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Interaction of seminalplasmin with chlortetracycline, a fluorescent chelate probe of Ca^{2+}

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The interaction of seminalplasmin with chlortetracycline, a fluorescent chelate probe of Ca^{2+}, was studied. The results indicate that seminalplasmin binds to chlortetracycline. The binding is not influenced by salt. Both Ca^{2+} and seminalplasmin probably bind to the same site on chlortetracycline. Seminalplasmin also reduced the Tb^{3+}-associated fluorescence of bovine spermatozoal plasma membrane. These results are discussed in relation to the inhibitory effect of seminalplasmin on the uptake of Ca^{2+} in bovine spermatozoa.

Seminalplasmin; Chlortetracycline; Terbium; Ca^{2+}-binding site; (Bovine spermatozoal membrane)

1. INTRODUCTION

Seminalplasmin (SPLN), an antimicrobial protein from bull seminal plasma, is a broad-spectrum antibiotic which inhibits the growth of gram-positive and gram-negative bacteria as well as yeast cells by specifically inhibiting the synthesis of RNA [1–7]. It is produced by the auxiliary sex glands and coats the surface of ejaculated spermatozoa [8]. Recent in vitro studies have indicated that SPLN binds to synthetic bilayer membranes of both negatively charged and neutral lipids [9] and also to the plasma and outer acrosomal membranes of bovine spermatozoa [10]. SPLN also inhibits the uptake of Ca^{2+} in bovine spermatozoa [11]. Ca^{2+} is known to influence the motility, acrosome reaction and fertilising ability of spermatozoa [12,13]. Hence, studies directed towards understanding the mechanism by which SPLN inhibits Ca^{2+} uptake would be important.

Here, the interaction of SPLN with chlortetracycline (CTC), a fluorescent chelate probe for divalent cations, was studied by monitoring the changes in fluorescence intensity of the CTC emission band (520 nm). The results indicate that SPLN, like Ca^{2+}, binds to CTC and the binding of SPLN to CTC is not influenced by salt. Tb^{3+} was also used as a fluorescent probe to monitor Ca^{2+}-binding sites on bovine spermatozoal plasma membrane (SPM) in the presence and absence of SPLN.

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2. MATERIALS AND METHODS

Plasma membrane of bovine spermatozoa was isolated as described [14,15]. SPLN was purified from bovine semen [1,16]. Protein was determined by the method of Lowry et al. [17].

Fluorescence measurements were recorded on a Hitachi 650-10S fluorescence spectrophotometer operated in the ratio mode, with 4 nm excitation and emission band pass. A 2 mM aqueous solution of CTC-HCl served as stock and the excitation wavelength was 400 nm. Terbium chloride was used as an aqueous solution and was excited at 295 nm. Fluorescence studies were carried out using 10 mM Tris-HCl buffer (pH 7.4). All chemicals used here were purchased from Sigma (USA).

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3. RESULTS AND DISCUSSION

CTC has been used as a fluorescent probe of Ca^{2+}-binding sites on membranes [18,19] and for following the migration of Ca^{2+} in sarcoplasmic reticulum, pancreatic acinar cells, brain synaptosomes, leukocytes, erythrocytes, platelets and mitochondria [20–27]. However, little is known about the interaction of CTC with membrane components such as lipids and proteins [28,29]. The emission spectra of CTC in the presence of SPLN and Ca^{2+} are shown in fig.1. Binding of CTC to SPLN was accompanied by a large enhancement of the intensity of the emission band at 520 nm, the increase in intensity being dependent on the amount of SPLN added and finally levelling off (fig.1A). Ca^{2+} also caused an increase in the fluorescence intensity of CTC in a concentration-dependent manner (fig.1B). This increase in CTC emission intensity in the presence of metal ions is well known and is attributed to the formation of a coordination site on the CTC molecule for binding of the cation [30]. The results in fig.1 indicate that SPLN binds to CTC. It was also observed that the binding was not influenced by high concentrations of salt (tested up to 10 mM NaCl), irrespective of whether it was added to CTC before or after the addition of SPLN. This would mean that the bind-

