Activation of bovine plasminogen by Streptococcus uberis

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Abstract: Culture filtrate from Streptococcus uberis was found to activate bovine and equine plasminogen but not that from rabbit, human or porcine plasma. In contrast, streptokinase from a Lancefield group C Streptococcus activated human plasminogen but not that from bovine, porcine and rabbit plasma. Very slight activity was observed against equine plasminogen. Plasmin was detected by hydrolysis of skimmed milk protein in agarose. The activation of bovine plasminogen by S. uberis culture filtrate resulted in the formation of three polypeptides with molecular masses of 56, 26 and 21 kDa. This is the first report of a streptokinase activity from this species.

Key words: Plasminogen; Streptococcus; Mastitis; Streptokinase

Introduction

Streptococcus uberis is a common cause of bovine mastitis, responsible for around 20% of all clinical cases in the UK [1]. The ability of this organism to infect the lactating mammary gland is dependent on its ability to grow in the secretion and avoid phagocytosis by bovine neutrophils [2].

The majority of nitrogen in bovine milk is present in the form of protein [3] and in the absence of proteolysis bacterial growth in milk is limited by the lack of free amino acids. This is highlighted by the dependence of the lactic streptococci on extracellular, caseinolytic proteinases for growth in milk [4]. The ability of bacteria to grow in mastitic milk is enhanced by the presence of the caseinolytic enzyme, plasmin [5]. The transformation of plasminogen to plasmin requires plasminogen activators which are known to occur in blood plasma and animal tissues [6]. Certain streptococci are capable of producing streptokinase which activates plasminogen to plasmin.

Streptokinase from Streptococcus equisimilis (Lancefield group C) activates human and feline plasminogen [7], whereas that from a Lancefield group E Streptococcus activated porcine plasminogen [8]. None of these bacterial proteins have been shown to activate bovine plasminogen. This communication reports the presence of such an activator in culture filtrates of S. uberis and its
effect on plasminogen from a variety of mammalian species.

Materials and Methods

Bacterial strains and growth conditions

All bacterial isolates were obtained from clinical cases of bovine mastitis. The isolates of *S. uberis* were shown to be distinct strains by the DNA fingerprinting method of Hill and Leigh [9]. Bacteria were stored at $-20^\circ\text{C}$ in Todd Hewitt broth (THB) containing 25% (w/v) glycerol. Cultures were grown at 37$^\circ$C in THB or on Todd Hewitt agar (THB containing 1.2% (w/v) Oxoid agar No. 3).

Chemicals and reagents

Plasminogen from rabbit, human, porcine, equine and bovine plasma was obtained from Sigma Chemical Co. (Poole, Dorset, UK) and reconstituted in sterile distilled water to a final concentration of 1.0 unit ml$^{-1}$. Streptokinase from a Lancefield group C *Streptococcus* was also

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Fig. 1. Hydrolysis of milk proteins by *Streptococcus uberis*, *Streptococcus agalactiae* and *Streptococcus dysgalactiae* in the presence of bovine plasminogen. *S. uberis* strain 01-40J (A and B) overlaid with agarose containing skimmed milk in the presence (A) and absence (B) of bovine plasminogen. *S. agalactiae* strain 411.07 (C) and *S. dysgalactiae* strain CE127 (D) overlaid with agarose containing skimmed milk and bovine plasminogen.
obtained from Sigma, reconstituted at a concentration of 1 mg ml\(^{-1}\) in phosphate buffered saline (pH 7.4). Plasminogen and streptokinase were stored at \(-70^\circ\text{C}\) and thawed only once prior to use.

**Preparation of culture filtrate**

Overnight broth cultures of *S. uberis* strain 0140J were centrifuged at 5000 \(\times\) g for 30 min at \(4^\circ\text{C}\). The resulting supernatant was sterilised by filtration and stored at \(-70^\circ\text{C}\).

**Detection of plasminogen activation by agarose / skimmed milk overlay**

Overnight cultures in THB were streaked onto Todd Hewitt agar and incubated at 37\(^\circ\text{C}\) for 18 h. Plates with isolated colonies were overlaid with 10 ml of molten agarose (10 mg ml\(^{-1}\)) containing NaCl (150 mM), Tris-HCl (50 mM, pH 8.1), OXoid skimmed milk (1% v/v) and bovine plasminogen (10 \(\mu\)g ml\(^{-1}\)), and incubated at 37\(^\circ\text{C}\). Controls were performed using overlays identical to that above except that plasminogen was omitted.

**Plasminogen activation and detection of plasmin**

Equal volumes of plasminogen (1.0 unit ml\(^{-1}\)) and *S. uberis* culture filtrate or streptokinase (1 \(\mu\)g ml\(^{-1}\)) from a Lancefield group C Streptococcus were mixed and incubated at 37\(^\circ\text{C}\) for 45 min after which 10 \(\mu\)l was assayed for the presence of plasmin by the detection of caseinolytic activity. Activity was detected by diffusion from wells cut in skimmed milk agarose (as overlays above containing no plasminogen) following 24 h incubation at 37\(^\circ\text{C}\).

**Conversion of plasminogen to plasmin and detection by polyacrylamide gel electrophoresis and protein staining**

Bovine plasminogen (0.005 unit in a volume of 5 \(\mu\)l) was mixed with 2 \(\mu\)l of *S. uberis* culture filtrate and incubated at 37\(^\circ\text{C}\) for 1 h. Samples were mixed with an equal volume of sample buffer containing sodium dodecyl sulphate (0.01% w/v) and 2-mercaptoethanol (0.2% v/v), and heated at 65\(^\circ\text{C}\) for 5 min. Proteins were separated by electrophoresis [10] and detected by the staining procedure of Oakley et al [11].

