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Deterioration of Goat Sperm Viability in Milk Extenders Is Due to a Bulbourethral 60-Kilodalton Glycoprotein with Triglyceride Lipase Activity[1]

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ABSTRACT

The aim of this work was to purify, identify, and characterize the component of goat bulbourethral gland secretion (BUS) responsible for goat sperm deterioration in skim milk extender. BUS extracts promote a decrease in the percentage of motile spermatozoa, deterioration in the quality of movement, breakage of acrosomes, and cellular death of goat spermatozoa diluted in skim milk. A 55- to 60-kDa monomeric glycoprotein (BUSgp60) was purified by cation-exchange, concanavalin A, and heparin-affinity chromatography and was identified as the only BUS component responsible for the deterioration of spermatozoa in milk. The BUSgp60 was shown to display triacylglycerol hydrolase activity, and partial sequences (37 amino acids in all) exhibited 50–70% homology with sequences of various types of pancreatic lipases (PLs), especially PL-related protein 2 (PL-RP2). In addition, porcine PL produced deterioration of goat sperm properties in milk as effectively as BUS and BUSgp60. These results support the preliminary identification of the goat BUSgp60 as a bulbourethral lipase belonging to the PL-RP2 family. Since seminal plasma also contains factors favorable for sperm survival, it is envisaged that, for the best preservation of goat semen, specific inhibitors of this family of lipase could be added into milk-based extenders without eliminating seminal plasma.

INTRODUCTION

Seminal plasma is formed by the mixture of secretions from cauda epididymis and from the various male accessory glands at the time of ejaculation. In mammals, the more representative male accessory glands are the prostate, the seminal vesicles, and the bulbourethral glands (Cowper's glands), and their secretions constitute most of the volume and chemical composition of seminal plasma. The available information concerning the type, number, size, and topographic localization of accessory glands as well as the volume, chemical composition, and functions of their secretions reveals enormous variations among species [1].

Goat seminal plasma is deleterious for the survival of goat sperm chilled or frozen in media containing egg yolk [2] or milk [3]. Among the various secretions of accessory glands, bulbourethral gland secretion (BUS) was found to be the component of goat seminal plasma responsible for this effect. In contrast, remaining goat accessory gland secretions appear to be beneficial to preservation of sperm integrity [4–6].

Roy [7] has attributed the problems originating from BUS, encountered with the storage of goat semen using egg yolk, to a phospholipase A named egg yolk coagulating enzyme. This enzyme was shown to hydrolyze egg yolk phospholipids into lysophospholipids such as lysolecithins, which are toxic to spermatozoa [8]. It was also found that egg yolk coagulating enzyme induced the coagulation of egg yolk diluents and catalyzed the release of fatty acids from egg yolk lipids, leading to a decrease in pH [9]. More recently, a 40- to 60-kDa protein fraction from goat BUS was found to be responsible for deleterious effects on sperm viability during storage of goat semen in skim milk [4].

Egg yolk and skim milk in semen extenders exert a protective effect against cold shock upon chilling or freezing of spermatozoa [10, 11]. Thus, the storage of goat semen in the presence of egg yolk or milk, especially at high concentrations, requires that most of the seminal plasma be removed before sperm dilution in order to reduce the deleterious effects associated with BUS. The removal of the seminal plasma is carried out by dilution of ejaculated sperm in an isotonic buffer followed by centrifugation (washing method). Washing increases the storage duration of chilled goat sperm and also improves the survival of spermatozoa after freezing and thawing [2, 3, 7]. Nevertheless, this is a complex and time-consuming method that causes loss and damage to spermatozoa [12, 13].

In order to eliminate the harmful effects of BUS without losing the positive effects of the secretions from seminal vesicles and prostate on the survival of goat spermatozoa, an alternative method would be to specifically inhibit the activity of the sperm-deteriorating goat BUS component in seminal plasma. The aim of the present study was thus to purify, identify, and characterize this component from goat BUS as a first step in an effort to devise such a strategy.

MATERIALS AND METHODS

Unless otherwise noted, all chemicals were from Prolabo (Paris, France) and were of the highest available grade. Milk extender was composed of 10 g dehydrated cow skim milk (obtained at a local supermarket) and 0.2 g D-(+)-glucose in 100 ml of distilled water; it was heated at 92°C for 10 min and allowed to cool off at room temperature before the addition of sperm.

BUS and Spermatozoa

Reproductive tracts from Alpine and Saânen male goats were collected during breeding season at a local abattoir. The BUS were diluted 1:4 (v:v) with 10 mM Tris-HCl (pH 7.4) buffer containing 150 mM NaCl and 0.1% sodium deoxycholate (Janssen Chimica, Geel, Belgium). Samples were incubated for 3 h at room temperature and then were

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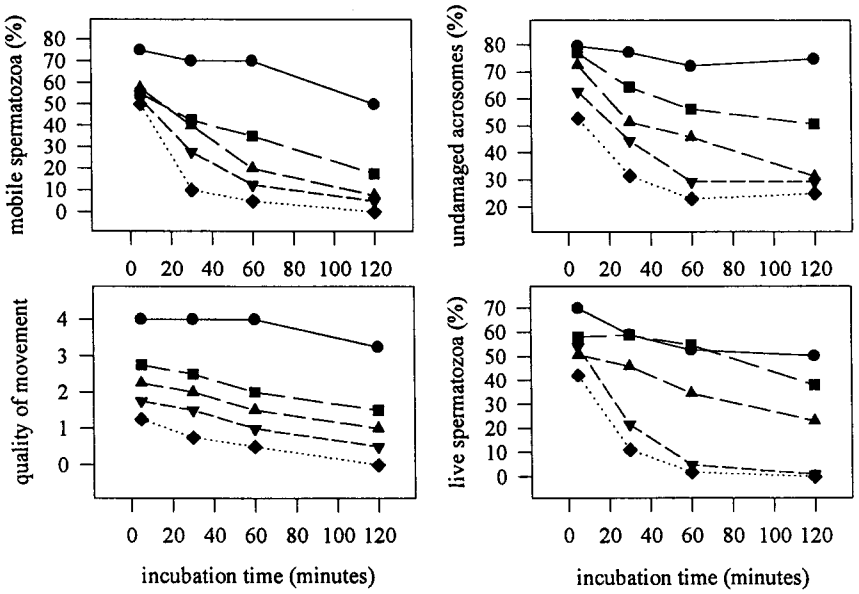


FIG. 1. Effects of BUS on the quality parameters of epididymal spermatozoa diluted in skim milk during 120 min at 37°C. Symbols indicate BUS dose: control 0.0 (circles), 6.2 μg/ml (squares), 12.5 μg/ml (triangles), 25.0 μg/ml (inverted triangles), 50.0 μg/ml (diamonds). Each value represent the mean of two replicates.

centrifuged at 10 000 × *g* for 30 min. The supernatant fluids were desalted by dialysis or gel-filtration in ammonium bicarbonate buffer, lyophilized, and stored at 4°C until use. Epididymal spermatozoa were expelled from cauda epididymis by retroflushing with paraffin oil and diluted to 10^8 spermatozoa/ml in milk extender at room temperature.

