Journal of Chromatography, 619 (1993) 306–309
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 7041

Short Communication

High-performance liquid chromatographic determination of histatins in human saliva

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(First received April 14th, 1993; revised manuscript received June 23rd, 1993)

ABSTRACT

A simple method for the assay of histatins in human saliva by high-performance liquid chromatography has been developed. The histatins in saliva were extracted with 0.1 *M* HCl–methanol, and histatins 1, 3, 5, and 6 were separated and concentrations were determined by reversed-phase chromatography. This simple method enabled the determination of levels of individual histatins from the saliva of normal subjects. The average concentration of histatins 1, 3, 5, and 6 in parotid saliva collected from 26 healthy volunteers aged from 20 to 30 years were 11.25 ± 5.65, 8.15 ± 3.08, 7.67 ± 3.12, and 1.56 ± 0.53 μmol/l (mean ± S.D.), respectively.

INTRODUCTION

Histatins are a family of histidine-rich polypeptides which have been identified in human saliva [1–3]. At the present time, histatins 1–12, which are mutually homogeneous, have been determined in human parotid secretion [4]. Histatins are though to be important components of the non-immune defense system in the oral cavity, having antibacterial and antifungal activity [5,6]. Histatins also possess histamine-releasing activity in mast cells [2], and high affinity for hydroxyapatite [7]. However, the precise biological functions of histatins are still poorly understood. No direct quantitative determination of the histatins has been made, except for an enzyme-linked immunosorbent assay (ELISA) [8].

It is important to determine quantitatively individual species of histatins, because the antimicrobial and histamine-releasing activities vary in different species of histatins [9,10]. This paper describes a reversed-phase high-performance liquid chromatographic (HPLC) procedure, which effectively separates histatins 1, 3, 5, and 6, and permits their quantitative determination.

EXPERIMENTAL

Samples

Histatins 1, 3, 5, and 6 were prepared accord-

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0378-4347/93/$06.00

ing to the method described previously [10]. Briefly, pooled saliva was heated for 5 min at 100°C and centrifuged at 10 000 *g* for 15 min, and the supernatant was applied to a heparin-Ultrogel column (80 mm × 15 mm I.D.) (IBF Biotechnics, Villeneuve-la-Garenne, France). The column was developed with a linear gradient from 0 to 1.5 *M* NaCl in a total volume of 200 ml of 0.01 *M* Tris–HCl (pH 7.0). The flow-rate of eluent was 0.5 ml/min. A histatin 1 fraction, from the second peak, and a fraction containing histatins 3, 5 and 6, from the third peak, of a heparin column were purified using reversed-phase HPLC.

Saliva samples were collected from 26 healthy students (20–30 years of age, 13 females and 13 males) at mid-morning. Parotid saliva flow was stimulated by 2% citric acid and was collected using double-walled suction cups. Saliva samples were immediately adjusted to pH 3 with 10% citric acid and stored at −20°C. Total protein was measured by the method of Lowry *et al.* [11].

Sample preparation

Saliva samples were prepared by dilution with an equal volume of distilled water. A 1-ml volume of diluted saliva was mixed with 4 ml of methanol containing 0.1 *M* HCl. After centrifugation for 10 min at 2000 *g*, the supernatant fraction was evaporated to dryness under vacuum. The samples were dissolved in 200 μl of 10% acetonitrile containing 0.05% trifluoroacetic acid (TFA) and passed through an ultrafiltration membrane (cut-off M_r 10 000, Japan Millipore, Tokyo, Japan) to eliminate high-molecular-mass substances. A 40-μl aliquot of each of the filtrates was injected onto the HPLC column.

HPLC separation

The HPLC system consisted of a Model CCPM pump (Tosoh, Tokyo, Japan), a TSK gel ODS-80 TM reversed-phase column (250 mm × 4.6 mm I.D.) packed with 5-μm particles (Tosoh), and a Model 80 UV absorbance detector set at 225 nm. The HPLC column was developed with a 30-min linear gradient from solvent 1 (0.05% TFA–10% acetonitrile) to solvent 2 (0.05% TFA–40% acetonitrile) at a flow-rate of 1.0 ml/min. The histatins were quantified by comparing the peak heights of the eluted histatins of samples with those of the histatin standards.

