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SHORT REPORT

The structure of platelet-activating factor acetylhydrolase (PAF-AH) isolated from boar seminal plasma and examined using mass spectrometry*

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Platelet-activating factor – acetylhydrolase (PAF-AH) – of boar seminal plasma is a heterogeneous protein consisting of four polypeptides with molecular weights of 43, 55, 65 and 100 kDa. In 2004 the authors of the current report demonstrated that the N-terminal amino acid sequence of 43 kDa polypeptide is homologous with the amino acid sequences of the IgG-binding proteins and *zona pellucida*-binding adhesion proteins. In the current report, due to the strongly blocked N-terminal amino groups of 55, 65 and 100 kDa polypeptides, their molecular structure was examined with mass spectrometry. In the PAF-AH complex structure the presence of epididymis-specific α-mannosidase, fibronectin, spermadhesins AWN-1 and PSP-II, and IgG-binding proteins was

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confirmed. Determinations of the molecular weights of 55, 65 and 100 kDa enabled the identification of their peptide sequences and site of post-translational modifications.

KEY WORDS: acetylhydrolase / boar / mass spectrometry / platelet-activating factor / seminal plasma

Platelet-activating factor (1-O-alkyl-2-acetyl-*sn*-glycero 3-phosphorylcholine – PAF), a compound naturally occurring in membrane acetylated glycerophospholipid, has been found to be implicated in a variety of reproductive functions [Harper 1989]. It has been suggested that PAF plays a special role in the regulation of sperm function. Accumulating evidence has been shown that PAF is important in fertilization processes, by enhancing sperm motility, capacitation, and the acrosome reaction [Kordan and Strzeżek 2002, Odech *et al.* 2003, Kumar and Sharma 2005, Zhu *et al.* 2006, Bathgate *et al.* 2007]. More recently, it has been demonstrated that there was an improvement in the farrowing rate, litter size and fertility index in sows inseminated (AI) with semen diluted in the PAF-supplemented extender, Gedil^{TM} [Genes diffusion 2005]. It has also been shown that under physiological conditions, the level of sperm-derived PAF is regulated by the activity of PAF acetylhydrolase (AH) that occurs mainly in the accessory sex gland fluids [Soubeyrand and Manjunath 1998, Kordan *et al.* 2003]. Kordan [2001] showed that purified PAF-AH comprised of four subunits with molecular weights of 43, 55, 65 and 100 kDa. Immunofluorescence studies by Kordan [2001] showed that PAF-AH was strongly bound to the entire surface of the sperm *plasmalemma*, while recently Kordan *et al*. [2004] demonstrated that the N-terminal amino acid sequence of 43 kDa polypeptide is homologous with the characteristic sequences of IgG-binding proteins and *zona pellucida*-adhesive proteins.

In our laboratory, digestion of PAF-AH with various chemical agents, such as protease V-8, cyanogen bromide and formic acid was unsuccessful because the N-terminal amino groups of the polypeptides analysed were strongly blocked and chemical cleavage did not generate any peptides (unpublished data). Therefore, in the current report mass spectrometry was used to characterize the structure of 55, 65 and 100 kDa polypeptides of PAF-AH isolated from boar seminal plasma.

Material and methods

The procedure used for the isolation of PAF-AH from the boar seminal plasma was that described by Kordan [2001]. The protein samples were subjected to sodium dodecyl sulphate gel electrophoresis (SDS-PAGE) in 12.5% slab gels, under reducing conditions with Tris-glycine buffer (pH 8.3), using Mini Protean II Electrophoretic Cell (BioRad, USA), as described by Laemmli [1970]. Following electrophoresis, the gels were stained in a solution containing 0.025% Coomassie Brilliant Blue R-200 (w/v), 4% methanol (v/v), and 7% acetic acid (v/v). The gels were destained in a solution containing 7% acetic acid (v/v) and 20% methanol (v/v). Subsequently, the gels were cut into slices (2 × 2 mm) and analysed with mass spectrometry in the Mass

Spectrometry Laboratory, Polish Academy of Sciences Institute of Biochemistry and Biophysics, Warsaw, Poland).