Analysis of Sperm Viability

The percentage of motile spermatozoa, the quality of movement estimated on a scale from 0.0 (all spermatozoa are immotile) to 5.0 (motile spermatozoa exhibit a fast progressive movement), the percentage of spermatozoa with an undamaged acrosome, and the percentage of live spermatozoa were estimated. These parameters were analyzed after incubating 1-ml sperm samples in a water bath at 37°C over 120 min. The sperm motility parameters (quality of movement and percentage of motile spermatozoa) were analyzed by observation under a coverslip on a warm stage at 37°C using a phase-contrast microscope. Acrosome morphology was observed in samples of sperm fixed in 2% glutaraldehyde-PBS solution examined under a phase-contrast microscope at ×64 magnification [14]. Sperm viability was assessed by eosin-nigrosin staining [15] under a bright-field microscope at ×100 magnification. For all these estimations, the assessor did not know the identity of the samples.

High-Performance Ion-Exchange Chromatography

BUS proteins (10–30 mg in 5 ml of 10 mM Tris buffer, pH 7.4) were separated by preparative ion-exchange HPLC (Waters 650 HPLC; Waters, Milford, MA) on an SP15HR column (Waters) over 45 min using a linear gradient from 0 to 0.4 M NaCl in Tris buffer at a flow rate of 1 ml/min. Immediately after recovery, the fractions were either stored frozen at −20°C or desalted on an AcA Ultrogel (Sepracor-IBF, Villeneuve-la-garenne, France) 202 column (2.5 × 15 cm) equilibrated with 100 mM ammonium bicarbonate buffer. Desalted samples were then lyophilized and stored at 4°C until use.

High-Performance Size-Exclusion Chromatography

Analytical size-exclusion HPLC was performed at a flow rate of 0.4 ml/min with approximately 10 μg of protein in 50 μl on a Superose 12HR 10/30 column (Pharmacia, Uppsala, Sweden) equilibrated with 200 mM ammonium bicarbonate buffer.

Concanavalin A (ConA)-Affinity Chromatography

Chromatography was carried out at room temperature with a ConA-sepharose (Pharmacia) column (1 × 3 cm) equilibrated with 20 mM sodium acetate buffer (pH 7.4) containing 500 mM NaCl, 1 mM $CaCl_2$, 1 mM $MnCl_2$, and 1 mM $MgCl_2$. BUS proteins (10 mg) dissolved in 5 ml of equilibration buffer were loaded onto the column, which was washed with the same buffer, to elute the unbound material. Bound proteins were eluted first with 100 mM methyl-α-D-glucopyranoside and then with 500 mM methyl-α-D-glucopyranoside.

Heparin-Affinity Chromatography

BUS proteins were chromatographed at room temperature on a heparin-sepharose CL-6B (Pharmacia) column (1 × 3 cm) equilibrated with 10 mM sodium phosphate buffer (pH 7.3) containing 150 mM NaCl. BUS proteins (7.5–10 mg in 5 ml of equilibration buffer) were loaded onto the column, which was washed with the same buffer. Then the bound proteins were eluted, first with 250 mM NaCl and second with 500 mM NaCl in the same buffer.

PAGE

Proteins were analyzed in reducing conditions by vertical discontinuous SDS-PAGE using 15% acrylamide separating gels according to standard methods [16].

Amino Acid Sequence Analyses

Amino acid sequence analyses of the N-terminal and an internal peptide of the BUSgp60 lipase were carried out by automatic Edman degradation using a 477A Protein Sequencer (Applied Biosystems, Foster City, CA) after puri-

fication of BUSgp60 by ion-exchange chromatography followed by vertical discontinuous SDS-PAGE under reducing conditions, electrotransfer to a polyvinylidiene difluoride membrane (Problott; ABI Analytical, Ramsey, NJ), and alkylation in situ with 4-vinylpyridine as previously described [17]. In order to obtain and purify internal peptides, the protein was incubated for 16–18 h at 37°C with Asp-N endoproteinase (Boehringer, Mannheim, Germany) at a molar enzyme:protein ratio of 1:60. The peptides were purified by reverse-phase chromatography on a C_{18} column at 40°C using an acetonitrile linear gradient in 0.1% trifluoroacetic acid.

Lipase Activity Analysis

Lipase activity determinations were performed using a commercially available quantitative titrimetric method (Sigma, St. Louis, MO) and using the type VI-S lipase (EC 3.1.1.3.) from porcine pancreas (Sigma; 34 000 U/mg protein) and the *Pseudomonas spp.* lipoprotein lipase (LpL) (EC 3.1.1.34.) (Merck, Rahway, NJ; 200 U per mg) as references.

Statistical Analysis

Data for comparative dose-dependent effects of BUS, porcine pancreatic lipase (pPL), and LpL on the sperm quality parameters were examined by one-way analysis of variance (multivariate test). Differences were considered to be statistically different at $p < 0.01$.

RESULTS

Purification of the Component from BUS Responsible for Sperm Deterioration in Milk Extender

Figure 1 shows that BUS in milk extender induces a dose- and time-dependent decrease in the percentage of motile spermatozoa, a deterioration of quality of movement, acrosome breakage, and cellular death of spermatozoa. Maximum decreases relative to control levels of each parameter were observed after 60 min with BUS protein concentrations higher than 25 μg/ml.

