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Bovine seminal plasma aSFP: localization of disulfide bridges and detection of three different isoelectric forms

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Abstract

Acidic seminal fluid protein (aSFP) is a major 13 kDa protein isolated from bull seminal plasma and characterized as a new growth factor which stimulates in vitro cell division and progesterone secretion by ovarian cells. Here, we establish that the four cysteines of oxidized aSFP form two disulfide bridges between nearest-neighbour residues. This pattern is conserved in boar spermadhesins, with which aSFP shares up to 50% amino acid sequence identity, and other proteins of the recently identified CUB domain family. Using isoelectric focusing in combination with sulfhydryl group-specific blotting, the three forms of aSFP were identified as completely oxidized (pI 4.7), partly reduced (pI 4.8) and fully reduced at pI 5.1. These results indicate that native aSFP possesses two pairs of cysteine residues of different reactivity. The observation that aSFP can protect sperm from oxidative damage might be explained by its reduction/oxidation behaviour.

Key words: Bovine seminal plasma; aSFP; Isoelectric focusing; Disulfide bridges; CUB domain

1. Introduction

Mammalian seminal plasma proteins interact with spermatozoa and exert multiple effects on sperm function [1]. Though many different active components have been described as enzymes, hormones and growth factors, the structure and the role of most of these seminal plasma proteins on sperm physiology are not known [2]. The recently discovered acidic seminal fluid protein (aSFP) is a major component of bovine seminal plasma. It is encoded by a single gene locus [3]. This 13 kDa protein is mainly synthesized by the ampulla and seminal vesicle epithelium of the bull in high concentrations (1–7 mg/ml) and has not been detected in any other bovine tissues nor in other mammalian species, such as goat, sheep, pig, rat, dog, or human [4,5].

The amino acid sequence of aSFP has been deduced by cDNA cloning [3]. It comprises 114 residues and shows up to 50% similarity with boar spermadhesins [6–10]. Unlike the porcine proteins, bovine aSFP does not tightly bind to the surface of spermatozoa [5], and therefore may not play a role in sperm–egg interaction. Interestingly, aSFP has been characterized as a new growth factor which stimulates the in vitro division of lymphocytes and progesterone secretion by ovarian granulosa cells [11]. In addition, this bovine protein, at its physiological concentration, seems to protect spermatozoa from oxidative damage by diminishing lipid peroxidation [12].

The molecular basis of the different functional properties of bovine aSFP has not been established. In this study we have investigated the number, location and reactivity of sulfhydryl residues and disulfide bridges of purified aSFP, and characterized three different isoforms. It is expected that these data are helpful towards a better understanding of structure–function relationship of bovine aSFP.

2. Materials and methods

aSFP was purified from bovine seminal fluid as described [11] with the following modification: after ion-exchange chromatography on a Mono Q column, purified aSFP was further subjected to gel filtration on a Superdex 75 column (Pharmacia, Uppsala, Sweden).

Protein concentration was determined using the bicinchoninic acid assay [13].

Isoelectric focusing in immobilized pH-gradients (IPG-IEF) in the pH-range 4.5–5.5 was carried out on a PHAST-system (Pharmacia). Gels (50 × 45 mm) were cut out from Immobiline DryPlate (Pharmacia) and rehydrated in 1.8 ml 8 M urea, 20% glycerol, 0.2% CHAPS and 0.5% Ampholine pH 4.0–6.5 for 2 h. For reduction/oxidation experiments 50 mM of either glutathione (GSSG), reduced glutathione (GSH), cysteine or DTT were included in the rehydration solution. Samples (1 μl containing 120 ng protein) were applied near the cathode

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and separated for 15 min at 150 V, 0.5 mA, 2 W and 15°C, followed by 90 min at 2,000 V, 0.5 mA, 2 W. Proteins were stained according to [14].

