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Effective method for purification of lysozyme from human urine

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Abstract

Lysozyme in the urine of a hemodialysis patient was purified in two steps: DEAE Sephadex chromatography followed by Sephacryl chromatography. The Sephacryl S-100 column chromatographed fraction showing lytic activity was proven to give one band on SDS-PAGE and to have a molecular mass of 14 500, in agreement with that of lysozyme. The N-terminal amino acid sequence of this purified protein was identical to that of lysozyme. These results indicate that the protein purified was indeed lysozyme. The specific affinity of lysozyme for Sephacryl S-100 may explain the greater purity of the same protein isolated by this method.

Keywords: Lysozyme; Proteins; Enzymes

1. Introduction

Lysozyme is a group of enzymes catalyzing hydrolysis of β-1,4 glycoside bonds of polysaccharides comprising the peptidoglycan [1]. It has been reported that lysozyme in the urine (molecular mass 14 000–15 000) is a useful indicator of proximal renal tubular damage or deterioration of renal function [2,3].

Attempts have been made to purify lysozyme from hen or goose egg white, animal tissues, human urine and human milk [1,4–12], but the methods used have not allowed sufficient purification. For example, the procedures using CM cellulose [6] or Bio-Rex 70 [7,8] require a high pH buffer for elution of lysozyme from the column. The chromatofocusing method [9] also requires a high pH (pH 9.5) buffer at the start. Therefore, in these methods, lysozyme eluted from the column may release lytic activity and precipitate because of the extremely high pH [10,11]. Moreover, lysozyme is only poorly isolated by DEAE cellulose [4,12].

Recently, we found that lysozyme was eluted from the Sephacryl S-100 column later than β_2-microglobulin whose molecular mass is less than that of lysozyme. Thus, assuming that the Sephacryl S-100 column has a specific affinity for lysozyme, we developed a simple and reliable method to purify lysozyme from urine at physiological pH. In the present study, firstly, lysozyme was isolated from the urine of hemodialysis patients using a DEAE Sephadex A-50 column in the first step, and a Sephacryl S-100 column in the second. Secondly, the molecular mass of the purified lysozyme was com-

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pared with the reported value, and the N-terminal amino acid sequence was determined.

2. Experimental

2.1. Measurement of lytic activity

Lytic activity was measured by the method of Litwak [13] with some modifications. Briefly, 10 mg of *Micrococcus lysodeikticus* cell walls (Seikagaku Kogyo, Tokyo, Japan) was suspended in 100 ml of 60 m*M* sodium phosphate buffer (pH 6.2), containing 17.1 m*M* sodium chloride. Then, 0.1 ml of the sample solution was added to 5.0 ml of the suspension solution, and the absorbance was measured at 650 nm at room temperature at 0.25-min intervals, 6 to 8 times, to obtain the slope of the regression line between the time (min) from the addition of a sample solution and the logarithmic value of l_0/l (dimensionless), which reflects the lytic activity, where l_0 is the absorbance of the solution when the sample solution was added, and l is the absorbance at a given time.

In the present study, one unit of lytic activity was defined as 0.001 min^{-1} of the slope of the regression line. As a standard, egg white lysozyme (×3 crystallized) (Sigma, St. Louis, MO, USA) was used.

2.2. Purification of lysozyme using DEAE Sephadex A-50 and Sephacryl S-100

Approximately 600 ml urine from a hemodialysis patient was dialysed against deionized water overnight and then lyophilized. The lyophilized urine was dissolved in 14.0 ml of 20 m*M* sodium phosphate buffer (pH 7.2), and the resulting precipitate was removed by centrifugation at 3000 *g* for 10 min.

The concentrated urine was applied to a DEAE Sephadex A-50 column (45×2.5 cm I.D.; Pharmacia, Uppsala, Sweden), which was equilibrated with 20 m*M* sodium phosphate buffer (pH 7.2). The column was washed with the same buffer, and every 5 ml of eluted solution was collected to measure lytic activity. The fractions showing lytic activity were dialysed against deionized water and condensed by lyophilization.

The lyophilized sample was dissolved in 3.0 ml of 20 m*M* sodium phosphate buffer (pH 7.2), containing 0.3 mol/l sodium chloride, and applied to a Sephacryl S-100 column (45×1.5 cm I.D.; Pharmacia, Lot No. FK09717), which was equilibrated with the same buffer. The flow-rate of the sodium phosphate buffer through the column was set at 4.0 ml/h. In order to obtain the fraction showing lytic activity, every 4.0 ml of the eluent was collected up to ten fractions, and thereafter, every 2.2 ml. The fraction showing lytic activity was condensed as a finally purified lysozyme solution.

All the above purification steps were carried out at 4°C.

2.3. SDS polyacrylamide gel electrophoresis of finally purified lysozyme solution

Polyacrylamide gel electrophoresis containing sodium dodecyl sulfate (SDS-PAGE) using a gel concentration of 8–17% was carried out by the method of Laemmli [14]. In the present study, the sample proteins were not reduced with 2-mercaptoethanol or dithiothreitol prior to electrophoresis.