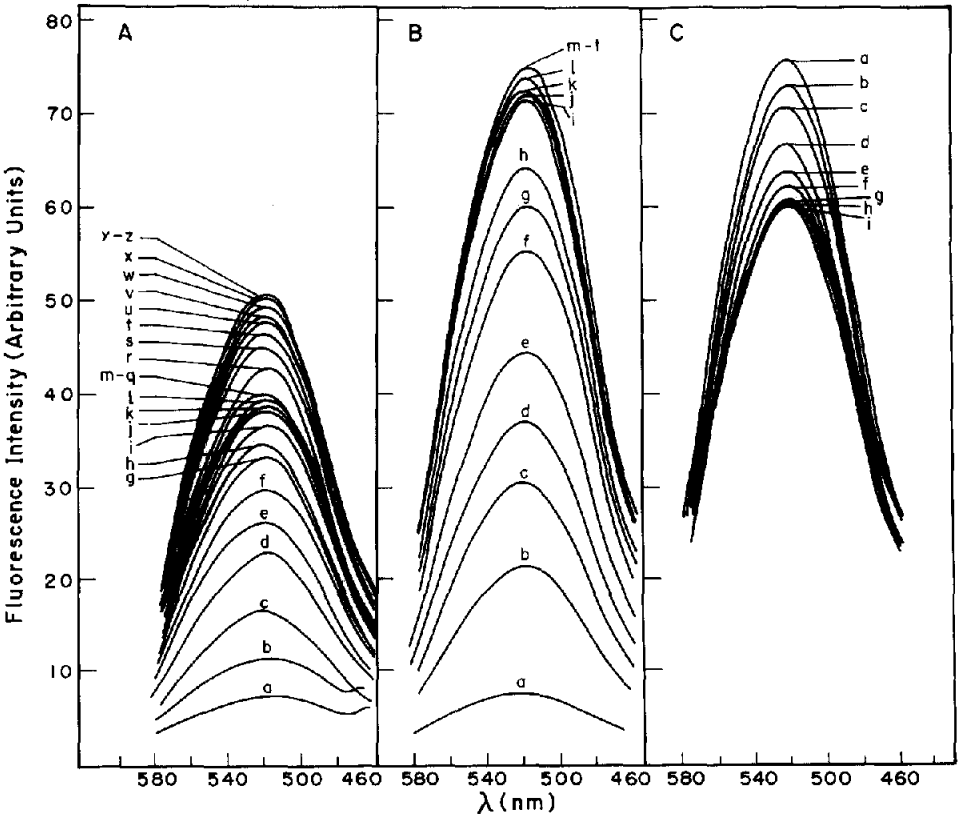

Fig.1. Fluorescence emission spectra of CTC (25 μM) in the presence of SPLN and Ca^{2+}. λ(excitation) = 400 nm. (A) Titration of CTC with SPLN. a, free CTC; b–q, successive addition of SPLN as follows: b,c, 100 μg; d–i, 200 μg; j–l, 100 μg and m–q, 50 μg; r–z, successive addition of Ca^{2+} to q such that the final concentration of Ca^{2+} was 0.1, 0.2, 0.3, 0.4, 0.6, 0.8, 1, 1.2, 1.4 and 1.6 mM from r to z, respectively. (B) Titration of CTC with Ca^{2+}. a, free CTC; b–t, successive addition of Ca^{2+} to a such that the final concentration of Ca^{2+} varied from 0.3 mM in b to 2.2 mM in m where CTC was completely saturated with Ca^{2+}; further increase in Ca^{2+} concentration to 2.5 mM as in t did not alter the fluorescence intensity. (C) a, same as t of B; b–i, successive addition of 100 μg SPLN to a, to a concentration of 800 μg SPLN in i.

ing of CTC to SPLN, a highly basic protein (p*I* 9.6), is not a mere charge-charge interaction. The possibility that the binding of CTC to SPLN is due to divalent cations already associated with SPLN is ruled out due to three reasons: (i) divalent cations do not bind to SPLN; (ii) EDTA does not bind to SPLN; and (iii) CTC binds to SPLN even in the presence of EDTA (not shown).