Standard preparation and quantitative determination

Standard solutions containing 0.625, 1.25 or 2.5 μg each of histatins 1, 3, 5, and 6 in a 40-μl volume containing 10% acetonitrile and 0.05% TFA were prepared. These solutions were injected into the HPLC apparatus. The histatins were quantitated from the peak-heights of the standards. The recovery from saliva was found by assaying the known concentrations of histatins 1, 3, 5, and 6 added to aliquots of saliva pools from healthy subjects. The amount assayed in the supplemented samples was deduced from the value of the saliva pool and compared with the known amount added.

RESULTS

Fig. 1 shows chromatograms for standards and salivary extract for histatins. Histatins 1, 3,

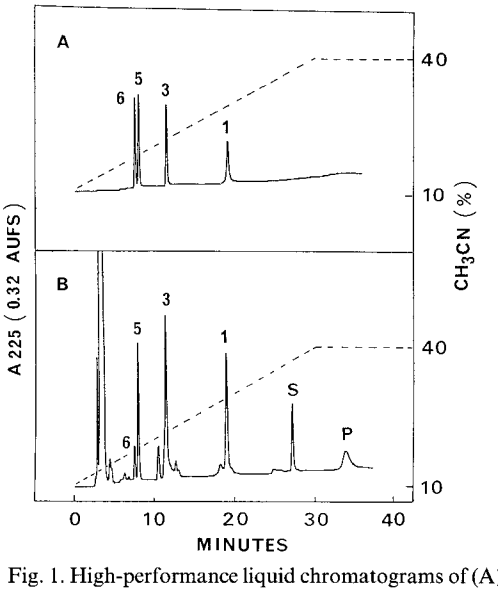

Fig. 1. High-performance liquid chromatograms of (A) 1 μg each of standard histatins; (B) a saliva sample extracted with 0.1 *M* HCl–methanol. For conditions, see Experimental. Peaks: 1 = histatin 1; 3 = histatin 3; 5 = histatin 5; 6 = histatin 6. S and P = unknown peptides.

TABLE I

RECOVERY OF HISTATINS ADDED TO HUMAN PAROTID SALIVA

Amount added, 2 μg.

Compound	Recovery (mean ± S.D., n = 5) (%)	C.V. (%)
Histatin 1	95.3 ± 3.6	3.8
Histatin 3	96.4 ± 7.2	7.5
Histatin 5	98.2 ± 3.1	3.2
Histatin 6	97.5 ± 2.6	2.7

TABLE II

CONCENTRATIONS OF HISTATINS IN HUMAN STIMULATED PAROTID SALIVA

Compound	Concentration (mean ± S.D., n = 26)	
	μM	μg/mg of protein
Histatin 1	11.25 ± 5.65	10.05 ± 4.12
Histatin 3	8.15 ± 3.08	7.65 ± 3.14
Histatin 5	7.65 ± 3.12	6.67 ± 2.35
Histatin 6	1.56 ± 0.53	1.18 ± 0.78
Total	28.63 ± 9.62	25.55 ± 9.78

5, and 6 were separated on an ODS column eluted with a linear gradient of 10 to 40% acetonitrile containing 0.05% TFA, generated over a 30-min period. The retention times of histatins 1, 3, 5, and 6 were 18.8, 11.8, 8.0, and 7.5 min, respectively (Fig. 1A). The separation of histatins was satisfactory, and the extract prepared from parotid saliva produced no interfering peaks (Fig. 1B)

Peak fractions corresponding to individual histatins were separately collected and subjected to amino acid analysis. Each peak fraction obtained from HPLC showed the same amino acid composition as those of the corresponding authentic histatin. In addition to the peaks for the histatins of salivary extract, two peaks (S and P) appeared after histatin 1 (Fig. 1B). Peaks S and P did not contain histatins.

The calibration curves were calculated by analysing histatins 1, 3, 5, and 6 with an ODS column. A linear correlation between the concentration of histatins and their peak heights was obtained with each standard curve in the concentration range 0.625–2.5 μg. Correlation coefficients for all lines were greater than 0.99.