For two-dimensional electrophoresis under non-reducing conditions, 2 mm strips cut from the unstained gels after electrofocusing of aSFP, were equilibrated for 15 min in 50 mM Tris-HCl, pH 6.8, 6 M urea, 30% glycerol. To maintain aSFP in the completely reduced form, gel strips were equilibrated with the same buffer containing 1% DTT and thiogroups subsequently alkylated with 260 mM iodoacetamide for 15 min [15]. After transfer to a PhastGel High Density SDS-gel (Pharmacia) electrophoresis was carried out for 1 h according the manufacturer's protocol.

Free sulfhydryl groups on electroblotted protein were detected using the digoxigenin-3-*O*-succinyl-(2-(*N*-maleimido))-ethyl-ami de/anti-digoxigenin-alkaline peroxidase system (Boehringer, Mannheim, Germany).

For quantitation of free cysteine residues and disulfide bridges in aSFP, the protein (2 mg/ml in 100 mM Tris-HCl, pH 8.6, 1 mM EDTA, 6 M guanidine-HCl) was incubated with either 10 mM iodoacetamide for 1 h at room temperature, or with 1% 2-mercaptoethanol for 2 min at 100°C, followed by addition of a 2.5 M excess of iodoacetamide over reducing agent for 1 h at room temperature. Then both samples were dialysed, lyophilized, hydrolysed with 6 N HCl at 110°C for 24 h and subjected to amino acid analysis using an Alpha Plus amino acid analyzer (Pharmacia).

For peptide mapping and localization of disulfide bridges, aSFP (2 mg/ml in 100 mM $NaHCO_3$ pH 8.0) was digested overnight at 37°C with TPCK-trypsin (sequencing grade, Boehringer) at an enzyme:substrate ratio of 1:100. The reaction mixture was lyophilized and peptides isolated by reversed-phase HPLC on a Lichrospher RP-100 column (C18, 25 × 0.4 cm, 5 μm size, Merck) eluting at 1 ml/ml with 15% acetonitrile in 0.1% TFA for 5 min, followed by a 15–50% gradient of acetonitrile within 55 min and 50–70% for 50 min. Peptides were detected at 220 nm and analyzed by amino acid analysis as described.

3. Results and discussion

3.1. Location of disulfide bonds in aSFP

The amino acid sequence of bovine aSFP contains four cysteine residues [3]. When aSFP isolated under non-reducing conditions was treated with iodoacetamide under denaturing conditions obviously no free cysteine residues were detected, whereas after reduction and alkylation 4 mol carboxymethylcysteine per mol protein were found in the acid hydrolysates. These results clearly indicate that isolated aSFP contains two intra or intermolecular disulfide bridges. The disulfide bonding pattern was established following tryptic digestion of the native protein (Fig. 1) and analysis of the purified peptides (Table 1). Two peptides corresponding to fragment ^{7}NTNCGGILK^{15} linked to ^{29}TNCVWTIQM-PPEYHVR^{44} and ^{45}VSIQ...MDYR^{83}, none of them containing free cysteine residues, were characterized (Fig. 1B). This indicates that oxidized fragments of aSFP contain two disulfide-bridged peptides and one having an intrachain disulfide bound, respectively. Therefore, we conclude that aSFP isolated under oxidizing conditions contains two intramolecular disulfide bonds between Cys^{10}–Cys^{31} and Cys^{54}–Cys^{75}.

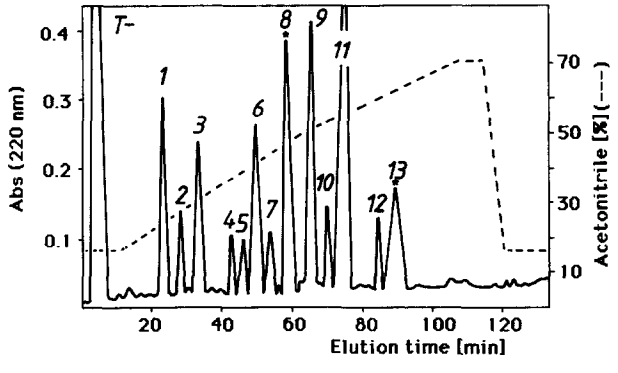

Fig. 1. Separation of tryptic fragments of aSFP by reversed-phase HPLC. Position of bridged peptides is indicated (*). Structural characterization of isolated peptides is shown in Table 1.

