 1B. These results suggest that both the OPA reaction and the ethyl acetate extraction were necessary in the method to determine Hyp in cell hydrolysates rapidly by HPLC.

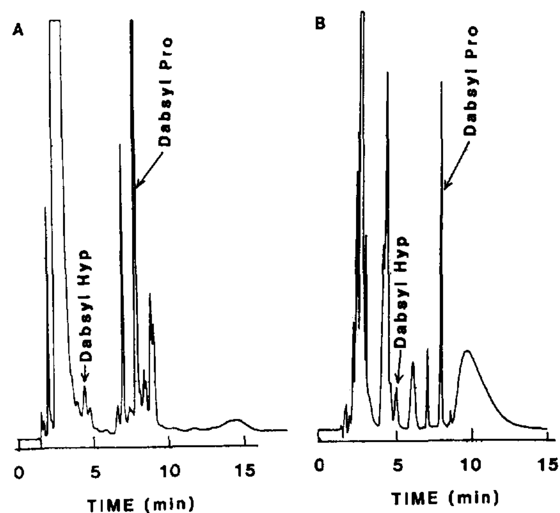

Fig. 1. Chromatograms of dabsylamino acids contained in cell hydrolysates: (A) with the OPA reaction but without ethyl acetate extraction; (B) without the OPA reaction but with ethyl acetate extraction. The amount of cellular protein applied to the column was 11 μg. The gradient elution programme is shown in Table 1.

When both the OPA reaction and the ethyl acetate extraction were applied before sample application to the column, a simple elution profile was obtained, as shown in Fig. 2. A small dabsyl-Hyp peak was clearly observed. This excellent result was due to the blocking of the primary amino acids and to the application of the ethyl acetate extraction.

In the present method, the extraction procedure with ethyl acetate was used to exclude the extra peaks. Therefore, 3,4-dehydroproline was added to the sample as an internal standard to obtain the correct value, and Hyp at different concentrations was also added according to the standard addition method. The relationship between the peak area of dabsyl-Hyp and the amount of Hyp added externally was investigated. The vertical axis (y) represented the ratio

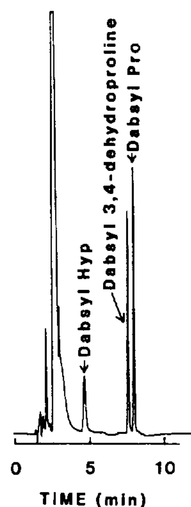

Fig. 2. Chromatogram of dabsylamino acids contained in cell hydrolysates. The sample was treated with OPA and ethyl acetate. The gradient elution programme is shown in Table 1.

of the peak area of Hyp to that of 3,4-dehydroproline as an internal standard, and the horizontal axis (x) the amount of Hyp added externally (Fig. 3). The relationship was linear and expressed by the equation $y = 0.0091x + 1.9175$, $r = 0.9967$. From this equation, the amount of Hyp without the external Hyp was 337 pmol. The concentration of Hyp in the cell hydrolysate

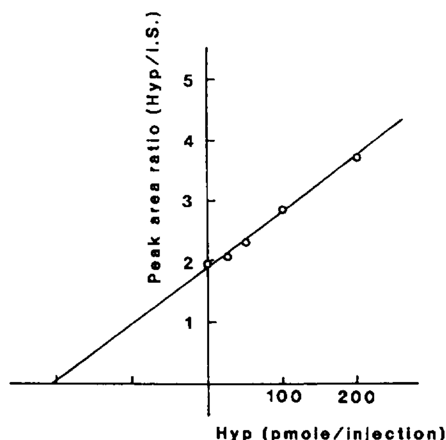

Fig. 3. Determination of Hyp content in M cell lysate by the standard addition method. The amount of 3,4-dehydroproline was 200 pmol and the protein content of M cells was 11 μg.

was 282 ± 40 μmol/l = 25.4 ± 3.6 pmol/μg protein. The value obtained with an amino acid analyser (Model L-8500; Hitachi, Tokyo, Japan) using the ninhydrin reaction and our previous method using HPLC and the phenyl isothiocyanate reaction [9] were 321 and 278 μmol/l, respectively, in the same sample.

4. Discussion

In a previous paper [10], we showed that our method was applicable to the selective determination of Hyp and Pro derivatized with dabsyl-Cl from rat tail collagen. This method was based on the blocking of the primary amino group with OPA and the selective derivatization of the secondary amino group with dabsyl-Cl. The combination of these two reactions excluded the peaks due to the primary amino acids on the HPLC elution profile. 9-Fluorenylmethyl chloroformate (FMOC) [13,14] or 4-chloro-7-nitrobenzofuran (NBD-Cl) [15] have been used instead of dabsyl-Cl as a derivatization reagent. We used dabsyl-Cl in the previous [10] and present studies, because the dabsylamino acid was stable. In addition, the detection wavelength for the dabsylamino acid (436 nm) was almost independent of the absorbance of other cellular components; only a few biomaterials have absorbance at 436 nm.

It is clear that the peaks of dabsyl-Hyp and -Pro were not disturbed by the other peaks, and that both of the dabsylamino acids remained in the aqueous phase. Dabsyl-Hyp with the hydroxy group in the side-chain was hardly extracted with ethyl acetate, whereas dabsyl-Pro without a hydroxy group was partially extracted with ethyl acetate. Therefore, the present method is applicable to the determination of Hyp, but not Pro. Although the sample without the ethyl acetate treatment showed dabsyl-Hyp and dabsyl-Pro, other unknown peaks were observed, as shown in Fig. 1B. These results indicate that the ethyl acetate extraction was useful for eliminating other peaks.

The values of Hyp obtained with the present method, our previous method and an amino acid

analyser were similar. This indicates that the present modified method is useful for the determination of Hyp contained in cultured cells because of the short analysis time and high sensitivity. In some experiments, however, the value obtained with the present method was slightly lower than that obtained with the amino acid analyser. The standard deviation (14% of the mean value) is relatively large. As the content of Hyp was less than 5% of the total amino acids in M cells [9], the large deviation might be due to this small value.

In order to investigate collagen metabolism, the content of Hyp is an important index, because the hydroxylation of Pro takes place in the procollagen molecule inside the cells. This paper shows that our modified method can determine Hyp within 10 min in cells that contain various known and unknown compounds.

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