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Simple two-step procedure for the preparation of highly active pure equine milk lysozyme

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Abstract

A fast, simple two-step purification scheme is presented for the isolation of lysozyme at a high yield from equine milk. In the first step, fluidized bed technology, using the Streamline system, was exploited. In the following step, advantage was taken of Ca^{2+}-induced conformational changes to obtain a pure, high specific activity, enzyme fraction by hydrophobic interaction chromatography.

1. Introduction

In the literature, many protocols have been described for the isolation of *c*-type lysozymes from a wide variety of mammalian species. In most preparative purification methods [1–6], caseins, which are abundant in milk, are first removed by salt or acid precipitation. Subsequently, the supernatant is extensively dialyzed, lyophilized and then subjected to at least two additional fractionation techniques, such as size-exclusion and ion-exchange chromatography. In between the various stages, additional dialysis and freeze-drying steps are often required. These various manipulations are time-consuming and can result in significant inactivation of the enzyme [6]. As a consequence, the development of a faster purification method for the production of a highly active enzyme preparation is highly desirable.

In the present work, we took up this challenge using skimmed equine milk as starting material. In the first step, we exploited the potential of the highly innovative Streamline system which, as far as we know from the open literature, is introduced here for the first time in the field of milk protein fractionation. A further increase in specific activity of the enzyme preparation was achieved using hydrophobic interaction chromatography (HIC), which was found to be highly suited to the isolation of lysozyme molecules, whose conformation changes in the presence or absence of Ca^{2+} ions.

2. Materials and methods

2.1. Materials

The Streamline system, Streamline-SP adsorbent gel, phenyl-Sepharose 6FF gel, low-molecular-mass protein calibration kit and Cleangel 10% were obtained from Pharmacia Biosystems (Uppsala, Sweden). Servalyt Precotes (3–10)

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and the isoelectric focusing reference proteins are Serva products (Heidelberg, Germany). *Micrococcus luteus* was purchased from Boehringer (Mannheim, Germany). All other chemicals were of analytical grade.

2.2. Sample preparation

Equine milk was defatted at 4°C by centrifugation at 7500 *g* for 10 min. Then about two volumes of concentrated Tris-HCl buffer (60 m*M* Tris) was added to a 1.6-l volume of the milk to give a final Tris-HCl concentration of 20 m*M*. The pH was adjusted to 8.0 with HCl.

2.3. Protein adsorption using the Streamline system

A 300-ml volume of Streamline-SP adsorbent gel was fluidized in a Streamline C-50 column (100 × 5 cm) using an upward linear flow of 300 cm h^{-1} of equilibration buffer, consisting of 20 m*M* Tris-HCl, 0.02% NaN_3, pH 8.0. In this flow regime, the expanded gel bed height was 40 cm (corresponding to a volume of 785 ml). After the fluidized bed was stabilized, the defatted and diluted milk (see above) was pumped into the column and the gel washed with the equilibration buffer until the non-bound proteins were completely removed. Subsequently, the gel beads were allowed to sediment (gel bed height: 17 cm), the adaptor lowered and elution performed in a downward direction using the above-mentioned equilibration buffer containing 1 *M* NaCl. This so-called "salt fraction" was quickly dialyzed against water.

2.4. Hydrophobic interaction chromatography

Hydrophobic interaction chromatography was performed on a 8 × 5 cm column of phenyl-Sepharose 6FF (high substitution type). The gel bed was first equilibrated using three column volumes of 50 m*M* Tris-HCl, 1 m*M* EDTA, adjusted to pH 7.5 with NH_4OH (linear flow-rate: 20 cm h^{-1}). Subsequently, 25 ml of the dialyzed "salt fraction" (protein concentration 8.22 mg/ml), containing a final concentration of 50 m*M* Tris, 10 m*M* EDTA (pH 7.5) was loaded onto the column and the non-bound proteins eluted using the 50 m*M* Tris buffer, 1 m*M* EDTA, pH 7.5. The bound proteins were released by substituting EDTA for Ca^{2+} (50 m*M*) in the elution buffer.