**Results and Discussion**

All five strains of *S. uberis* (0140J, EF20, ST10, C216, C197C) produced zones of caseinolytic activity in skimmed milk, bovine plasminogen, agarose overlays within 4 h at 37\(^\circ\text{C}\) (Fig. 1A). No zones were detectable around isolated colonies in overlays in the absence of bovine plasminogen (Fig. 1B). *S. agalactiae* (strain 411.07) and *S. dysgalactiae* (strain CE127) did not induce the formation of caseinolytic activity in the presence of bovine plasminogen (Fig. 1C and D, respectively) even after incubation for 24 h. The ability of *S. agalactiae* and *S. dysgalactiae* to activate plasminogen from other species was not investigated.

Culture filtrate from *S. uberis* 0140J or streptokinase from a Lancefield group C *Streptococcus* was incubated with plasminogen from a variety of mammalian species and plasmin formation detected by diffusion through skimmed milk agarose (Fig. 2). Culture filtrates from *S. uberis* activated bovine and equine plasminogen but the activity with the latter was only apparent after incubation of the skimmed milk agarose for around 18 h compared to 2 h when using bovine plasminogen. This suggests either a lower activity of *S. uberis* streptokinase for this substrate or the poor activity of the resulting plasmin molecule for bovine milk proteins. *S. uberis* culture filtrate failed to activate plasminogen from human, rabbit or pig. In contrast, streptokinase from the Lancefield group C *Streptococcus* activated human plasminogen and showed a trace of activity towards equine plasminogen but no activity towards that from rabbit, bovine or porcine plasma. Neither *S. uberis* culture filtrate nor streptokinase from the Lancefield group C *Streptococcus* showed any caseinolytic activity in the absence of plasminogen.

The conversion of bovine plasminogen to plasmin by *S. uberis* culture filtrate was analyzed by SDS-PAGE (Fig. 3). The plasminogen used during this investigation contained contaminating proteins, although the suppliers (Sigma) claim the plasmin contamination to be less than 5% of the total protein. Incubation of the plasminogen preparation with a 1:10 dilution of *S. uberis*
culture filtrate (Fig. 3, Track D) resulted in the loss of the 91 kDa protein band. This protein is also lost from the preparation following plasminogen activation by urokinase (data not shown) and is assumed to be the plasminogen protein. This is also inferred since the molecular mass of this protein agrees closely with that proposed for bovine plasminogen [12]. Activation of bovine plasminogen by culture filtrate of S. uberis 0140J also resulted in the loss of another protein (48 kDa) from the plasminogen preparation. It is not clear whether the disappearance of this protein was a result of the direct action of the culture filtrate or of the resulting plasmin activity. It is possible that the 48 kDa protein represents a fragment of the plasminogen molecule which contains the activation site required by the plasminogen activator of S. uberis. The disappearance of the 91 kDa plasminogen band and the 48 kDa

Fig. 3. SDS-PAGE of bovine plasminogen following incubation in the presence of S. uberis culture filtrate. Tracks show bovine plasminogen in the presence of 2 µl of phosphate-buffered saline (A), S. uberis culture filtrate diluted 1:1000 (B), 1:100 (C) and 1:10 (D) in phosphate-buffered saline (PBS). Arrows indicate the position of the plasmin associated polypeptides and an asterisk indicates the position of the 91 kDa plasminogen protein band. No protein bands were detected following electrophoresis of culture filtrate diluted 1:5 in PBS (data not shown). Numbers indicate the position of proteins of known molecular mass.

Fig. 2. Detection of caseinolytic activity following activation of plasminogen with streptokinase from Lancefield group C Streptococcus or S. uberis culture filtrate. Wells contain a mixture of streptokinase from Lancefield group C Streptococcus (column 1), S. uberis culture filtrate (column 2), or phosphate-buffered saline (column 3) and either human (row H), rabbit (row R), porcine (row P), equine (row E) or bovine (row B) plasminogen. The row labelled PBS contained phosphate-buffered saline in place of plasminogen.
proteins corresponded with the formation of three smaller polypeptides with apparent molecular masses of 56, 26 and 21 kDa.

Since no protein bands were seen following electrophoresis of culture filtrate at a concentration 2-fold higher than that used to achieve total plasminogen activation, the presence of an additional polypeptide (21 kDa) cannot be explained simply by the presence of bacterial proteins. The cumulative total of the molecular masses of the three polypeptides observed following plasminogen activation during this investigation showed a significant discrepancy from that of bovine plasminogen. One possible explanation for this is that the 21 kDa polypeptide is a product of the degradation of the 48 kDa protein which contaminates the plasminogen preparation and which is depleted during activation. The determination of the molecular masses of the products of the activation of purified plasminogen by purified S. uberis streptokinase will resolve this discrepancy.

The streptokinase activity present in S. uberis culture filtrate differed from that isolated from Lancefield group E streptococci which is reported to activate porcine plasminogen [8]; S. uberis culture filtrate failed to activate this molecule (Fig. 2). It also differed from similar activities from S. equisimilis and S. pyogenes, both of which activate human but not bovine plasminogen [7,13], whereas streptokinase from S. uberis activates bovine but not human plasminogen. This is the first report of the presence of plasminogen activating activity in S. uberis.

The concentration of plasminogen in normal bovine milk is around 1.3 µg ml⁻¹ [14]. Activation of this to plasmin during the early stages of infection, prior to an inflammatory response and plasma leakage into the gland, could result in hydrolysis of milk proteins and thus increase the availability of peptides and amino acids for bacterial growth. As such, streptokinase which activates bovine plasminogen may be an essential virulence determinant of S. uberis which allows more rapid bacterial growth in the lactating, bovine mammary gland during the very early stages of infection.

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References