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The calcium-binding property of equine lysozyme

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It was found that equine lysozyme binds one Ca^{2+}. It was eluted with equimolar Ca^{2+} from a Bio-Gel P-4 column. Human lysozyme did not behave similarly. Equine lysozyme is concluded to be a calcium metalloprotein like α-lactalbumin, which is a homologue of hen egg white lysozyme.

Lysozyme; Ca^{2+} binding; α-Lactalbumin

1. INTRODUCTION

Chicken type lysozyme and α-lactalbumin have undoubtedly evolved from a common ancestral protein because of the similarity of their amino acid sequences [1] and intron-exon constitutions of their genes [2]. However, their functions are different. Lysozyme is a lytic enzyme decomposing peptidoglycan, a constituent of bacterial cell walls, while α-lactalbumin acts as a specificity modifier to convert galactosyltransferase to lactose synthase. Many comparative studies have been reported on similarities or differences between the structures of lysozyme and α-lactalbumin. An essential difference between them has been considered to be the calcium-binding ability [3]. All α-lactalbumins which have been analysed thus far are calcium metalloproteins, although the role of Ca^{2+} in α-lactalbumin has not yet been established. On the other hand, chicken type lysozymes are known to have no strong calcium-binding site at all.

The results of high-resolution X-ray structure analysis of baboon α-lactalbumin were recently published [4] and the calcium-binding site was revealed. It consists of two backbone carbonyl groups of residues 79 and 84 and three side chain carboxyl groups of Asp-82, Asp-87 and Asp-88. These three aspartyl residues are all conserved in the α-lactalbumin subfamily. In the case of hen egg white lysozyme, it is shown that the backbone conformation of the α-lactalbumin elbow [4], the binding site, is essentially conserved, although the side chains are radically altered. Furthermore, it is pointed out that the above three aspartyl residues are all conserved in equine lysozyme and the possibility is suggested that equine milk lysozyme might bind calcium. In addition, from preliminary results, equine lysozyme is suggested actually to bind Ca^{2+} [5]. In the case of human lysozyme, one of the three residues is also conserved and therefore it seems to contain a partially formed (or destroyed) calcium-binding site [4].

The aim of this study is to examine and to quantitate the calcium binding of the lysozyme from these two mammalian species.

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2. MATERIALS AND METHODS

Lysozyme of horse (*Equus caballus*) was prepared from pooled equine milk of Percheron mares according to Bell et al. [6]. Prepared equine lysozyme was dissolved in 50 mM Tris-HCl buffer (pH 7.6), dialysed exhaustively against deionized water after the addition of a slight excess of $CaCl_2$,

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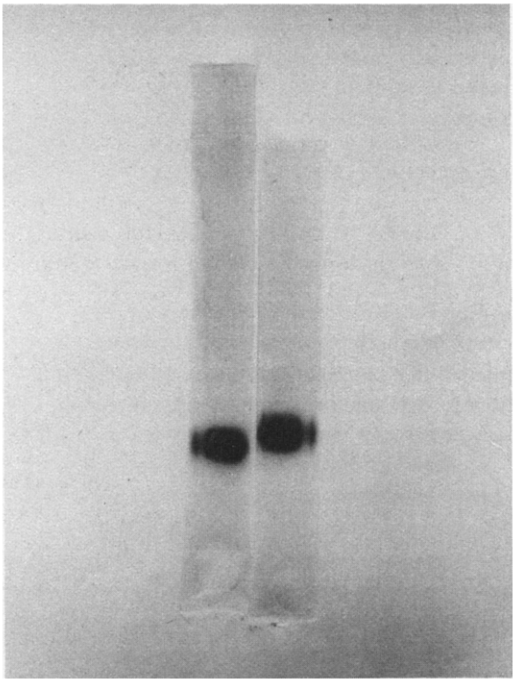

Fig.1. SDS-PAGE [12.5% poly(acrylamide) gel] of equine and human lysozyme. Left, human lysozyme; right, equine lysozyme.

and lyophilized. The homogeneity was checked on poly(acrylamide) gel electrophoresis as shown in fig.1 and enzymatic activity was followed using a cell suspension of *Micrococcus luteus* as a substrate. Human lysozyme was prepared from human milk using essentially the same method as in the case of the equine enzyme [7]. Bovine α-lactalbumin was prepared as in [8] and hen egg white lysozyme was purchased from Seikagaku Kogyo (Tokyo, lot no.E7302). The concentration of equine or human lysozyme was determined from the absorbance at 280 nm using the absorption coefficient expected from their amino acid sequences [9,10] according to the method described in [11]. The concentrations of calcium and magnesium were determined with an atomic absorption spectrometer (Hitachi 170-10) equipped with a hollow cathode lamp (Hitachi HGA-45S Ca-Mg).

3. RESULTS

3.1. *Metal ion analysis*

Equine lysozyme was dissolved in 50 mM Tris-HCl buffer (pH 7.6) to various concentrations from 0 to 20 μM. The concentration of calcium was determined by atomic absorption spectroscopy and plotted vs that of lysozyme determined from the absorbance at 280 nm, as shown in fig.2. The content of calcium was estimated to be 0.94 for equine lysozyme from the slope. The content of magnesium for equine lysozyme and those of calcium and magnesium for human lysozyme were also determined, the results being summarized in table 1.