2.5. Purity and catalytic properties at the different stages of preparation

At regular intervals during the loading, washing and elution stages, samples were collected from the effluent. The protein composition of the different fractions was checked using both isoelectric focusing on Servalyt Precotes and SDS-PAGE on Clean Gel 10%. The protein content was determined using the Lowry method [7]. The recovery of active lysozyme was calculated from its lytic activity on *Micrococcus* cell walls, as described by Kikuchi et al. [8].

3. Results and discussion

3.1. Fractionation in the Streamline setup

The Streamline system, which exploits fluidized, or expanded-bed, technology, was originally developed to purify proteins from crude feed stocks in fermentors [9,10]. A major advantage of this technique over classical packed-bed chromatographic methods is that soluble products can be directly adsorbed from an unclarified feed stream. Thus, particulate material (e.g. yeast cells, cell debris, aggregates such as large micelles, etc.) do not foul or block the column. We profited from this feature by directly pumping skimmed milk into a fluidized bed of Streamline SP adsorbent gel with strong cation-exchange properties [11]. Since lysozymes are cationic proteins ($pI > 10$), and since the gel type used has a very high binding capacity (approximately 70 mg lysozyme per ml adsorbent [11]), relatively large amounts of skimmed milk could be treated in a short time. In our experimental conditions, linear flow-rates up to 400 ml h^{-1} (corresponding to a feed flow of 7.85 l h^{-1}) could be easily applied.

The elution profile monitored at 280 nm, showed two fractions (Fig 1). The first, eluting

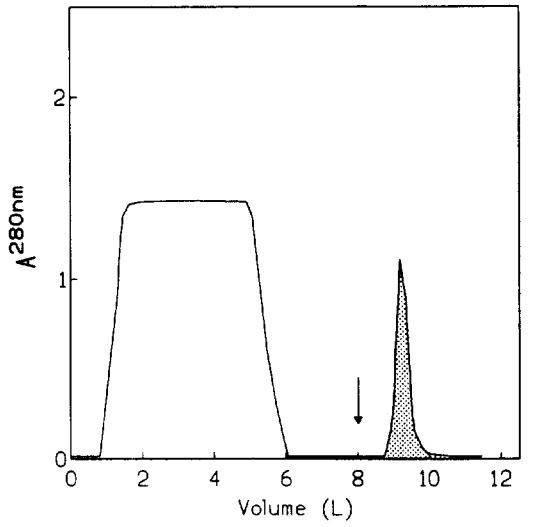

Fig. 1. Elution profile following application of skimmed milk into a fluidized bed of Streamline-SP gel (C-50 type column). The initial buffer used was 20 m*M* Tris-HCl, 0.02% NaN_3, pH 8.0. At the arrow, it was changed to 20 m*M* Tris-HCl/1 *M* NaCl and the flow direction reversed from an upward direction (expanded bed) to a downward direction (after settling of the gel beads). The speckled area represents the lysozyme-containing fraction.

over five gel-bed volumes, was slightly translucent; probably as a result of light scattering brought about by casein micelles. Isoelectric focusing of samples from this fraction (see Fig. 3, lane c) revealed a series of proteins with isoionic points below pH 8. The second peak was eluted by the addition of 1 *M* NaCl to the elution buffer. As expected, this fraction only contained a protein (or proteins) with isoionic point(s) above pH 8.0 (see Fig. 3, lane d). By SDS-PAGE (see Fig. 4, lane d), however, a mixture of proteins with different molecular masses could be detected. This so-called "salt fraction" contained only 10% of the original amount of protein, but up to 89% of the lytic activity (Table 1).