Samples were analysed with liquid chromatography coupled to mass spectrometry (LC-MS/MS). Prior to analysis the gel slices (2 × 2 mm) were subjected to standard "in-gel digestion" procedure in which the proteins were reduced, alkylated and digested with trypsin. Briefly, the gel slices were destained in 50% acetonitrile solution (ACN) in 50 mM NH_4HCO_3, dehydrated in 100% ACN and reduced in 10 mM dithiothreitol (DTT) for 30 min at 56°C. The DTT solution was removed and the samples were subjected to alkylation with 50 mM iodoacetamide in darkness for 45 min. The gel slices were then washed with 25 mM NH_4HCO_3 and again dehydrated in 100% ACN. Next, the ACN was removed by vacuum centrifugation. Following digestion of proteins with 10 ng/µl trypsin solution (overnight at 37°C), the formed peptides were extracted in a solution containing 0.1% formic acid (FA) and 2% ACN. The extracted peptides were applied to RP-18 precolumn (WATERS), using 0.1% FA as a mobile phase, and then transferred onto the UPLC RP-18 column (WATERS) using ACN gradient (5-30% in 45 minutes) in the presence of 0.1% FA, with a flow rate of 250 nl/min. Column outlet was directly coupled to Finningan Nanospray ion source of LTQ-FT (THERMO) mass spectrometer, working in the regime of data-dependent MS to MS/MS switch. Resulting raw data were pre-processed with MASCOT Distiller and the output list of precursor and product ions was compared to NCBI nr database with MASCOT local server (www.matrixscience.com). A blank run ensuring a lack of cross-contamination from previous samples preceded each analysis.

Results and discussion

Out of four polypeptide fractions obtained with SDS-PAGE by Kordan *et al*. [2004] and shown in the current report in Photo 1 (reprinted from the former) only three (55, 65 and 100 kDa) were examined with mass spectrometry analysis. The results are shown in Table 1 of the current report. Computerized mass spectrometry analysis of each polypeptide, which was obtained after trypsin digestion, showed that the amino acid sequence of 100 kDa polypeptide (fraction I) was homologous with that of epididymis-specific α-mannosidase precursor. It should be noted that the amino acid sequence of 42 peptides of fraction I exhibited 43% identity with seminal epididymis-specific α-mannosidase precursor. When present in the semen, mannosidases hydrolyze the glycosidic bonds, resulting in the release of terminal mannosyl residues. Moreover, two isoforms of α-mannosidases with molecular weights of 135 kDa and 320 kDa, which have been identified in the epididymis, exhibit distinct substrate specificity and optimum pH. The former α-mannosidase has been suggested to be involved in sperm maturation processes and sperm-egg recognition [Okamura *et al.* 1995]. It has been suggested that sperm surface mannosidase may act as a receptor for mannose-containing oligosaccharides located on the *zona pellucida*.

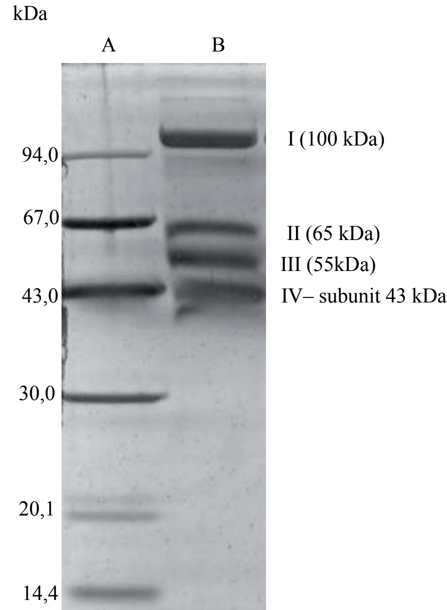

Photo 1. SDS-PAGE protein fractions of platelet-activating factor acetylhydrolase (PAF-AH). Reprinted from Kordan *et al*. [2004]. Results of analyses of fractions I, II and III obtained using mass spectrometry are given in Table 1 of the current report.
Lane A – molecular weight standards (Amersham). Lane B – protein fractions of PAF-AH.