2.4. Analysis of amino acid sequence

The N-terminal amino acid sequence of the purified lysozyme was determined using the Applied Biosystems Model 470-A sequencer (Foster City, CA, USA).

2.5. Measurement of protein concentration

The protein concentration was determined in each purification step by the method of Bradford [15] using a Bio-Rad protein assay kit (Richmond, CA, USA). Bovine serum albumin (fraction V) (Miles, Kankakee, IL, USA) and egg white lysozyme were used as a standard.

3. Results

Fig. 1 shows the DEAE Sephadex A-50 chromatogram of the concentrated urinary proteins of the hemodialysis patient, together with the lytic activity in each fraction. Lytic activity was found in the very early eluted fraction. When the fraction with lytic

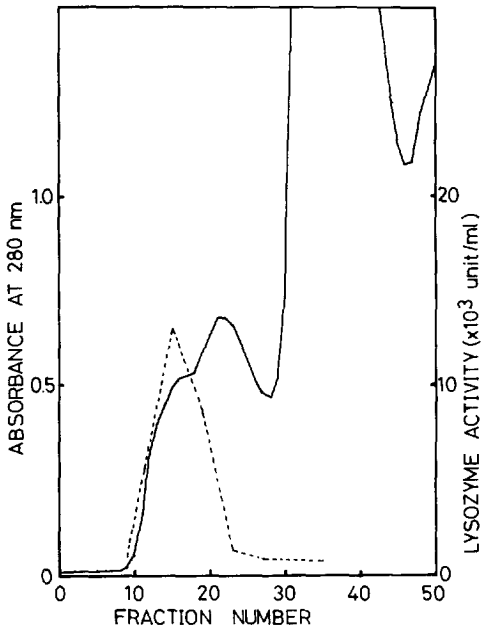

Fig. 1. DEAE Sephadex A-50 chromatography of condensed urine proteins of hemodialysis patient. A 14-ml sample containing 534.1 mg of protein was applied to DEAE Sephadex A-50 column (45×2.5 cm I.D.), which was equilibrated with 20 m*M* sodium phosphate buffer (pH 7.2). The proteins were eluted with the same buffer, while monitoring absorbance at 280 nm. Every 5 ml of eluted solution was collected to determine lytic activity. Absorbance at 280 nm is indicated by the solid line and lytic activity by the broken line.

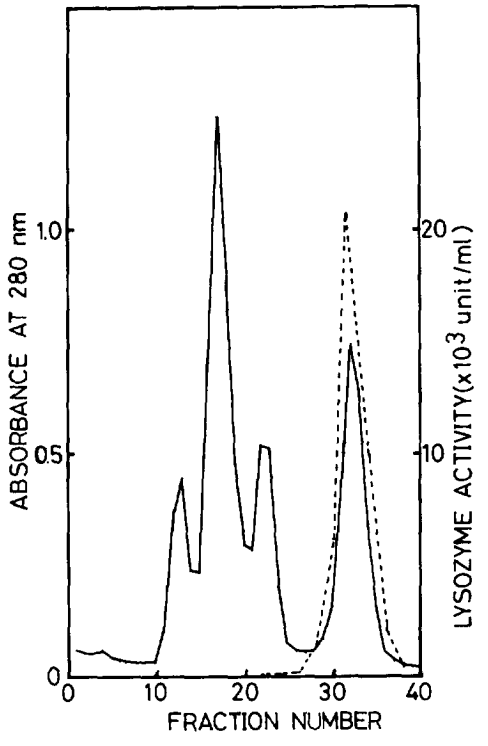

Fig. 2. Sephacryl S-100 chromatography of DEAE Sephadex A-50 chromatographed fraction, which showed lytic activity. DEAE Sephadex A-50 chromatographed fraction (3.0 ml) showing lytic activity was applied to a Sephacryl S-100 column (45×1.5 cm I.D.), which was equilibrated with 20 m*M* sodium phosphate buffer (pH 7.2), containing 0.3 mol/l sodium chloride. Then, the same buffer was flowed at a constant rate of 4.5 ml/h. Every 4 ml of the eluent was collected to measure lytic activity at first up to 10 fractions, and thereafter, every 2.2 ml.

activity was further analyzed by SDS-PAGE, the electrophoretic pattern showed the lysozyme band and two other protein bands with respective molecular masses of ca. 25 000 and 50 000 (lane 2 of Fig. 3). These proteins were identified as IgG heavy chain and light chain by two-dimensional electrophoresis.

Fig. 2 shows the Sephacryl S-100 chromatogram of the DEAE Sephadex A-50 chromatographed fraction which indicated lytic activity. In the Sephacryl S-100 chromatogram, lytic activity was found in the fraction corresponding to the last peak.

As shown in Fig. 3, the protein in the Sephacryl S-100 chromatographed fractions showing lytic activity gave one band by SDS-PAGE, and its molecular mass was estimated to be 14 500, in agreement with the previously reported molecular mass of lysozyme.