It is possible that Ca^{2+} and SPLN, which bind to CTC, bind at the same site. If this is so, it would appear that saturation of CTC with any one of the two molecules should prevent the binding of the other. The results in fig.1A indicate that addition of Ca^{2+} to the CTC-SPLN complex at saturation increased the fluorescence intensity from 40 to 50 units at a final concentration of 1.2 mM Ca^{2+}. Further addition of Ca^{2+} had no effect. If Ca^{2+} and SPLN were binding to different sites on CTC then, as indicated in fig.1B, CTC should have accommodated 2.2 mM Ca^{2+} and also increased the fluorescence intensity to 75 units. Addition of SPLN to the CTC-Ca^{2+} complex at saturation reduced the fluorescence intensity from 75 to 60 units (fig.1C). It appears that the above effects may be due to the formation of a ternary complex between CTC, SPLN and Ca^{2+}.

That SPLN and Ca^{2+} bind at the same site on CTC was further indicated by determining the concentration of SPLN and Ca^{2+} required to bring about half-maximum change in fluorescence intensity of the CTC emission band. At this concentration (0.9 mM for Ca^{2+} and 400 μg/ml for SPLN), addition of SPLN to CTC-Ca^{2+} complex or Ca^{2+} to CTC-SPLN complex increased the intensity in either case to around 45 units (fig.2A,B).

In the light of the observation that CTC binds to SPLN, and previous findings that SPLN binds to bovine spermatozoal plasma membrane (SPM)

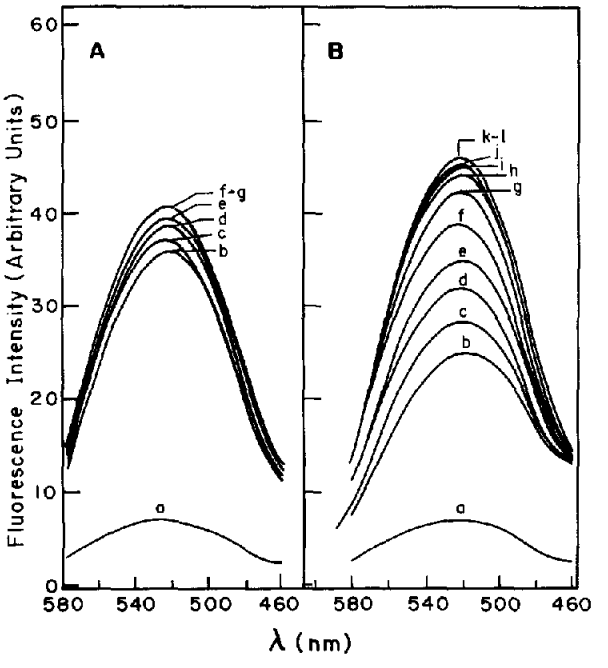

Fig.2. (A) Binding of SPLN to CTC (25 μM) in the presence of 0.9 mM Ca^{2+}. a, free CTC; b, a + 0.9 mM Ca^{2+}; c–g, successive addition of 100 μg SPLN to b to a concentration of 500 μg SPLN in g. (B) Binding of Ca^{2+} to CTC (25 μM) in the presence of 400 μg of SPLN. a, free CTC; b, a + 400 μg SPLN; c–l, successive addition of Ca^{2+} to b such that the final concentration of Ca^{2+} was 0.2, 0.4, 0.6, 0.9, 1.5, 2.0, 2.2, 2.4, 2.6 and 2.8 mM from c to l, respectively.

