The soluble hyaluronidase from bull testes is a fragment of the membrane-bound PH-20 enzyme

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Abstract The membrane-bound PH-20 hyaluronidase is known to be essential for fertilization. Here we addressed the question whether the soluble hyaluronidase from bull teste is related to the PH-20 polypeptide. The sequence of the membrane-bound PH-20 hyaluronidase from bovine sperm was determined via cDNA cloning. In parallel, from a commercial preparation of bovine hyaluronidase the major 60-kDa form was purified to apparent homogeneity. The soluble enzyme was digested with two different proteases and with cyanogen bromide and the amino acid sequence of 44 different fragments was determined. All the peptide sequences could be aligned to the sequence deduced from the cloned cDNAs. Our results thus show that the soluble 60-kDa hyaluronidase from bovine testes is a glycoprotein derived from the sperm PH-20 enzyme. As compared to the primary translation product of the PH-20 mRNA, it lacks the signal peptide at the amino terminus and 56 amino acids at the carboxyl end. These results demonstrate that the soluble 60-kDa enzyme is a fragment of the PH-20 hyaluronidase. It is currently not known whether the soluble testes hyaluronidase has a distinct biological function.

Key words: Hyaluronidase; PH-20; Testes; Fertilization

1. Introduction

It has been known for a long time that extracts from mammalian testes contain hyaluronidase activity. The testes enzymes are endo-*N*-acetylhexosaminidases (EC 3.2.1.35) which hydrolyze hyaluronan, a glycosaminoglycan with a molecular mass of up to several million Da, to tetrasaccharides as the main end product [1]. The hyaluronidases present in the venom of snakes, bees and wasps as well as the lysosomal enzymes also belong to this group. Partial purification of these enzymes from testes as well as from other sources has been achieved by several groups [2]. In addition, hyaluronidase preparations from bovine and ovine testes are available from several companies. However, until recently, no structural data for any of these enzymes have been forthcoming.

Starting from the amino-terminal sequence of the bee venom hyaluronidase [3,4], the structure of this enzyme could be established by cDNA cloning [5]. It was then found that significant sequence homology existed between the soluble insect enzyme and PH-20, a protein with a glycosyl-phosphatidylinositol (GPI) anchor present on the head and the acrosomal membrane of mammalian sperm [6,7]. This protein plays an essential role in fertilization; guinea pigs immunized with their own PH-20 protein are sterile [8]. It could then be shown that expression of the cloned cDNAs encoding the human [9], monkey, and mouse PH-20 proteins [10] in rabbit kidney RK13 or insect cells yielded polypeptides with hyaluronidase activity.

These findings raised the question whether the different forms of soluble hyaluronidase present in testes extracts are related to the PH-20 enzyme. We have now addressed this by comparing the structure of the bovine PH-20 protein deduced from cloned cDNAs with the amino acid sequence of the major hyaluronidase present in extracts from bull testes. As is shown in this communication, the latter enzyme is a fragment of the PH-20 hyaluronidase.

2. Materials and methods

2.1. Isolation of mRNA from bull testes and cDNA synthesis

RNA was isolated from bull testes [11] and poly(A)$^+$ RNA was obtained using Oligotex-dT beads (Diagen). First strand cDNA was synthesized with 5 μg of RNA and 50 pmol of the primer-adaptor GGAATTCTCGAGCTCAAGC$(T)_{18}$ [12] and reverse transcriptase (Superscript, GIBCO-BRL). Aliquots of this cDNA pool were used for the PCR experiments. As primers we used the oligonucleotides GGGGTTATTATCTATTTCCTG and GGAATTCTCGAGCTCAAGC (Ada4). The former corresponds to nucleotides 998–1018 of the guinea pig PH-20 cDNA [7], a sequence which is highly conserved in several mammalian mRNAs encoding PH-20 poly-peptides [9,10]. This yielded an 1100 bp DNA fragment which was derived from the 3′-end of the mRNA encoding the bovine PH-20. This was inserted into the plasmid pBsII and two clones were sequenced by the chain termination method using the Sequenase 2.0 kit (US Biochemicals). The 5′-end of the PH-20 mRNA was amplified in a second PCR with the degenerate oligonucleotides AGTGTA/GTAA/GGAAGA/GAAT/AA from the 5′-end of the guinea pig PH-20 cDNA (nucleotides 209–226) and ATTTCTCCTTTTTTCTACAT (antisense, nucleotides 72–91 of the 1100-bp fragment from the first PCR). This second reaction yielded a 780-bp fragment which was inserted in the pBsII plasmid and sequenced.

