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Short Communication

Separation of human tear proteins with ceramic hydroxyapatite high-performance liquid chromatography

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ABSTRACT

Human tear protein, which consists mainly of albumin, lysozyme, and lactoferrin, was assayed with high-performance liquid chromatography using a new ceramic hydroxyapatite column. Proteins were eluted at room temperature using a 20-min linear gradient from 95:5% A/B buffer to 0:100% A/B buffer (buffer A, distilled water; buffer B, 400 m*M* KH_2PO_4 containing 240 m*M* NaOH). The proteins eluted at 1.2 min for albumin, 8.5 min for lysozyme, and 20.4 and 21.7 min for lactoferrin, respectively. The assays may be performed in 30 min.

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INTRODUCTION

It is well known that over 80% of the total tear protein consists of four components: lactoferrin, albumin, lysozyme, and tear-specific pre-albumin [1]. The analysis of these proteins in tear fluid has been studied extensively using electrophoretic techniques such as cellulose acetate gel-electrophoresis, one- and two-dimensional polyacrylamide gel-electrophoresis, and isoelectric focusing crossed immunoelectrophoresis [27]. In order to develop a new method with a high sensitivity for the identification and quantification of the major protein components, recently high-performance liquid chromatography (HPLC) has been investigated, using ceramic hydroxyapatite chromatography as an example. This technique is characterized by its convenient measurement of both acidic and basic proteins and by the preservation of the protein activities during the measurement [8,9]. However, this type of packing does not seem to be suitable for the separation of tear proteins because of the poor mechanical strength and poor reproducibility during preparation of ceramic hydroxyapatite crystals in the form of flat plates. To overcome this disadvantage, a spherical packing with a microporous structure has been prepared successfully by sintering small crystals of ceramic hydroxyapatite. This paper describes a simple and rapid HPLC assay with the new ceramic hydroxyapatite packing for the analysis of tear proteins, especially albumin, lactoferrin and lysozyme.

EXPERIMENTAL

Reagents and materials

Human serum albumin (HSA) and lysozyme (egg white) were purchased from Wako Pure Chemical Industries (Tokyo, Japan). Lactoferrin from human milk was obtained from Sigma (St. Louis, MO, USA). Other chemicals were of analytical reagent grade.

Sample preparation

Standard protein samples containing 2.0 mg of HSA, 2.0 mg of lysozyme, and 2.0 mg of lactoferrin were charged into a glass vial and then 1 ml of an aqueous solution containing 1% polyethylene glycol 4000 as a nonionic surfactant was added to obtain a homogeneous solution. The human tear fluid was collected from normal volunteers, using sterile 10 μl microcapillary pipettes.

Chromatography

The HPLC system consisted of an intelligent pump (Hitachi L-6200, Hitachi, Tokyo, Japan), a UV detector (Hitachi L-4000), a chromato-integrator (Hitachi I-2500), and a ceramic hydroxyapatite column 100 mm × 7.5 mm I.D. (Pentax SH-0710F with a particle size of approximately 2 μm; Asahi Optical Co., Tokyo, Japan). The mobile phase is composed of distilled water (buffer A) and a mixture of 400 m*M* KH_2PO_4 and 240 m*M* NaOH (buffer B). Ten μl of aqueous protein solution, consisting of 1 ml of distilled water, 10 mg of polyethylene glycol 4000, 2.0 mg of HSA, 2.0 mg of lysozyme, and 2.0 mg of lactoferrin, was injected into the HPLC system with the Pentax SH-0710F ceramic hydroxyapatite column under the following conditions: flow-rate 1.0 ml/min, pressure 50 kg/cm^2, emission wavelength 230 nm (0.2 AUFS), and room temperature.