The recoveries were calculated by adding histatins 1, 3, 5, and 6 at a concentration of 2 μg/ml to the parotid saliva samples (Table I). The percentage recovery of histatins from saliva was in the range 95.3–98.2%. The coefficients of variation (C.V.) of the peak-height ratios in assays of calibration standards in saliva were less than 10%.

Histatin concentrations in stimulated parotid saliva from 26 healthy subjects are shown in Table II. The concentration of histatin 1 was higher than those of histatins 3, 5, and 6. No significant difference was observed between histatins 3 and 5. Total histatin values (histatins 1, 3, 5, and 6) ranged from 12.5 to 46.8 μM, with a mean value of 28.6 μM ± 9.6 S.D.

DISCUSSION

Twelve components (histatins 1–12) of the histatin family in human salivary secretions have been isolated and their primary structures determined [4]. Of these, histatins 1, 3, and 5 comprise 85–90% of the total histatin and are called the major histatins [3]. However, little is known about the quantitative determination of histatins in human saliva, except for an ELISA assay. Atkinson *et al.* [8] determined the histatin concentration in human saliva by ELISA using antibodies against histatin 1. Despite its high sensitivity, this method did not permit the determination of individual histatins, because the antibodies also reacted with histatins 3 and 5.

Histatins gave various activities (antimicrobial, histamine-releasing activity and inhibition of hydroxyapatite crystal growth), and these activities vary for individual histatins. Histatin 1 inhibited hydroxyapatite crystal growth more effectively than histatin 5, which has a higher potency to kill *Candida albicans* than histatin 1 [3,9].

The histamine-releasing activity of histatin 3 and 5 was ten-fold higher than that of histatin 1 [10]. Moreover, Azen [12] reported that histatins displayed a genetic polyporphism. Therefore, the determination of the content of individual histatins in human saliva is required to elucidate the biological role of histatins in the mouth.

In this study, we have developed a more convenient method for the evaluation of individual histatins in human saliva. Our procedures involve extraction with acidic methanol, followed by reversed-phase HPLC. We found methanol containing 0.1 *M* HCl to be more quantitative than methanol alone for extraction of the histatins from human saliva [13]. Following a simple extraction from a 0.5-ml sample of saliva, the histatins were separated and detected by reversed-phase chromatography on an ODS column with a 30-min linear gradient from 10 to 40% acetonitrile containing 0.05% TFA. Because histatins 1, 3, 5, and 6 were eluted as single peaks under these conditions, quantification of individual histatins from saliva samples was possible (Fig. 1B). This method showed a favourable average recovery (95–98%) and good reproducibility.

The present results indicate that stimulated parotid saliva was found to contain 25.55 ± 9.78 μg (mean, $n = 26$) of total histatin (histatins 1, 3, 5, and 6) per milligram of saliva protein. Atkinson *et al.* [8] reported that the average total concentration of histatin was 3.1 and 11 μg/mg saliva protein in stimulated parotid and submandibular/sublingual saliva, respectively. We found much higher values, perhaps owing to the extraction by acidic methanol. Because histatins are cationic peptides, they bind to acid mucopolysaccarides, such as heparin [10]. Thus, possibly the histatins may have been bound to the high-molucular-mass acid components in saliva and have been eliminated from the saliva samples as a precipitate. However, the character of the histatins in human saliva is as yet unknown.

One of the minor histatins, histatin 6, is identical with histatin 5 except for an additional arginine residue in the C-terminal, and it was eluted before histatin 5 on an ODS column (Fig. 1A). We did not estimate the minor histatins other than histatin 6, because their concentrations in saliva are very much less than those of the major histatins [4]. An unknown fraction, peak S (Fig. 1B), showed an amino acid composition very similar to that of statherin [14]. Peak P fraction contained peptides similar to basic proline-rich polypeptide [15]. However, no further attempt was made to identify peaks S and P.

ACKNOWLEDGEMENT

This work was supported in part by a Grant-in-Aid from the Scientific Research from the Ministry of Education, Science and Culture of Japan.

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