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Cloning and seasonal secretion of the pancreatic lipase-related protein 2 present in goat seminal plasma

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Abstract

The storage of frozen semen for artificial insemination is usually performed in the presence of egg yolk or skimmed milk as protective agents. In goats, the use of skimmed milk extenders requires, however, that most of the seminal plasma is removed before dilution of spermatozoa because it is deleterious for their survival. It has been previously demonstrated that a lipase (BUSgp60) secreted by the accessory bulbourethral gland was responsible for the cellular death of goat spermatozoa, through the lipolysis of residual milk lipids and the release of toxic free fatty acids.

This lipase was purified from the whole seminal plasma of goat and was found to display both lipase and phospholipase A activities, this latter activity representing the main phospholipase activity detected in goat seminal plasma. Based on its N-terminal amino acid sequence, identical to that of BUSgP60 purified from bulbourethral gland secretion, and the design of degenerated oligonucleotides, the lipase was cloned from total mRNA isolated from bulbourethral gland. DNA sequencing confirmed it was the goat pancreatic-lipase-related protein 2 (GoPLRP2).

The physiological role of GoPLRP2 is still unknown but this enzyme might be associated with the reproductive activity of goats. A significant increase in lipase secretion was observed every year in August and the level of lipase activity in the semen remained high till December, i.e., during the breeding season. A parallel increase in the plasmatic levels of testosterone suggested that GoPLRP2 expression might be regulated by sexual hormones. The lipase activity level measured in goat seminal plasma, which could reach 1000 U/ml during the breeding season, was one of the highest lipase activity measured in natural sources, including gastric and pancreatic juices.

Keywords: Goat; Lipase; Lipolysis; Pancreatic lipase-related protein; Phospholipase; Reproduction; Seminal plasma; Hormonal regulation

1. Introduction

In goats, like in many other mammals, seminal plasma is a complex mixture of secretions from the epididymis and various accessory sex glands, including the secretion from the bulbourethral gland (Fig. 1). The bulbourethral gland, also named Cowper's gland, is a paired organ that produces a mucoid secretion that cleans the urethra prior to semen passage.

In 1997, Pellicer-Rubio et al. [1] identified a 60-kDa glycoprotein from bulbourethral gland secretion (BUSgp60) responsible for the detrimental effect of the whole seminal plasma on spermatozoa survival during the cryoconservation process of sperm. From N-terminal sequencing of Busgp60, as well as sequences of internal peptides, it was shown that BUSgp60 had a significant homology (50%–70%) with members of the pancreatic lipase family, the closest homology being observed with pancreatic-lipase-related proteins 2 (PLRP2) [1]. The

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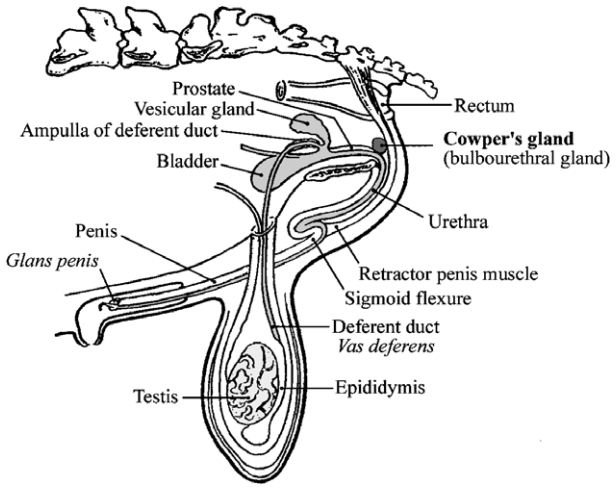

Fig. 1. Male reproductive organ of the goat. Adapted from Ref. [43].

presence of this lipase in seminal plasma induced a decrease in the motility of spermatozoa, an acrosome deterioration and the death of epididymal spermatozoa when skimmed milk was used as an extender for cryoconservation. It was further demonstrated that the deterioration of goat spermatozoa in skimmed-milk-based extenders was due to the release of fatty acids upon the hydrolysis of residual milk lipids by BUSgp60 [2]. It was shown that BUSgp60 was able to hydrolyze both triolein and milk triglycerides, and that oleic acid, the major fatty acid (30–40%) present in milk triglycerides, exerted a clear dose-dependent adverse effect on goat spermatozoa viability. A similar effect was observed when spermatozoa were mixed with the bulbourethral gland secretion and milk extender [2].