The N-terminal amino acid sequence of the above purified protein was determined as Lys-Val-Phe-Glu-Arg-( )-Glu-Leu-Ala-Arg-( )-Leu-Lys-Arg-Leu-Met-Asp-Glu-(brackets denote the residues of uncertain sequence data). This was identical to the previously reported amino acid sequence of lysozyme [3], except for the unidentified residues.

The molar absorptivity of the purified protein at 280 nm was calculated to be 36 000 M^{-1} cm^{-1}.

All these results indicate that the protein purified by the present procedures was indeed lysozyme.

Table 1 indicates the protein amounts in the various fractions showing lytic activity at each purification step. From 600 ml of the urine of the hemodialysis patient, 9.96 mg of total protein was obtained in the finally purified sample. This is 1.86% of the total protein amount (534.1 mg) in the original

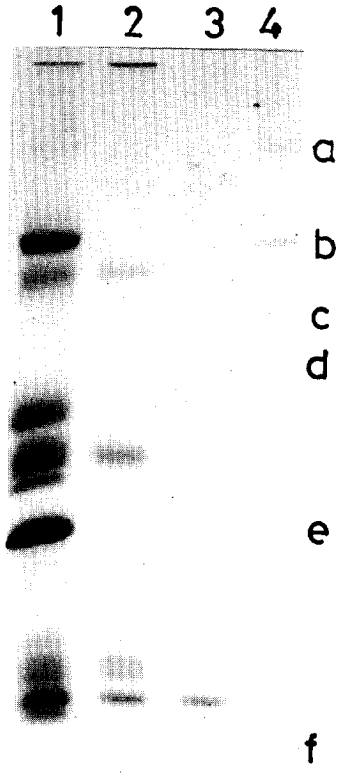

Fig. 3. SDS-PAGE patterns of the sample at each purification step. Lane 1: 30 μg of proteins in the original urine; lane 2: 10 μg of DEAE Sephadex A-50 fraction; lane 3: 5 μg of finally purified protein. Lane 4 shows marker proteins: a, phosphorylase b (94 000); b, BSA (67 000); c, ovalbumin (45 000); d, carbonic anhydrase (30 000); e, soybean trypsin inhibitor (20 100); f, α-lactalbumin (14 000).

urine. On the other hand, when the concentration of lysozyme was estimated on the basis of lytic activity using egg white lysozyme as a standard, the total amount of lysozyme was 29.4 mg in the original urine, against 10.8 mg in the finally purified sample (36.7% recovery).

4. Discussion

Many procedures for lysozyme purification have been reported. In most of these, a high pH buffer is needed, which causes precipitation and inactivation of the lysozyme. In the procedure proposed here, lysozyme in the urine was purified by DEAE Sephadex A-50 and Sephacryl S-100 chromatography using a buffer with physiological pH. From the DEAE Sephadex A-50 column, lysozyme was washed out with IgG heavy chain and light chain (data not shown), whereas other urinary proteins and pigments were retained in the column. Lysozyme thus washed out with IgG heavy chain and light chain was then applied to the Sephacryl S-100 column, resulting in the complete purification of lysozyme.

The complete purification of lysozyme with the method proposed here may be explained by its molecular affinity for Sephacryl S-100, which is usually used for gel permeation chromatography because of the minimal affinity for proteins. In an unpublished study, we found the K_{av} determined for lysozyme by chromatography on Sephacryl S-100 was 1.2, while that for β_2-microgobulin was 0.75, when 20 m*M* sodium phosphate buffer (pH 7.2), was flowed through the column. Thus, the K_{av} of lysozyme was more than 1.0 and greater than that for β_2-microgobulin which was expected to show little adsorption to Sephacryl and whose molecular mass was smaller than lysozyme. These findings suggest the affinity of lysozyme for Sephacryl S-100.

Fernandez-Sousa et al. [16] reported one-step

Table 1
Recovery of lysozyme at each purification step

	Volume (ml)	Total protein (mg)	Protein recovery (%)	Activity ($\times 10^3$ units/ml)	Total activity ($\times 10^3$ units)	Activity recovery (%)	Specific activity
Condensed urine	1.4	534.10[a]	100.00	48.1	673.4	100.00	1.26
DEAE fraction	3.0	22.16[b]	4.15	95.6	286.8	42.59	12.94
S-100 fraction	3.0	9.96[b]	1.86	98.0	294.0	43.66	29.52

[a] BSA was used as a standard.
[b] Egg white lysozyme was used as a standard.

purification of lysozyme using Bio Gel A-0.5 m (Bio Rad, Hercules, CA, USA), which is an agarose gel. In this procedure, the elution volume of lysozyme was larger than the column volume. However, when one attempts to purify lysozyme from urine by this method, lysozyme may be poorly isolated because of the great amount of urinary pigments, as reported by Gachon et al. [9]. It may be especially difficult to purify lysozyme from the urine of a renal failure patient, since such urine contains much more protein other than lysozyme. In our preliminary experiment, we found that direct application of a urine sample to a Sephacryl S-100 column resulted in elution of lysozyme with urinary pigments.

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