3.2. Hydrophobic interaction chromatography

For further downstream processing of the mixture, we decided to use hydrophobic interaction chromatography, the rationale being that the hydrophobic/hydrophilic nature of equine milk lysozyme can be modified depending on the presence or absence of Ca^{2+} ions [12][1]. In fact, the usefulness of this particular feature in protein

[1] Besides the equine type, pigeon lysozyme too demonstrates Ca^{2+}-binding properties with affinities of $10^{6.4}$ and $10^{7.2}$ M^{-1}, respectively [13]. In human milk and egg white, non-Ca^{2+}-binding species were detected [14].

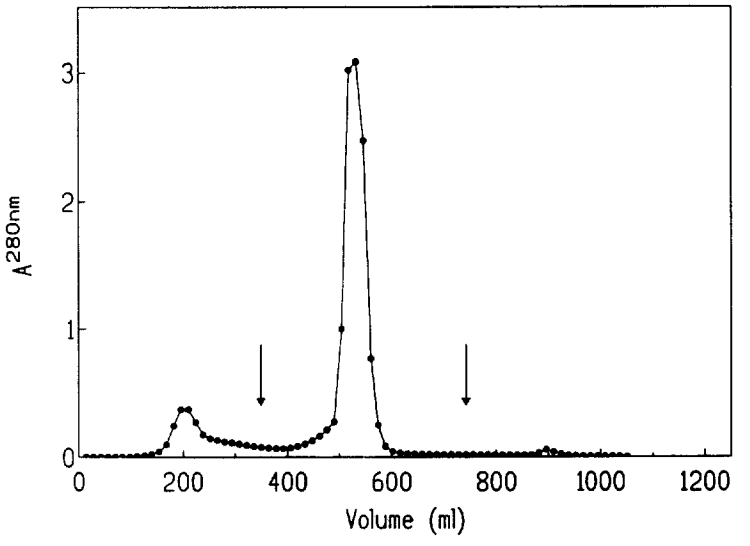

Fig. 2. Hydrophobic interaction chromatographic profile of the lysozyme-containing fraction (see Fig. 1) on a phenyl-Sepharose 6FF support. The initial buffer used was 50 m*M* Tris-HCl, 1 m*M* EDTA, pH 7.5. At the first arrow, this was changed to 50 m*M* Tris-HCl, 50 m*M* Ca^{2+}. At the second arrow, the elution solvent was changed to water. Flow-rate: 400 ml h^{-1}.

Table 1
Purification of equine lysozyme from skimmed milk

	Protein concentration (mg/ml)	Volume (ml)	Amount of protein (mg)	Specific activity (U/mg)[a]	Total activity U (10^6)	Purification factor	Yield
Skimmed milk	8.61	4990	42 964	1335	57.35	1.0	100
Salt fraction (Streamline)	8.22	560	4603	11 075	50.98	8.3	88.9
Final product (HIC)	0.56	2750	1547	26 939	46.88	20.2	81.2

[a] One unit of lysozyme activity was defined as the amount of protein that decreased the $A^{450\ nm}$ by 0.001 units per minute at 25°C.

purification by hydrophobic interaction chromatography was first exploited by Lindahl and Vogel [15], who demonstrated a significant reduction in the hydrophobicity of α-lactalbumin upon Ca^{2+} binding.

Fig. 2 shows a typical elution profile of the "salt fraction" on a phenyl-Sepharose column. In the presence of an excess of EDTA, which converts the enzyme into its apo-form, only one fraction eluted from the column; as shown by SDS-PAGE (Fig. 4, lane e), this contained the