2.2. Purification of hyaluronidase from bull testes

A commercial preparation of bovine testicular hyaluronidase (Biochemie Ltd, Kufstein, Austria) was fractionated on a Sephacel S-200 column. Further purification was achieved by reversed phase HPLC. Proteins were collected and assayed for hyaluronidase activity. Enzymatic activity was detected with a spreading assay [13].

2.3. Deglycosylation

For N-deglycosylation, recombinant peptide-*N*-glycosidase was used as recommended by the supplier (Boehringer Mannheim). Chemical deglycosylation was carried out with the glyco-free deglycosylation kit (Oxford Glycosystems).

2.4. Proteolytic digestion, cyanogen bromide cleavage, and analysis of fragments

Purified hyaluronidase was cleaved with trypsin (pH 8.0, 14 h at 37°C) or endoproteinase Glu-C (pH 4.0, 15 h at 20°C). Cyanogen bromide was dissolved in 70% formic acid (10 mg/ml). To 0.2 mg of protein, 0.1 ml of this solution were added and incubated over night. Reduction and alkylation was performed as follows: 0.2 mg of dried digest was dissolved in 40 μl of Na-borate (pH 9.0 containing 1 mM

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1                                   35          ▪                60
MRMLRRHHISSRSFAGSSGTPQAVFTFLLLPCCLALDFRAPPLISNTSFLWAWNAPVERC
                                      LDFRAPPLISNTSFLWAWNAPVERC

                                                                120
VNRRFQLPPDLRLFSVKGSPQKSATGQFITLFYGDRLGYYPHIDEKTGKTVFGGIPQLGN
VNRRFQLPPDLRLFSVK.....SATGQFITLFYGDRLGYYPHIDEKTGKTVFGGIPQLGN

                  *                                             180
LKSHLEKAKNDIAYYIPNDSVGLAVIDWENWRPTWARNWKPKDVYRDESVELVLQKNPQL
LKSHLEKAKNDIAYYIPNDSVGLAVIDWENWRPTWARNWKPKDVYRDESVELVLQKNPQL

                                                                240
SFPEASKIAKVDFETAGKSFMQETLKLGKLLRPNHLWGYYLFPDCYNHNYNQPTYNGNCP
SFPEASKIAKVDFETAGKSFMQETLKLGKLLRPNHLWGYYLFPDCYNHNYNQP.......

                                                                300
DVEKRRNDDLDWLWKESTALFPSVYLNIRLKSTQNAALYVRNRVQEAIRLSKIASVESPL
.....RNDDLDWLWKESTALFPSVYLNIRLKSTQNAALYVRNRVQEAIRLSKIASVESPL

                                                  ▪             360
PVFVYARPVFTDGSSTYLSQGDLVNSVGEIVSLGASGIIMWGSLNLSLSMQSCMNLGTYL
PVFVYAR.............................MWGSLNLSLSM....NLGTYL

▪       ▪                 ▪                                     420
NTTLNPYIINVTLAAKMCSQVLCHDEGVCTRKHWNSSDYLHLNPMNFAIQTGEGGKYTVP
NTTLNPYIINVTLAAKMCSQVLCHNEGVCTRKHWNSSDYLHLNPMNFAIQTGEGGKYTVP

                                  +                             480
GTVTLEDLQKFSDTFYCSCYANIHCKKRVDIKNVHSVNVCMAEDICIDSPVKLQPSDHSS
GTVTLEDLQKFSDTFYCSCYANIHCK..VDIKNVHSVNVCMAEDICIDSPVKLQPSDH

                                                              534
SQKEASTTTFSSISPSTTTATASPCTPEKHSPECLKVRCAERHPQRHPKGVSKC
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Fig. 1. Amino acid sequence of the bovine PH-20 polypeptide, deduced from cloned cDNAs (upper line) and of peptides from the soluble hyaluronidase (lower line). Asn residues which are N-glycosylated are marked (■), the one which is not glycosylated is marked (*), Asn-458 (see text) is marked (+).

EDTA and 1% SDS). Then, 40 μl of an ethanol solution of tris-(2-carboxyethyl)-phosphine-HCl (Pierce) and methyl-4-nitrobenzene-sulfonate (Fluka, both 1 mg/ml) were added and heated for 5 min at 100°C.