BUS was fractionated by ion-exchange chromatography with a SP15HR column (Fig. 2A). Fifteen percent by weight of total proteins were eluted in the SPII fraction, which was shown by SDS-PAGE (Fig. 2B) to be essentially composed of a protein with a molecular mass of 60 kDa. The SPII fraction was also analyzed by size-exclusion chromatography on a Superose 12HR column, either in native conditions immediately after its purification (Fig. 3A), after storage at 4°C for 24 h (Fig. 3B) or −20°C for 24 h (Fig. 3C), or after lyophilization (Fig. 3D). A major elution peak at the retention time of 34.2 ± 0.1 min corresponding to a molecular mass of 54.6 kDa was observed in samples A, B, and C (peak 3), representing, respectively, 82.3%, 77.2%, and 77.7% of the total profile area of each chromatogram. After lyophilization, this peak represented only 31.6% of total area, whereas peak 1 (20.8 ± 0.2 min), corresponding to proteins with molecular mass > 1000 kDa, was much higher (62.2% of the total elution area). These results together with those obtained from SDS-PAGE show that SPII contained a major monomeric protein that has an estimated molecular mass of 55–60 kDa under native conditions but undergoes aggregation upon lyophilization.

The dose-dependent effects of BUS and of the SPII fraction in milk extender on the four parameters of sperm quality were compared. The dose-response curves for BUS and

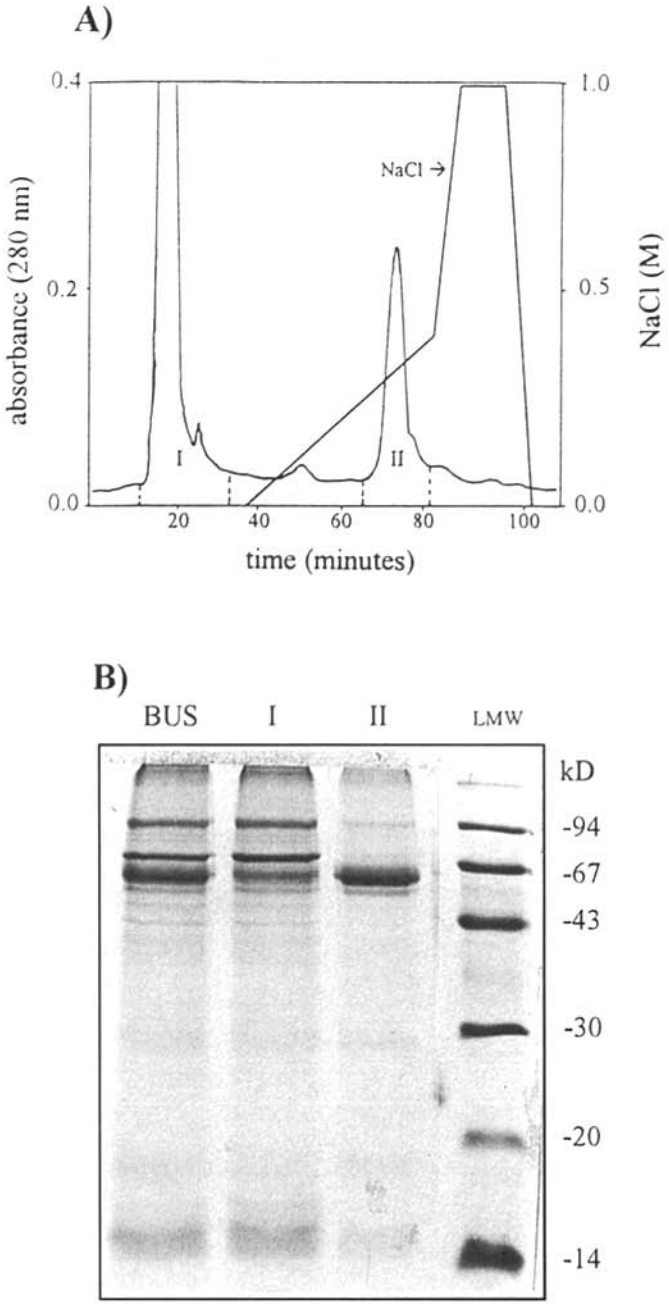


FIG. 2. **A**) SP15HR column chromatography of BUS (10 mg) in 10 mM Tris-HCl (pH 7.4) buffer at 1 ml/min. **B**) SDS-PAGE (15% acrylamide) of reduced samples of BUS and SPI and SPII fractions (20 μg/lane). LMW: molecular standards (×10 ^{3}).

SPII, normalized on the basis of their content of 60-kDa protein (Fig. 4), were superimposed with regression coefficients r_2 of 0.939, 0.944, 0.908, and 0.835, respectively, for the percentage of motile spermatozoa, the quality of movement, the percentage of spermatozoa with undamaged acrosome, and the percentage of live spermatozoa. Maximum deleterious effects on spermatozoa were observed with 6 μg/ml of the 60-kDa protein, which appears to be the only BUS protein responsible for sperm deterioration in skim milk extenders.

Chromatography of BUS on ConA-sepharose gave three peaks (Fig. 5A), which were analyzed by SDS-PAGE under reducing conditions (Fig. 5B). The major fraction of the 60-kDa protein identified above was eluted in peak II with