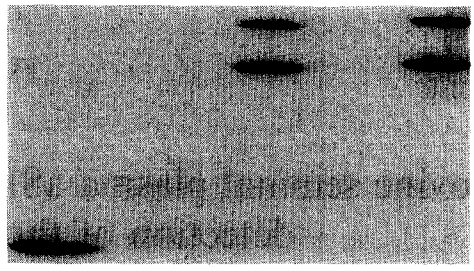

Fig. 2. Isoelectric focusing (pH range 4.5–5.5) of pure aSFP in gels containing 8 M urea, 20% glycerol, 0.2% CHAPS, 0.5% Ampholine, pH 4.0–6.5 (A), and in the same buffer but containing 50 mM of either GSH (B) or cysteine (C). Protein was stained with Coomassie brilliant blue.

3.2. Isoelectric forms of aSFP and different reactivity of its disulfide bonds

Isoelectric focusing of aSFP yielded two bands with isoelectric points at pH 4.7 and 4.8 (Fig. 2A). After treatment with reduced glutathione (GSH) or cysteine, the protein focused at a single band of pI 5.1 (Fig. 2B,C). The same result was obtained when dithiothreitol (DTT) was employed for reduction (data not shown). Following electroblotting, free sulfhydryl groups were only detected at pI 4.8 and 5.1 (Fig. 3). Thus, under the chosen experimental conditions isolated aSFP represents a mixture of fully oxidized (pI 4.7) and partly reduced (pI 4.8)

Table 1
Characterization of the tryptic peptides of aSFP by amino acid analysis.

Fragment	Corresponding aSFP-sequence	
T-1	YGPK	25–28
T-2	SGSIMTVK	85–92
T-3	FQDPQA	109–114
T-4	SSGSIMTVK	84–92
T-5	TIQM...YHVR	34–44
T-6	EVLY	105–108
T-7	EESGVIATY	16–24
T-8*	NTNCGGILK	7–15
	TNCV...YHVR	29–44
T-9	YIRE...ASFY	93–104
T-10	EVLY...DPQA	105–114
T-11	MDWLPR	1–6
T-12	ESLE...VLGK	57–73
T-13*	VSI...NCN...ICE...DYR	45–83

*Indicated peptides did not contain free sulphydryl groups.

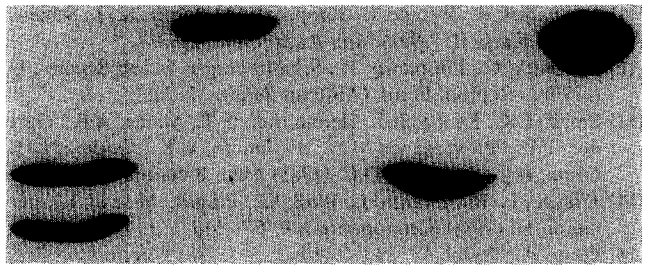

Fig. 3. Isoelectric focussing (pH 4.5–5.5) of non-reduced (−) or completely reduced (+) aSFP. A: Coomassie brilliant blue-stained gel. B: Detection of free sulfhydryl groups after blotting of focussed aSFP as in (A).

isoforms. The completely reduced protein appeared as a single band at pI 5.1. Additionally it was observed that reduced aSFP is readily oxidized to the pI 4.8 and pI 4.7 isoforms upon prolonged standing even in the presence of an excess of DTT. This observation might be an indication for the antioxidative potential of reduced or partly oxidized aSFP. As aSFP is synthesized in the nearly anaerobic environment of bovine seminal vesicles and ampulla it might be expected that its active form could be the totally reduced (pI 5.1) or partly oxidized one (pI 4.8). After isolation under oxidizing conditions, aSFP appears in the completely oxidized (pI 4.7) and partly oxidized (pI 4.8) form.