2.5. Sequence analysis

Proteins were separated by SDS-PAGE and electroblotted on glassybond (Biorad) [15]. Protein bands were cut out and directly used for sequencing. Peptides were separated by reverse phase HPLC on a C18 column (Vydac, 218TP) with a gradient from 5% to 70% acetonitrile in 0.1% trifluoroacetic acid. Protein and peptides samples were applied to polybrene coated glass filter disks [14]. Automated sequence analysis was performed on a model 470A gas phase sequencer coupled to an on-line PTH amino acid analyzer (Applied Biosystems).

3. Results

Poly(A)-rich RNA was isolated from bovine testes and transcribed into single stranded cDNA. By PCRs with suitable primers, cDNAs containing the complete coding region for the bovine PH-20 protein could be obtained. The cloned cDNAs have an open reading frame encoding a polypeptide which comprises 534 amino acids (see Fig. 1). The deduced sequence of bovine PH-20 is 54–58% identical to the guinea pig [7] and human [9,16] sequences; this figures rise to 59 and

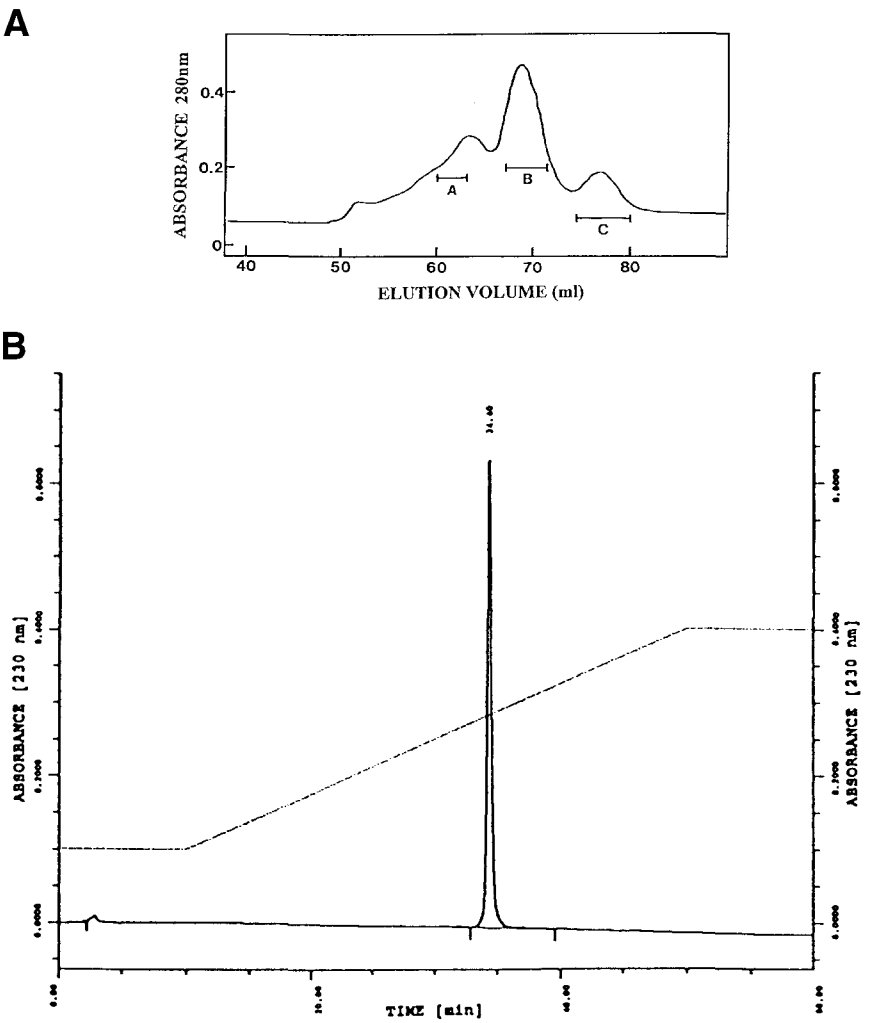

Fig. 2. Purification of bovine testicular hyaluronidase from a commercial enzyme preparation. (A) Protein (5 mg) was loaded onto a Sephacel S-200 column (1.6×60 cm, Pharmacia) and eluted with 0.3 M NaCl. (B) Reverse phase HPLC of fraction B (Vydac 214TP, 4.6×150 mm, gradient of 20–60% acetonitrile in 0.1% trifluoro acetic acid).

63% if the carboxy-terminal region is excluded from this comparison.