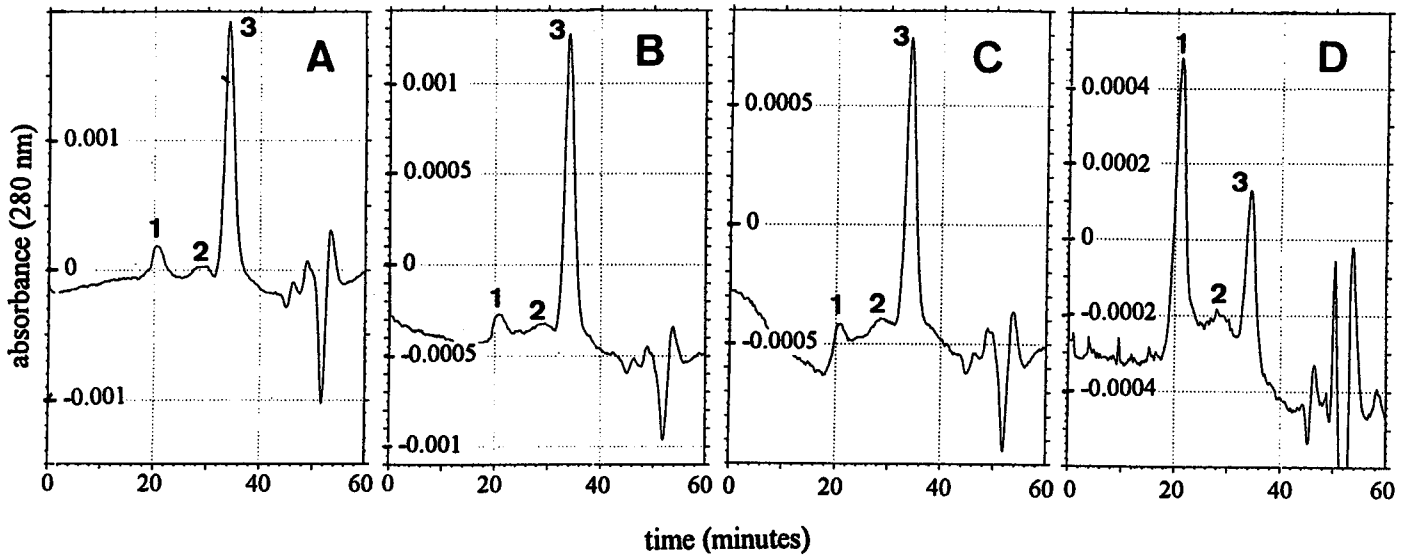


FIG. 3. Superose 12HR column chromatography with 200 mM ammonium bicarbonate at 0.4 ml/min of BUSgp60 (from SPII fraction) immediately after its purification (**A**), after storage at 4°C for 24 h (**B**) or at −20°C for 24 h (**C**), or after lyophilization (**D**).

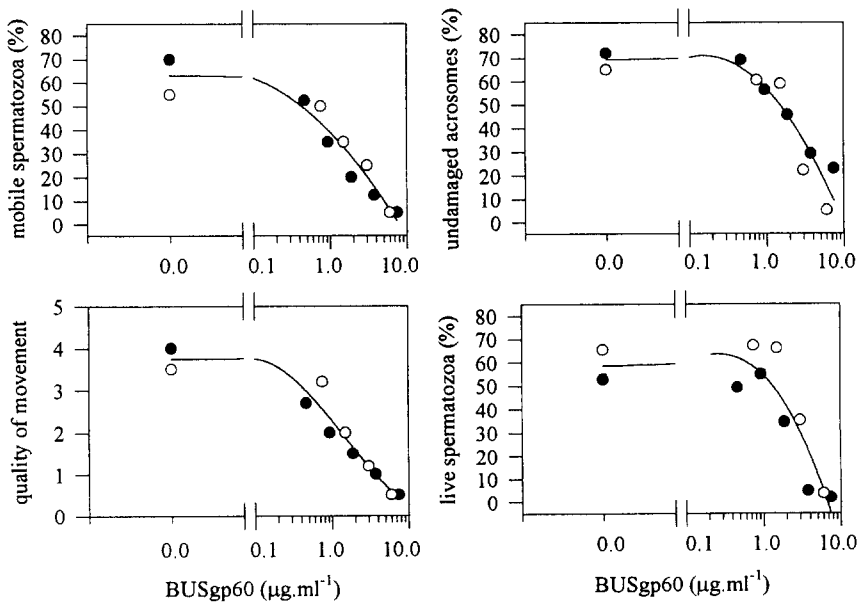
100 mM of methyl-D-glucopyranoside, indicating that it is a glycoprotein we call BUSgp60. Minor isoforms of this glycoprotein were eluted in peak III with 500 mM methyl-D-glucopyranoside. Finally, another minor fraction of the 60-kDa protein did not bind to ConA (peak I). SDS-PAGE analyses indicated that the major isoform of BUSgp60 can be obtained in a highly purified form in peak II of ConA-affinity chromatography. The BUS sperm-deteriorating activity in the presence of skim milk was identified in peaks II and III.

The heparin-affinity properties of BUS proteins were also examined by heparin-sepharose chromatography. BUS proteins were eluted in three peaks (Fig. 6A) that were analyzed by SDS-PAGE under reducing conditions (Fig. 6B). Major nonretained proteins in peak I exhibited apparent molecular masses of 94 and 68 kDa. Essentially, only one heparin-bound protein was eluted in peaks II and III with 250 mM NaCl and 500 mM NaCl, respectively. Its mobility in SDS-PAGE was identical to that of the major protein from SPII retained on ConA-sepharose. These results indicate that BUSgp60 exhibits strong affinity for heparin. Therefore, heparin-sepharose affinity chromatography is a very efficient method for isolating this protein.

Amino Acid Sequence Analysis of BUSgp60

The N-terminal sequence analysis of BUSgp60, purified from the SPII fraction by SDS-PAGE, revealed a unique sequence of 21 amino acid residues: Lys-Glu-Ile-Cys-Tyr-Glu-Pro-Phe-Gly-Cys-Phe-Ser-Asp-Glu-Lys-Pro-Trp-Thr-Gly-Thr-Leu. This sequence exhibited 61 ± 8% identity with the pancreatic lipase (PL) N-terminal sequence (Table 1). This homology increased to 79 ± 5% when amino acid functional analogies were considered. The sequence of an internal peptide of the 60-kDa glycoprotein (-Asp-Ser-Ala-Ser-Ile-Ile-Pro-Phe-Leu-Ser-Leu-Gly-Ile-X-Gln-Lys-Val-) exhibited 54 ± 8% amino acid identity and 78 ± 5% homology (including amino acid analogies) with PL sequenc-

FIG. 4. Comparative dose-dependent effects of BUS and its SPII fraction on the quality parameters of epididymal spermatozoa diluted in skim milk after incubating for 60 min at 37°C. Doses of SPII fraction (open circles) represent isolated BUSgp60. Doses of BUS (solid circles) are expressed as equivalent doses of BUSgp60 (0.15 mg BUSgp60/mg BUS as deduced from the percentage of total BUS protein eluted in SPII fraction). Each value represents the mean of two replicates.