Nevertheless, from this reduction/oxidation behaviour it may be concluded that the four cysteine residues of aSFP possess an unequal tendency to oxidation. To establish which pair of cysteines is easily oxidized, leading to the pI 4.8 isoform, needs further investigation.

According to the heterogeneity found by isoelectric focusing, two dimensional electrophoresis under non-reducing conditions revealed that both isoforms at pI 4.7 and pI 4.8 have a tendency to form dimers at 26 kDa via intermolecular disulfide exchange (Fig. 4A). As expected, a single 13 kDa band was obtained for the fully reduced (pI 5.1) aSFP molecule (Fig. 4B).

3.3. An emerging model for aSFP

Recently, a structure prediction approach, based on a search for conserved amino acid sequence features, has

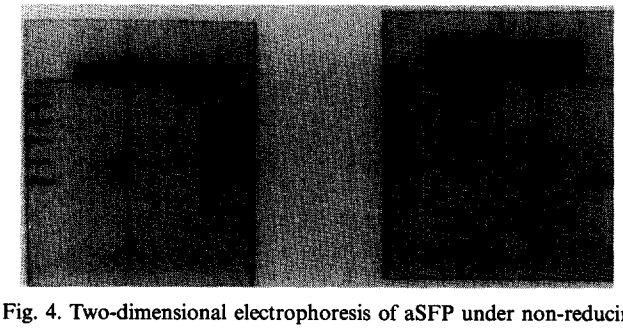

Fig. 4. Two-dimensional electrophoresis of aSFP under non-reducing (A) and reducing (B) conditions in both dimensions. First dimension: isolelectric focusing as in Fig. 1A. Second dimension: SDS-PAGE 16%. Proteins were stained with Coomassie brilliant blue.

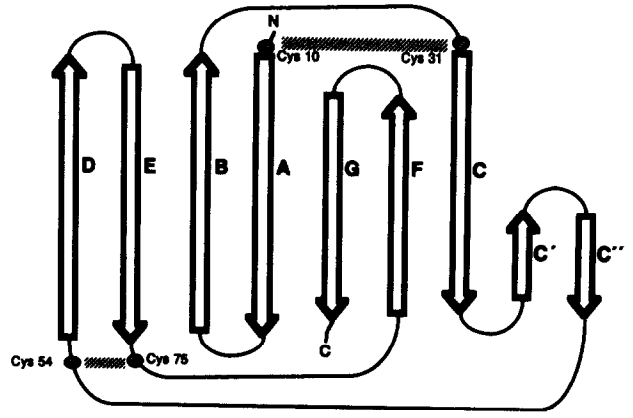

Fig. 5. Schematic representation of the proposed topology of aSFP showing the position of its two disulfide bridges. The N- and C-terminal residues are indicated.

revealed that bovine aSFP may belong to a large family of developmentally regulated proteins sharing the CUB domain [16]. The predicted topology for this 110 residues spanning structural module is an antiparallel beta-barrel similar to that found in immunoglobulins. The bonding pattern between nearest-neighbour cysteine residues found in aSFP is conserved in other members of the CUB family, including isolated boar spermadhesins AQN-1 [7], AQN-3 [6] and AWN [8], and the complement subcomponents C1s and C1r [17]. The disulfide bonds in isolated aSFP between Cys^{10}–Cys^{31} and Cys^{54}–Cys^{75} are linking the N-terminal parts of strands 1 and 3, and the N-terminal part of strand 6 with the end of strand 7 (see Fig. 5), fitting well with the hypothetical structure predicted for aSFP.

Finally, the results indicate that, under the experimental conditions chosen in this study, purified aSFP is able to exist in isoforms with different reduction and aggregation status. Whether these properties of aSFP are existing in vivo or are correlating with the observed ability of the protein to prevent lipid peroxidation awaits further research. Work is in progress to assess this possibility and to test the current structural model of aSFP.

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