RESULTS AND DISCUSSION

The standard protein samples dissolved in distilled water are subject to absorption on the surface of the glass vial when they are stored in the vial at temperatures of 4, 10, 20, 30, and 37°C, resulting in a poor reproducibility of the analysis of the sample. To overcome this problem, the sample was treated with aqueous surfactant solutions. A nonionic surfactant, polyethylene glycol 4000 (in an optimal concentration of 1%) was found to be suitable, giving a complete absence of absorption of the proteins on the surface of the vial. The protein samples were eluted within 30 min using a 20 min linear gradient from 95:5% A/B to 0:100% A/B, as can be seen in Fig. 1. Fig. 2 shows a characteristic separation pattern for standard protein samples such as a single HSA peak, a single lysozyme peak, and two lactoferrin peaks, which eluted at 1.2, 8.5, and 20.4 and 21.7

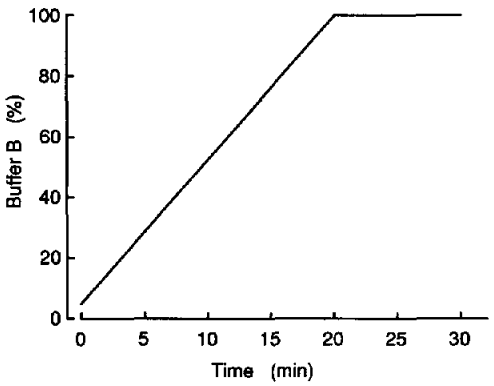

Fig. 1. A gradient curve of the A/B buffer, where A is distilled water and B is a mixture of 400 m*M* KH_2PO_4 and 240 m*M* NaOH.

min, respectively. Lactoferrin has also been analyzed by other chromatographic techniques, *e.g.* hydrophobic interaction chromatography, ion-exchange chromatography, reversed-phase chromatography, and gel-permeation chromatography. In these techniques lactoferrin is known to give a characteristic single peak [10]. In the ceramic hydroxyapatite chromatographic technique used in this study, lactoferrin gives two peaks. At present this reason is not clear, but the peak separation is presumably due to the existence of two types of charged lactoferrin which are separated only by ceramic hydroxyapatite chromatography. This technique has two advantages: injection can be done at intervals of 30 min and its high reproducibility (R.S.D. within 3% per 10 assays).

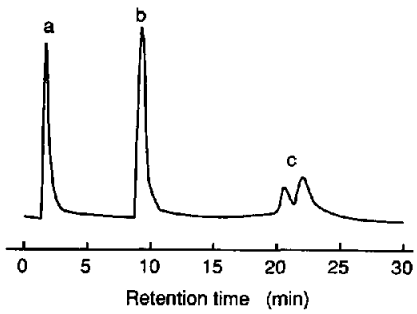

Fig. 2. Ceramic hydroxyapatite chromatograms of standard protein samples (one-fold dilution) such as (a) HSA (1.4 min), (b) lysozyme (8.9 min), and lactoferrin (20.6 and 21.9 min).

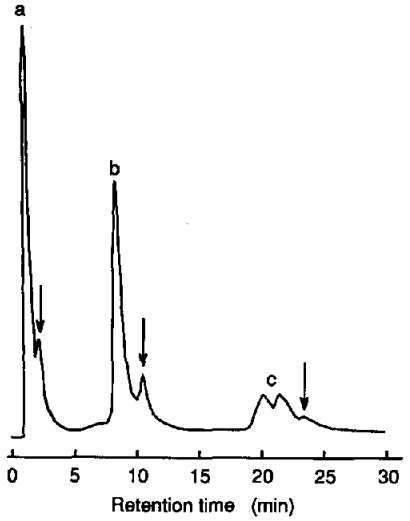

Fig. 3. Ceramic hydroxyapatite chromatograms of human proteins in tear fluid (one-fold dilution). Numbers in figure refer to kind of protein given in Fig. 2.

Fig. 2 shows a representative separation profile of tear proteins by HPLC on a Pentax ceramic hydroxyapatite SH-0710F column. It was found that albumin, lysozyme, and lactoferrin in tear fluid can be detected by this technique. The proteins eluted at 1.2 min for albumin, 8.5 min for lysozyme, and 20.4 and 21.7 min for lactoferrin. Based on this result, the total amount of each protein in 10 μl of tear fluid was estimated and was found to be 64, 27, and 21 μg, respectively. These values roughly agreed with those determined in a test of 101 normal adults [11]. As can be seen in Fig. 3, 3 peaks of unknown proteins could be detected by this technique, each as a shoulder of respectively the albumin peak (2.7 min), the lysozyme peak (11.3 min), and the lactoferrin peak (24.3 min). This means that the HPLC system with a ceramic hydroxyapatite column significantly improves the separation and analysis of tear proteins.

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