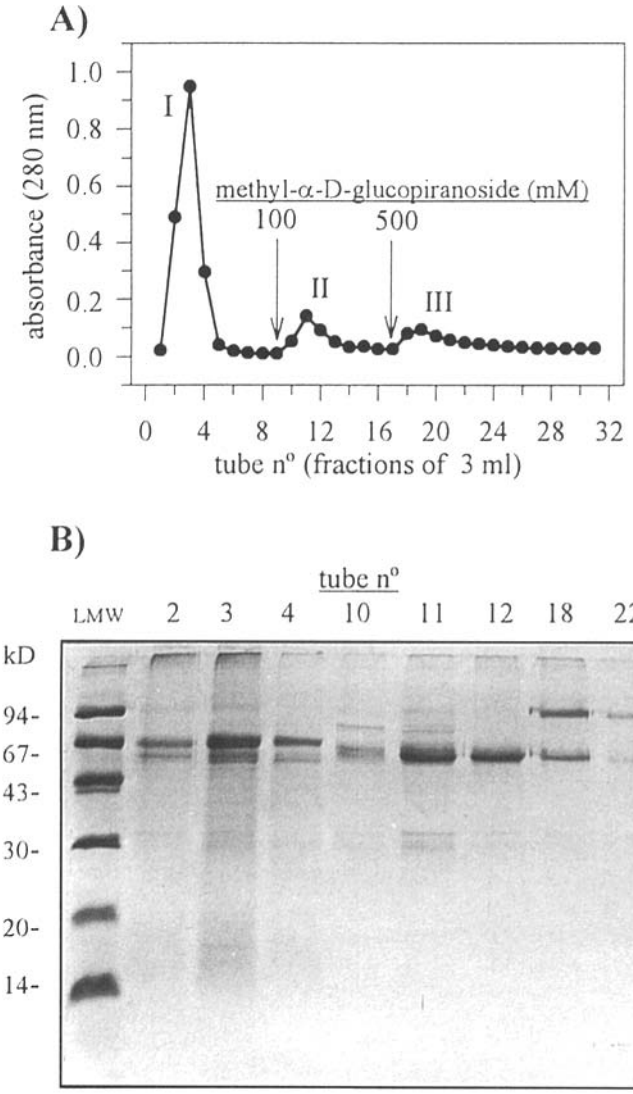


FIG. 5. **A**) ConA-sepharose column (3 ml) chromatography of BUS (10 mg) with acetate buffer (20 mM sodium acetate, pH 7.3, 500 mM NaCl, 1 mM $NaCl_2$, 1 mM $MgCl_2$ and 1 mM $MnCl_2$) at 6 ml/h. Peak I: unbound material. Peaks II and III: bound material eluted, respectively, with 100 and 500 mM methyl-α-D-glucopyranoside. **B**) SDS-PAGE (15%) of reduced samples of BUS fractions after ConA-sepharose chromatography. Peak I: tube 2 (20 μl), 3 (20 μl), and 4 (20 μl). Peak II: tube 10 (40 μl), 11 (40 μl), and 12 (40 μl). Peak III: tube 18 (40 μl) and 22 (40 μl). LMW: molecular standards ($\times 10^{-3}$).

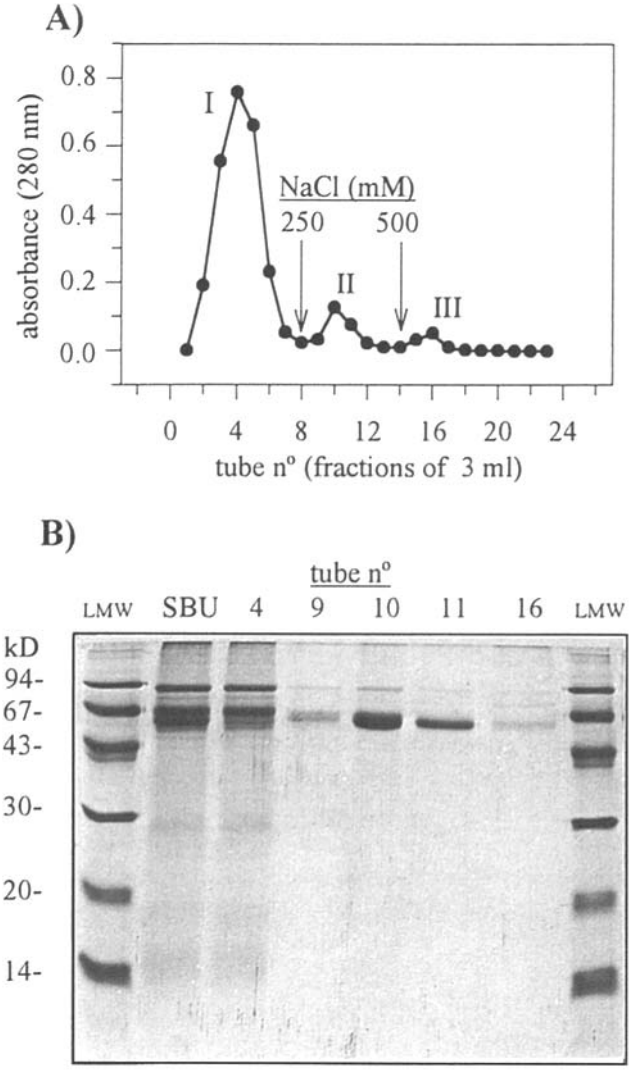


FIG. 6. **A**) Heparin-sepharose CL-6B column chromatography of BUS (10 mg) with phosphate buffer (10 mM phosphate, pH 7.3, and 150 mM NaCl) at 6 ml/h. Peak I: unbound material. Peak II and III: bound material eluted, respectively, with 250 and 500 mM NaCl. **B**) SDS-PAGE (15%) of reduced samples of BUS fractions after heparin-sepharose chromatography. Peak I: tube 4 (20 μl). Peak II: tube 9 (40 μl), tube 10 (40 μl), and tube 11 (40 μl). Peak III: tube 16 (40 μl). LMW: molecular standards ($\times 10^{-3}$).

es (Table 2). The highest sequence homologies of these two partial sequences (37 amino acids in all) from BUSgp60 were observed with PL-related protein 2 (PL-RP2; 64% identical amino acids vs. 52% and 59% with classical and PL-RP1 enzymes, respectively), supporting its identification as a novel lipase, most probably belonging to the PL-RP2 family.

Lipase Activity Analysis of BUSgp60

We examined the triacylglycerol hydrolase activity of BUSgp60 in order to confirm its identification as a lipase. Its enzymatic activity was compared to that of a commercial classic PL from porcine pancreas (pPL) and of a commercial LpL from *Pseudomonas spp.*, both exhibiting triacylglycerol hydrolase activity but showing specific differences in their primary structure and enzymatic properties [18, 19]. Figure 7 clearly shows that BUSgp60 exhibits

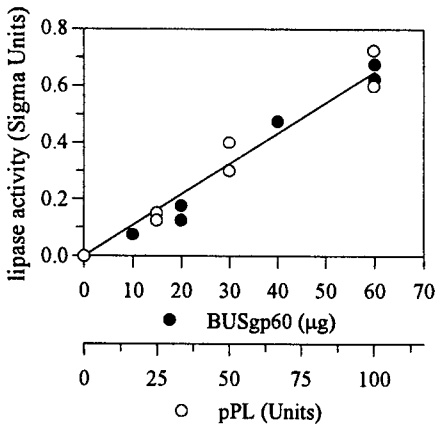


FIG. 7. Lipase activity of BUSgp60 (from desalted and lyophilized SPII fraction) and pPL, using standard olive oil emulsion as the substrate (6-h incubation at pH 8.0 and 37°C).