Table 1. Analysis of the protein complex of PAF-AH using mass spectrometry

Subunit number (fraction)	Protein	Molecular weight of peptide (Da)	Homologous amino acid sequence
I (100 kDa)	epididymis-specific α-mannosidase precursor	from 938 to 3052	42 peptides (sequence coverage: 43%)
	fibronectin (FN)	1430	VPGTSASATLTGLTR
	AWN-1 spermadhesin	919	SSSNIATIK
II (65 kDa)	human serum albumin (chain A)	1148	LVNEVTEFAK
		1442	YICENQDSSISSK
		1497	TCVADESAENCDK
		1510	VPQVSTPTLVEVSR
		1638	DVFLGMFLYEYAR
		1909	RPCFSALEVDETYVPK
		2044	VFDEFKPLVEEDQNLIK
III (55 kDa)	epididymis-specific α-mannosidase precursor	1300	FIAVEQEYFR
		1497	ASALLYAGESLFTR
		1694	DMFVEHLTTGMAGVR
	AWN-1 spermadhesin	1814	EYVELLDGPPGSEIIGK
	PSP-II spermadhesin	1151	DTSGSISNTDR

From the current report it is clear that fraction I shows homology with fibronectin and AWN-1 spermadhesin. Besides their lectin-like properties, spermadhesins have been shown to possess binding affinity for heparin, serine proteinase inhibitor, phospholipids and carbohydrates [Strzeżek 1999]. The above-mentioned compounds are thought to consist of two fibronectin domains. Earlier studies demonstrated that fibronectin type II, a dimeric glycoprotein structure with adhesion properties, displayed strong affinity for glycosoaminoglycans and gangliosides and binds receptors of different cells, thereby facilitating sperm:egg interaction [Strzeżek 2002]. Moreover, the sperm surface-associated AWN-1 spermadhesin may be responsible for modulating the fertilizing ability of epididymal spermatozoa [Calvete *et al.* 1995].

Mass spectrometry showed that 65 kDa polypeptide (fraction II) exhibited characteristic sequence similar to that of human serum albumin, whereas the amino acid sequence of 55 kDa polypeptide (fraction III) was homologous with that of epididymis-specific α-mannosidase precursor, AWN-1 spermadhesin and PSP II spermadhesin. It is noteworthy that PSP II spermadhesin, isolated from the boar seminal vesicle fluid, can associate with PSP I into a non-heparin-binding heterodimer complex, PSP I/PSP II, which displays *zona pellucida* glycoprotein-binding capacity and affinity for proteinase inhibitors [Kwok *et al.* 1993]. It should be emphasised that Fractions I and III also showed homology with IgG-binding proteins, similarly to 43 kDa polypeptide analysed earlier by Kordan *et al.* [2004].

In the current report, the amino acid sequences of 55, 65 and 100 kDa polypeptides appear homologous neither with the sequences of PAF-AH isolated from bovine seminal plasma [Hough and Parks 1997] nor with the sequence of PAF-AH originating from other animal species (according to NCBI data base). The sequences of 100 and 55 kDa polypeptides (fractions I and III, respectively) homologous with that of epididymis-specific α-mannosidase precursor or AWN-1 spermadhesin probably indicate that the secretory components of seminal plasma PAF-AH complex are formed in the epididymis. On the other hand, immunofluorescence studies did not confirm the presence of PAF-AH neither in the epididymal tissues nor in the epididymal fluid. Moreover, it has been confirmed that PAF-AH of seminal plasma is secreted mainly by the seminal vesicles and prostate as reported by Kordan [2001].

The results presented here indicate that PAF-AH activity in boar seminal plasma might result from the post-translational protein splicing. It seems that the fluids of the accessory sex gland are involved in this process.

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Zastosowanie spektrometrii mas do charakterystyki struktury acetylohydrolazy płytkowego czynnika aktywującego (PAF-AH) plazmy nasienia knura

S t r e s z c z e n i e

Acetylohydrolaza PAF (PAF-AH) plazmy nasienia knura jest heterogennym białkiem zbudowanym z czterech polipeptydów o masach cząsteczkowych 43, 55, 65 i 100 kDa. W roku 2004 trzej autorzy bieżącego doniesienia określili N-końcową sekwencję aminokwasów polipeptydu 43 kDa, stwierdzając jego podobieństwo do białek wiążących IgG oraz białek adhezyjnych. Charakterystyka struktury molekularnej pozostałych trzech polipeptydów (z silnie zablokowaną N-końcową grupą aminową), wymagała posłużenia się spektrometrią mas, czemu poświęcono kolejne badania, przedstawione w doniesieniu bieżącym. Na podstawie widma masowego stwierdzono występowanie w strukturze PAF-AH najądrzowego prekursora α-mannozydazy, a także fibronektyny, spermadhezyn AWN-1 i PSP-II oraz białek wiążących IgG. Zidentyfikowane masy cząsteczkowe pozwolą na określenie sekwencji peptydów w omawianych fragmentach peptydowych PAF-AH i poznanie miejsc potranslacyjnych modyfikacji białka.