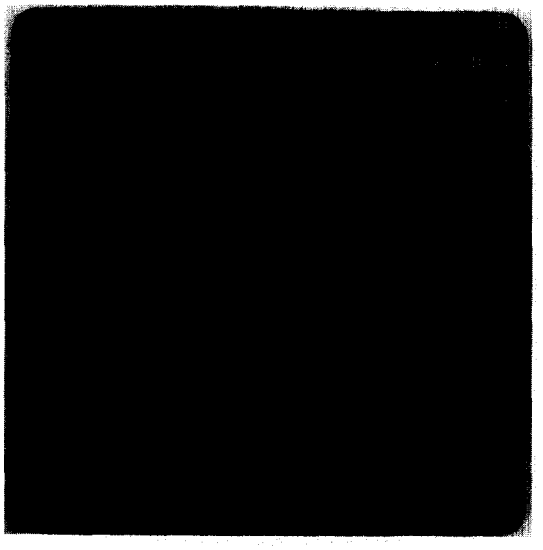

Fig. 3. Isoelectric focusing patterns on Servalyt Precotes (3–10). Lane a: IEP protein calibration kit; 1 = cytochrome *c*, p*I* 10.65; 2 = ribonuclease A, p*I* 9.45; 3 = lectin, p*I* 8.3, 8.0, 7.75; 4 = horse myoglobin, p*I* 7.35, 6.9; 5 = carbonic anhydrase, p*I* 6.0; 6 = β-lactoglobin, p*I* 5.3, 5.15; 7 = trypsin inhibitor, p*I* 4.5; 8 = glucose oxidase, p*I* 4.2; 9 = amyloglucosidase, p*I* 3.5. Lane b: defatted milk; lane c: non-bound proteins from the Streamline column; lane d: "salt fraction" eluted from the Streamline adsorbent gel; lane e: proteins in the salt fraction which are not adsorbed on phenyl-Sepharose support in the presence of EDTA; lane f: purified equine lysozyme after elution from phenyl-Sepharose by Ca^{2+}.

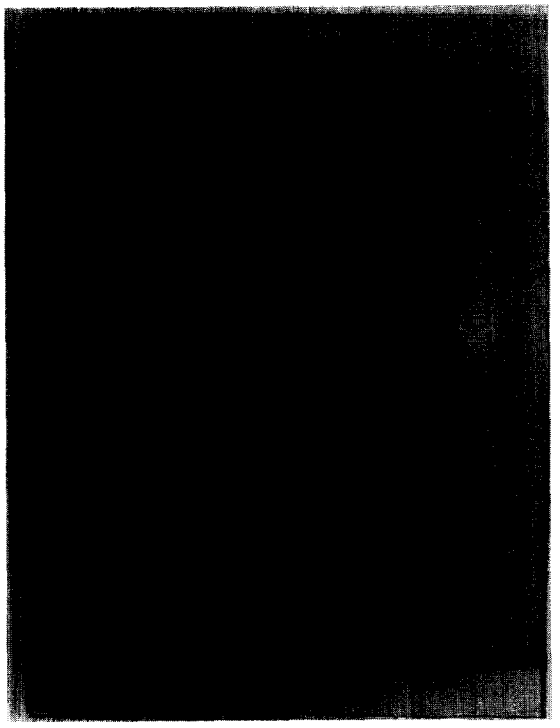

Fig. 4. SDS-PAGE of milk proteins at different stages in the lysozyme purification scheme. Lane a: molecular-mass marker kit (Pharmacia): phosphorylase (M_r 94 000), bovine serum albumin (M_r 67 000), ovalbumin (M_r 43 000), carbonic anhydrase (M_r 30 000), trypsin inhibitor (M_r 20 100) and α-lactabumin (M_r 14 400). The samples in lane b–f are the same as in Fig. 3.

higher-molecular-mass components. A second peak was eluted when EDTA was replaced with Ca^{2+} (Fig. 2, first arrow); it contained only a single protein on isoelectric focusing (Fig. 3, lane f) and SDS-PAGE (Fig. 4, lane f). The value found for the molecular mass (≈14 500) is consistent with that reported in the literature [4]. In this second step, the specific activity was enhanced 2.5-fold (Table 1). When the buffer was replaced by distilled water (Fig. 2, second arrow), no further enzymatically active material was eluted.

In summary, the two-step fractionation method that we present here yields a pure enzyme that still retains its Ca^{2+}-binding capacity which may be of interest in studying e.g. spectrophotometric characteristics of the protein. The overall recovery of enzymatic activity was greater than 80% of the value, originally found in the starting batch of skimmed milk.

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