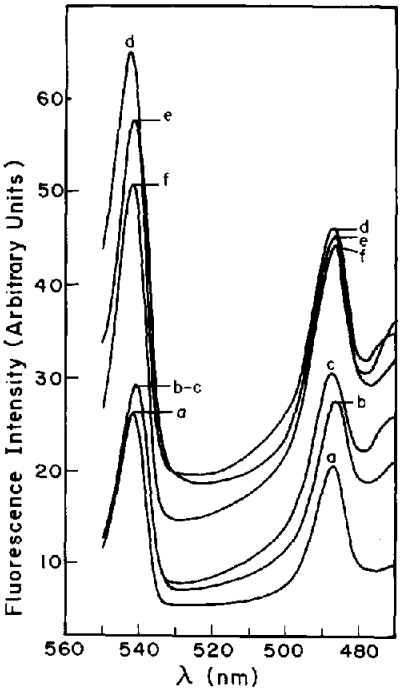

Fig.3. Fluorescence emission spectra of Tb^{3+} in the presence of SPM and SPLN. λ(excitation) = 295 nm. a, free Tb^{3+} (30 μM); b, a + 100 μg SPLN; c, b + 100 μg SPLN; d, 100 μg SPM + 30 μM Tb^{3+}; e and f, 100 μg SPM plus 100 and 200 μg SPLN, respectively, to which Tb^{3+} (30 μM) was added subsequently.

[10] and inhibits uptake of Ca^{2+} in bovine spermatozoa [11], it is tempting to predict that SPLN binds to Ca^{2+}-binding sites on the spermatozoal surface, thereby inhibiting the uptake of Ca^{2+}. If this prediction were to be correct, treatment of SPM with SPLN should reduce the binding of Ca^{2+} to the membrane. For this purpose Tb^{3+} was used as a fluorescent probe for monitoring Ca^{2+}-binding sites on the membrane [31–33], since CTC, if used, would bind not only to the membrane but also to SPLN. The emission spectra of Tb^{3+} in the presence of SPM and SPLN are shown in fig.3. The fluorescence of Tb^{3+} is considerably enhanced at 542 nm on binding to the membrane, thus indicating the presence of Ca^{2+}-binding sites. Enhancement of the fluorescence intensity of Tb^{3+} at 542 nm on binding to bovine SPM and the number of binding sites, on the membrane, have been reported earlier [33]. Addition of SPLN to the membrane decreased the fluorescence intensity of Tb^{3+} at 542 nm by about 20% at the maximum concentration of SPLN tested. This same concentration in fact increased the intensity of Tb^{3+} fluorescence only marginally. Thus it appears that SPLN does decrease the binding of Tb^{3+} to spermatozoal plasma membranes.

The above results indicate that SPLN binds to CTC and also competes with Ca^{2+} for this binding. The formation of the fluorescent chelate between CTC and Ca^{2+} is facilitated by the oxygens at positions 11 and 12 of the CTC molecule, and at positions 2 and 3 which form a coordination site for the binding of the cation [30]. Since SPLN inhibits Ca^{2+} uptake in bovine spermatozoa [11] it would appear that SPLN, which is known to bind to spermatozoal membranes, would, on binding, prevent or reduce the binding of Ca^{2+} to the membranes. The results with Tb^{3+} do indicate a reduction in the binding of Tb^{3+} to SPM on treatment with SPLN. Thus it appears that both SPLN and Ca^{2+} bind to similar sites on SPM, but the above results do not indicate whether SPLN affects the translocation of Ca^{2+} from the membrane into the cell. The mechanism of Ca^{2+} uptake in spermatozoa is not yet clearly understood. Ca^{2+} is, however, known to influence functions associated with ejaculated spermatozoa such as motility, acrosome reaction and fertilizing ability [12,13]. Further studies on the effect of SPLN on the binding of Ca^{2+} to the surface of intact spermatozoa and on the translocation of Ca^{2+} into the sperm cell may reveal the mechanism by which SPLN inhibits the uptake of Ca^{2+} in bovine spermatozoa [11].

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