TABLE 1. Comparison of the N-terminal sequence (1–21 residues) of BUSgp60 with those of classic PLs, PL-RP1, and PL-RP2 from different species.*

Protein	Species	Ref.	Homology (%) Id	Id + An	Residue (BUSgp60 from goat) 1 K	2 E	3 I	4 C	5 Y	6 E	7 P	8 F	9 G	10 C	11 F	12 S	13 D	14 E	15 K	16 P	17 W	18 T	19 G	20 T	21 L
PL	Human	[22]	62	81			**V**				R	**L**						**D**	S			**S**		I	T
	Horse	[51]	57	76	N		**V**				R	**L**						**D**	S			A		I	**V**
	Pig	[22]	48	71	S		**V**		**F**	P	R	**L**						**D**	A			A		I	**V**
	Guinea pig	[25]	57	71	G		**V**		**F**	**D**	R	**L**						N				A			S
	Rat	[22]	57	90			**V**		**F**	**D**	K	**L**						**D**	A			**S**			**I**
	Rabbit	[21]	48	71	L		**V**				R	**L**				**G**	**N**	R	I			**S**		G	T
PL-RP1	Human	[22]	67	86			**V**				D	**L**						T	E			**G**			**A**
	Dog	[22]	67	81			**V**				Q	**I**						A	E			A			**A**
	Rat	[22]	62	81			**V**			**D**	N	**L**						A	E			A			**A**
PL-RP2	Human	[22]	76	86			**V**			G	Q	**L**										A			
	Mouse	[22]	57	81			**V**			G	H	**L**					**N**	**D**				A		M	**I**
	Guinea pig	[24]	67	76	A		**V**			S	H	**L**										A			S
	Coypu	[26]	71	81	N		**V**			S	H	**L**										A			
	Rat	[23]	62	76			**V**			G	H	**L**					**N**	**D**				A		M	

* Blank indicates identical amino acids; bold indicates residues with similar functions.

triacylglycerol hydrolase activity. In addition, skim milk added to standard olive oil emulsion enhanced the lipase activity of BUS and pPL as well as that of purified BUSgp60 but did not affect that of LpL (Table 3).

Comparative Effects of BUS and pPL on the Viability of Sperm Diluted in Milk

In order to complete the identification of BUSgp60, we measured the lipase activities of BUS, pPL, and LpL in the presence or absence of 1% dehydrated skim milk to compare their effects on sperm quality parameters after incubating at 37°C for 60 min using skim milk as diluent (Table 4). The presence of BUS or pPL in sperm samples produced a dose-dependent loss of motile spermatozoa and of spermatozoa with undamaged acrosome. A deterioration of quality of spermatozoa movement was also observed, and these effects were accompanied by cellular death. Moreover, when equivalent lipase doses of BUS and pPL were measured in the presence of milk, a highly significant correlation between lipase activity and the deteriorating effects on sperm quality parameters was observed. In contrast, LpL promoted only a very moderate dose-dependent deterioration in sperm motility at equivalent lipase doses. This confirms the identification of BUSgp60 as a PL-like lipase.

DISCUSSION

Isolation and Characterization of BUSgp60

In the present work we have identified a 55- to 60-kDa glycoprotein lipase from goat BUS (BUSgp60 lipase) as responsible for the deteriorating effects of seminal plasma on spermatozoa in milk-based extenders. In agreement with previous studies, the results presented here show that goat BUS promotes a decrease in the percentage of motile spermatozoa, a deterioration in the quality of sperm movement, breakage of acrosomes, and cellular death of goat epidid-

TABLE 2. Comparison of an internal BUSgp60 peptide sequence with the corresponding fragments of classic PL, PL-RP1, and PL-RP2 from different species.*

Protein fragment	Species	Ref.	Homology (%) Id	Id + An	Residue (BUSgp60 from goat) D	S	A	S	I	I	P	F	L	S	L	G	I	X	Q	K	V
PL																					
(206–222)	Human	[22]	50	81		**G**		P		**V**		N		**G**	**F**		**M**	S		V	
(206–222)	Horse	[51]	44	75		I		P	**F**			N		**G**	**F**		**M**	S		T	**A**
(205–221)	Pig	[22]	56	75		A		P				N		**G**	**F**		**M**	S		T	
(206–222)	Guinea pig	[25]	50	75		**G**	N	P				N		**G**	**F**		**M**	S		T	
(206–222)	Rat	[22]	56	75		A		P				N		**G**	**F**		**M**	S		T	
(208–224)	Rabbit	[21]	44	75		A		P	**M**	**V**		N		**G**	**F**		**M**	S		T	
PL-RP1																					
(206–222)	Human	[22]	56	75		A		P	**L**					**G**	**F**		T	N		Q	**M**
(206–222)	Dog	[22]	50	75		A		P	**L**					**G**	**F**		T	S		Q	**M**
(206–222)	Rat	[22]	50	75		A		P	**L**					**G**	**F**		T	N		M	S
PL-RP2																					
(207–223)	Human	[22]	56	81			S	P		**V**		S		**G**	**F**		**M**	S			
(207–223)	Mouse	[22]	69	88				P				**Y**		**G**	**F**		**M**	S			
(207–223)	Guinea pig	[24]	56	81		I	S	P		**L**		S		**G**	**F**		**M**	S			
(207–223)	Coypu	[26]	56	81		I		P				S	**F**	**G**	**F**		**M**	S			
(208–224)	Rat	[23]	69	88				P				**Y**		**G**	**F**		**M**	S			

* Blank indicates identical residues; bold indicates residues with similar functions.

TABLE 3. Effect of milk on the lipase activity of BUS, pPL, and LpL determined after 20-h incubation at pH 8.0 and 37°C using olive oil emulsion as the substrate in the presence of increasing concentrations of dehydrated skim milk.