The aim of the present study was to isolate the BUSgp60 mRNA from goat bulbourethral gland in order to confirm the previous identification of this enzyme as a PLRP2. This lipase was also purified from the whole seminal plasma of goat and some of its biochemical properties were determined. From seminal plasma samples collected for 2 years, it was investigated whether the lipase secretion undergoes seasonal variation and might be associated with the reproductive activity of goats.

2. Materials and methods

2.1. Semen collection

Semen was collected from 10 Alpine and Saanen adult male goats, every month for 2 years with the aid of an artificial vagina at 37 °C. Semen was centrifuged at 800×*g* for 10 min. After sperm separation, plasma seminal was further centrifuged at 800×*g* for 20 min at 5 °C. The supernatant was mixed with protease inhibitors (40 μl/ml of semen) and stored at −20 °C till lipase activity was measured. The solution of protease inhibitors was prepared by dissolving a pellet of inhibitors (Complete™ protease inhibitor mix from Roche) in 2 ml distilled water. Samples of seminal plasma for the lipase purification procedure were freezed and lyophilized during 24 h. The dried material was then stored at −20 °C until used.

2.2. Lipase purification

The purification procedure was started by the solubilization of 2 g of seminal plasma powder in 20 ml distilled water, for 1 h under stirring at 4 °C. The solution was then centrifuged at 20,000 rpm, for 20 min at 4 °C. It was checked that all the lipase activity was found in the supernatant and the pellet was removed. The protein extract was then fractionated with 20% and 50% w/v ammonium sulfate ($AmSO_4$) precipitation. The major part of the lipase activity (around 90%) was found to precipitate with 50% w/v ammonium sulfate. After centrifugation at 10,000×*g* for 15 min at 4 °C, the protein pellet was dissolved in 10 ml distilled water under stirring for 1 h, at 4 °C. The solution obtained was dialyzed twice against water for 1 h and then against 10 mM MES buffer, 50 mM NaCl, pH 6.8, overnight. After centrifugation (5500×*g* for 30 min), the insoluble pellet was discarded and the clear supernatant was applied to a SP-sepharose gel column (1.2×7 cm, flow rate 1 ml/min) equilibrated with the same buffer. It was checked that all the lipase activity remained bound to the column upon loading. The column was then washed with 10 mM MES, 0.1 M NaCl, pH 6.8 buffer to release some bound proteins, and then, other proteins including the lipase were eluted using a linear NaCl concentration gradient ranging from 0.1 to 0.4 M NaCl in 60 min. Conductivity and absorbance at 280 nm were measured continuously at the column output and 2-ml fractions were collected. The absorbance at 280 nm and lipase activity were also measured on each fraction. The fractions containing lipase activity were pooled and concentrated with a Diaflo cell equipped with an Amicon PM 30 ultrafiltration membrane. The concentrated sample was dialyzed against 10 mM Tris buffer, 150 mM NaCl, pH 7.3, and then loaded onto a Heparin-sepharose CL-6B (Amersham-Pharmacia) column (1×10 cm, flow rate 1 ml/min, fraction volume 2 ml) equilibrated with the same buffer, at 4 °C. A stepwise elution procedure was then performed using 10 mM Tris buffer, pH 7.3, containing 250 and 500 mM NaCl. Bound proteins were eluted first with 250 mM NaCl and second with 500 mM NaCl in the same buffer. Lipase activity was found to be eluted during the first wash and fractions with the highest lipase activity were pooled and concentrated with a Diaflo cell equipped with an Amicon PM 30 ultrafiltration membrane. The concentrated sample was then loaded on a Hiload 16/60 Superdex 200 (Amersham-Pharmacia) column equilibrated with 10 mM Tris buffer, 150 mM NaCl, pH 7.3, at a flow rate of 0.5 ml/min and at 4 °C. Proteins were eluted at the