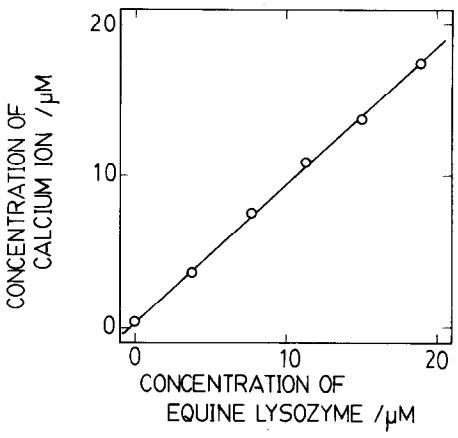

Fig.2. Concentration of Ca^{2+} in a solution of equine lysozyme. Slope: 0.94 ± 0.06.

3.2. *Bio-Gel P-4 chromatography*

5 ml of ~10 μM equine lysozyme solution was applied to a Bio-Gel P-4 column (1.7 × 15 cm) equilibrated with 0.05 M Tris-HCl buffer (pH 7.6) and eluted with the same buffer. The resulting chromatographic pattern is shown in fig.3a. Equine lysozyme was eluted with equimolar Ca^{2+}, indicating that equine lysozyme does bind one Ca^{2+}. Bovine α-lactalbumin, which is known to be a calcium metalloprotein, was also applied to the Bio-Gel P-4 column and the same result was ob-

Table 1

Observed numbers of metal ions bound to lysozyme

	Calcium	Magnesium
Equine lysozyme	0.94	0.04
Human lysozyme	0.03	0.02

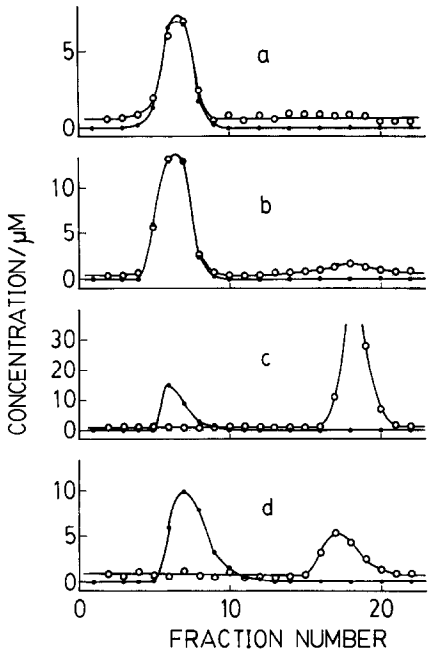

Fig.3. Bio-Gel P-4 chromatography of (a) equine lysozyme, (b) bovine α-lactalbumin, (c) human lysozyme with a 3-fold excess of $CaCl_2$, and (d) hen egg white lysozyme with equimolar $CaCl_2$. (○—○) Calcium, (●—●) lysozyme. Column dimensions: 1.7 × 15 cm (a,b,d) or 1.5 × 30 cm (c). Elution buffer: 0.05 M Tris-HCl (pH 7.6). One fraction consists of 50 drops (~2 ml) for a, b and d, or 75 drops for c.

tained as for equine lysozyme, as shown in fig.3b. Human lysozyme was chromatographed with a 3-fold excess of $CaCl_2$ and the result is shown in fig.3c. Human lysozyme and calcium were clearly separated, indicating that human lysozyme does not bind Ca^{2+}. Hen egg white lysozyme, which is not a calcium-binding protein, was also chromatographed with equimolar $CaCl_2$ and the result was the same as that obtained for human lysozyme as shown in fig.3d.

4. DISCUSSION

Equine lysozyme is a chicken type lysozyme in respect of its amino acid sequence [9]. At neutral pH, equine lysozyme binds one Ca^{2+}, whereas human and hen egg white lysozymes do not. After gradient elution from CM-Sephadex C-25 at acidic pH (pH 5.0), equine lysozyme contained only half of the Ca^{2+} in molar ratio. Ca^{2+} might have been depleted partly by carboxylate groups of CM-Sephadex. Exhaustive dialysis after the addition of Ca^{2+} at neutral pH does not eliminate Ca^{2+}.

The ancestral protein of the present lysozyme and α-lactalbumin should be lysozyme, because chicken type lysozyme appears widely among amniotes and even in insects [12] but α-lactalbumin is found only in mammals. From the results of this study, binding of Ca^{2+} is concluded not to be a characteristic property of the α-lactalbumin subfamily, but some chicken type lysozymes, such as equine lysozyme, also bind Ca^{2+}. The possibility that the ancestral lysozyme was a calcium metalloprotein can be suggested from the evidence shown here and that the backbone conformation of the α-lactalbumin elbow is conserved even in hen egg white lysozyme [4]. Because lysozyme and α-lactalbumin diverged in their function, it is unlikely that the bound Ca^{2+} contributes to each specific function. The bound Ca^{2+} must be involved principally in stabilization of their structure, which can be replaced by other stabilizing factors during evolution.

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