Enzyme	Dehydrated skim milk*							
	0% Control		0.01%		0.1%		1%	
BUS (20 μg)	0.544 ± 0.028[a]	(1.0)	1.100 ± 0.044[b]	(2.0)	1.550 ± 0.016[c]	(2.8)	1.481 ± 0.027[c]	(2.7)
pPL (25 U)	0.362 ± 0.021[a]	(1.0)	0.994 ± 0.037[b]	(2.7)	2.256 ± 0.010[c]	(6.2)	1.775 ± 0.022[d]	(4.9)
LpL (12.5 mU)	0.481 ± 0.049[a]	(1.0)	0.537 ± 0.051[a]	(1.1)	0.487 ± 0.073[a]	(1.0)	0.387 ± 0.058[a]	(0.8)

* In parentheses: lipase activity relative to the corresponding control.
[a,b,c,d] Values in the same row with different superscripts differ significantly ($p < 0.01$); each value represents mean ± SEM of 4 replicates.

ymal spermatozoa diluted in skim milk and incubated at 37°C.

This sperm-deteriorating activity of BUS was closely associated with the presence of BUSgp60. This lipase can be easily purified from BUS either by cation-exchange or heparin-sepharose chromatography. In addition, its behavior in ConA-affinity chromatography suggests the existence of several isoforms with different carbohydrate structures. The major BUSgp60 isoform can be readily isolated from other BUS proteins by one-step ConA-affinity chromatography. Alternatively, BUSgp60 can be purified first by cation-exchange or heparin-sepharose chromatography of BUS and then submitted to affinity chromatography on ConA-sepharose in order to isolate the different BUSgp60 isoforms: 1) the major BUSgp60 isoform, eluted with 100 mM methyl-D-glucopyranoside—suggesting that its carbohydrate chains are of the bi-antennary type; 2) a BUSgp60 population with higher ConA affinity, eluted only with 500 mM methyl-D-glucopyranoside, that probably bears carbohydrates with high-mannose structures and/or hybrid structures and/or bi-antennary structures deficient in galactose and/or *N*-acetylglucosamine; and 3) a minor fraction of BUSgp60 that does not bind to ConA and thus either bears tri- and tetra-antennary structures or is totally devoid of carbohydrate chain. This latter hypothesis is unlikely, as the molecular mass of this fraction was not smaller than those of the other isoforms in SDS-PAGE.

BUSgp60 exhibited triacylglycerol hydrolase activity, and accordingly its N-terminal sequence (21 amino acid residues) and the sequence of an internal peptide (17 amino acid residues) showed a significant homology (50–70% of identical amino acids) with various PLs: classical PL-RP1 and PL-RP2 from different species. In addition, pPL, but not another lipase (LpL from *Pseudomonas spp.*), promoted deterioration of goat sperm quality in a way similar to that of BUSgp60 with the use of skim milk as sperm extender. Moreover, BUSgp60 exhibited strong homologies with the PLs with respect to its molecular mass [20–26], glycoprotein nature [20, 23, 27–30], and heparin affinity [31], thus supporting its classification in the PL family.

The amino acid sequences of PLs are used to classify them into various groups. The percentage of amino acid identities within each group was on the order of 70–80% and was close to 55% between the groups [26]. The partial sequence of BUSgp60 (37 amino acids in all) exhibited the highest homology with PL-RP2 molecules from different species (64% identity), supporting its preliminary identification as a novel PL-RP2. Moreover, PL-RP1 members show no activity toward triolein [22, 27], in contrast to BUSgp60, indicating that BUSgp60 probably does not be-

TABLE 4. Correlation between the lipase activities of BUS, pPL, and LpL and their effects on goat sperm quality parameters in the presence and absence of 1% milk.*

Enzyme doses (Sigma Units)		Motile spermatozoa (%)	Quality of movement	Undamaged acrosomes (%)	Live spermatozoa (%)
1% Milk					
Control	0.0	78.7 ± 1.2[a]	3.8 ± 0.1[a]	89.8 ± 1.8[a]	88.0 ± 0.7[a]
BUS	0.7	52.5 ± 1.4[bc]	2.0 ± 0.1[cd]	79.3 ± 3.1[a]	82.2 ± 2.3[a]
	2.5	30.0 ± 2.0[d]	1.2 ± 0.1[ef]	42.7 ± 8.8[b]	60.8 ± 1.1[b]
	6.0	18.7 ± 4.2[d]	0.7 ± 0.2[f]	14.2 ± 2.1[c]	5.3 ± 1.4[c]
pPL	0.7	47.5 ± 2.5[c]	1.8 ± 0.1[de]	81.1 ± 1.3[a]	83.6 ± 4.9[a]
	2.5	22.5 ± 3.2[d]	1.0 ± 0.1[f]	37.8 ± 6.4[b]	51.6 ± 4.8[b]
	6.0	15.0 ± 2.0[d]	0.7 ± 0.1[f]	7.0 ± 3.8[c]	11.6 ± 1.9[c]
LpL	0.7	75.0 ± 2.8[a]	3.1 ± 0.1[b]	92.6 ± 2.1[a]	85.3 ± 0.4[a]
	2.5	66.2 ± 3.7[ab]	2.6 ± 0.1[bc]	92.6 ± 0.5[a]	88.1 ± 1.3[a]
	6.0	51.2 ± 3.1[bc]	1.8 ± 0.1[de]	76.8 ± 4.8[a]	84.0 ± 2.8[a]
No Milk					
Control	0.0	77.5 ± 1.4[a]	4.1 ± 0.1[a]	94.6 ± 0.3[a]	76.7 ± 1.0[a]
BUS	0.3	25.0 ± 5.4[bc]	1.3 ± 0.2[b]	64.0 ± 2.7[bc]	72.1 ± 2.6[a]
	1.2	32.5 ± 4.3[b]	1.5 ± 0.1[b]	55.5 ± 9.2[c]	66.8 ± 4.9[a]
	4.8	20.0 ± 5.4[bc]	0.8 ± 0.2[b]	26.2 ± 2.7[d]	23.0 ± 7.1[b]
pPL	0.3	22.5 ± 4.3[bc]	1.1 ± 0.1[b]	79.7 ± 4.2[ab]	77.6 ± 3.5[a]
	1.2	27.5 ± 3.2[bc]	1.1 ± 0.1[b]	8.0 ± 2.5[ed]	17.6 ± 2.8[b]
	4.8	7.5 ± 2.5[c]	0.5 ± 0.1[b]	3.1 ± 1.1[e]	5.7 ± 1.7[b]
LpL	0.3	70.0 ± 2.0[a]	3.8 ± 0.1[a]	95.1 ± 0.5[a]	80.6 ± 0.5[a]
	1.2	62.5 ± 3.2[a]	3.2 ± 0.3[a]	95.3 ± 1.4[a]	84.3 ± 2.0[a]
	4.8	32.5 ± 7.2[b]	1.2 ± 0.2[b]	84.3 ± 3.3[ab]	83.0 ± 1.1[a]

* Lipase activity of each enzyme was measured in 20-h incubations at pH 8.0 and 37°C using an olive oil emulsion as the substrate in the absence (bottom) or presence (top) of 1% dehydrated skim milk; sperm quality parameters were estimated in 60-min incubations at 37°C (means ± SEM of 4 replicates).
[a–i] Values in the same column of each experiment with different superscripts differ significantly ($p < 0.01$).

long to this subfamily. Members of the classical PL and PL-RP2 groups exhibit marked differences in their enzymatic properties regarding 1) interfacial activation, 2) bile salt and colipase effects, and 3) ability to display phospholipase A [24, 26, 32] and galactolipase [33] activities. Therefore, the study of these properties for BUSgp60 will permit its classification more precisely either as a genuine member of the PL-RP2 subfamily or as a new type of lipase.

Mechanism of the Sperm-Deteriorating Effect of BUSgp60

In this work we found a highly significant agreement between the lipase activities of BUS and pPL and their deteriorating effects on sperm quality. The latter represents a classical PL, exhibiting only lipase activity without any phospholipase or galactolipase activity. This suggests that only the lipase activity of BUSgp60 is involved in sperm deterioration. Phospholipids represent the major components of goat mature cauda sperm membranes (70%), followed by neutral lipids (28%) and glycolipids (2%). Sterols represent as much as 80% of neutral lipids whereas triglycerides constitute only a mere 1% of neutral lipids [34]. Therefore, it cannot be totally ruled out that the deleterious effect of BUSgp60 on spermatozoa occurs through the hydrolysis of cell membrane triglycerides, but this is very unlikely.

Moreover, the presence of milk together with BUS is indispensable for inducing the deterioration of motility and acrosome integrity of epididymal spermatozoa [4, 6]. Nunes [4] suggested that either an enzyme from BUS would act on a milk substrate to give a toxic product or that an enzyme from milk would act on a BUS component to give such a product. BUSgp60 lipase activity is strongly increased by milk. This suggests that it could hydrolyze residual triglycerides (approximately 1 mg/ml) in skim milk and produce fatty acids exhibiting toxic effects toward spermatozoa. For example, lysolecithin exerts such an action because of its detergent-like effects [8]. We have found that oleic acid released from triolein by BUSgp60 exerted a strong deteriorating effect on goat spermatozoa (unpublished results).

Washing of goat semen by centrifugation improves sperm viability during storage in media containing skim milk. The involvement of BUSgp60 in the deleterious effects of skim milk on the survival of spermatozoa, and its tentative identification as a PL-RP2, open the possibility of inhibiting its activity with highly specific inhibitors. This would avoid the harmful step of washing goat sperm and would retain the factors from seminal plasma exerting favorable effects on sperm motility and survival. Alternatively, it would be possible to keep seminal plasma and avoid the deteriorating effects of BUSgp60 on sperm functions by using milk devoid of substrates that give rise to toxic products for the constitution of goat sperm extenders.

Physiological Role(s) of BUSgp60

Lipases of the PL-RP2 subfamily exhibit dual lipase and phospholipase A activities [24, 26, 32]. If BUSgp60 does indeed belong to this subfamily, it could also display phospholipase activity and thus could take part in the acrosome reaction of spermatozoa, promoting sperm membrane fusion as described for seminal phospholipases A2 from various species [35–37]. Indeed, these enzymes could hydrolyze triglycerides and/or phosphoglycerides from the seminal plasma or the female reproductive tract fluids or in sperm membranes. Thus they could release fusogene lipids such as *cis*-unsaturated fatty acids and lysophospholipids and/or sperm phospholipase A2 activators such as diglycerides [38–40]. Moreover, BUSgp60 exhibited heparin affinity like many other seminal plasma proteins that have been shown to attach to the sperm surface and that play a role in the acrosome reaction or serve as receptors for zona pellucida recognition [41–43].

The possibility that BUSgp60 is involved in sperm energy metabolism cannot be excluded. Lipases and phospholipases A2 have a significant role in the energy metabolism of sea urchin spermatozoa following spawning in sea water [44]. In mammals, sperm phospholipases also are involved in producing the energy substrates for spermatozoa during maturation in the epididymis [45, 46]. After ejaculation, fructose (secreted by goat accessory glands) represents the major glycolytic substrate for spermatozoa in seminal plasma [1, 47]. However, it is rapidly diluted in the female reproductive tract, where triglycerides or phospholipid-derived fatty acids could be the main sources of metabolic energy of ejaculated spermatozoa.

In all species, the enzymes of the PL family are expressed only in the pancreas except in the case of PL-RP2 members, which are also expressed in mouse cytotoxic T lymphocytes and in mouse testis [23, 48]. The biological significance of the expression of a PL and especially of a PL-RP2 lipase in the goat bulbourethral glands is unknown. Guinea pig and coypu pancreas produce no specific phospholipase A2, suggesting that PL-RP2 lipases could mainly act as a real phospholipase under physiological conditions in these species [26]. Specific phospholipases A2 have been reported in the seminal plasma and/or accessory secretions from the human, bull, ram, and boar, whereas lipase activity has not been detected in these species [49, 50]. Phospholipase A activity originating from BUS in the seminal plasma has also been reported with egg yolk used as substrate and measurement of the amount of fatty acids released [9]. However, egg yolk contains both phospholipids and triglycerides. Inspection of the reported experimental conditions for phospholipase activity measurement reveals that the liberation of fatty acids from egg yolk triglycerides by a lipase cannot be excluded. The present study demonstrates the presence of a lipase related to the PL-RP2 subfamily in BUS that could display phospholipase activity. Since the question remains as to whether goat seminal plasma possesses a specific phospholipase A2, it could be that secretion of the BUSgp60 lipase constitutes an alternative way to express phospholipase A activity in goat semen.

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