A commercial preparation of bovine testes hyaluronidase was fractionated on a Sephacel S-200 column. Activity could be detected in Fractions A and B (see Fig. 2A). As tested by SDS-PAGE under reducing conditions, major polypeptides with an apparent molecular mass of 75 kDa and 60 kDa were present in fractions A and B, respectively. The amino-terminal sequence of both proteins were identical up to residue 20. After enzymatic and chemical deglycosylation, the molecular mass of the 60-kDa and 75-kDa proteins were reduced by about 15 kDa (see Fig. 3). This indicates that both have similar carbohydrate moieties. The structure of the 75-kDa protein remains to be established. The major 60-kDa form was further purified by HPLC (see Fig. 2B) and its amino acid sequence was investigated in detail. The protein was cleaved with proteases as well as with cyanogen bromide. Fragments were separated by HPLC and their sequence was determined (see Table 1). As shown in Fig. 1, all the 44 peptides could be aligned with the sequence deduced from the cloned cDNA. The only difference detected by protein sequencing was Asn in position 385 instead of Asp encoded by the cDNA.

The amino-terminal sequence of the soluble hyaluronidase starts with residue 36 of the precursor. The first 35 amino acids thus represent the signal peptide, as is also predicted by the algorithm of von Heijne [17]. The soluble enzyme apparently terminates with His-478. The sequence of the COOH-terminal tryptic peptide T29 was confirmed by mass spectroscopy ($MH^+ = 696.4$, calculated monoisotopic $M = 695.3$). In the hydrolysates we could not detect any peptides which were derived from the last 56 amino acids of the polypeptide encoded by the cloned cDNA. It is noteworthy that no similarity is discernible between the carboxy-terminal parts of the bovine and e.g. the human and guinea pig PH-20 polypeptides. Evidence has been presented that this part has a separate activity required for binding of sperm to the zona pellucida [18].

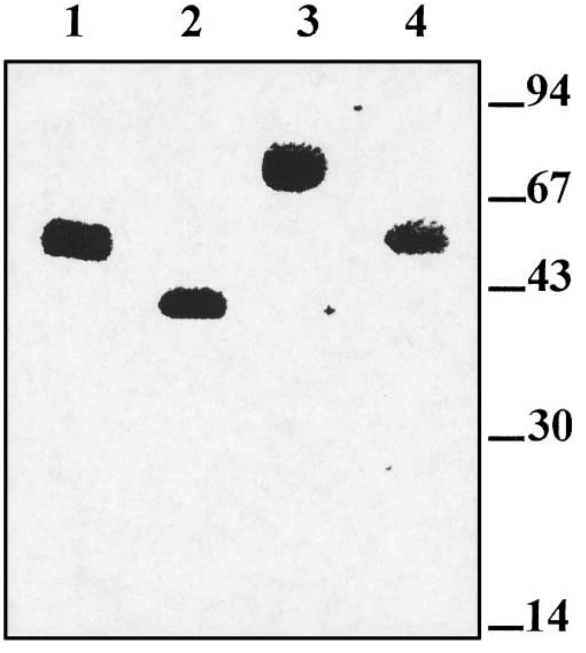

Fig. 3. Polyacrylamide gel electrophoresis of the 60 and 75-kDA hyaluronidases. Proteins were separated on 12.5% gels using a phast system (Pharmacia). Lanes: 1, 60-kDa enzyme; 2, 60-kDa form, deglycosylated; 3, 75-kDa enzyme; 4, 75-kDa form deglycosylated. Molecular mass (kDa) of standards are indicated.

Table 1
Peptide fragments from bovine testes hyaluronidase

T1	(36–39)	LDFR
T2	(40–59)	APPLISNTSF LWAWNAPVER
T3	(65–72)	FQLPPDLR
T4	(73–77)	LFSVK
T5	(83–96)	SATGQFITLF YADR
T6	(97–106)	LGYYPHIDEK
T7	(110–125)	TVFGGIP QLGNLK
T8	(123–127)	SHLDK
T9	(128–129)	AK
T10	(130–134)	NDIAY
T11	(153–157)	PTWAR
T12	(158–162)	NWKPK
T13	(163–176)	DVYRDESVEL VLQK
T14	(177–187)	NPQLSFPEAS K
T15	(188–190)	IAK
T16	(191–198)	VDFETAGK
T17	(199–206)	SFMQETLK
T18	(210–233)	LLRPNHLWGY YLFPDCYNHN YNQP
T19	(246–269)	RNDDLEWLWK ESTALFPSVY LNIR
T20	(272–281)	STQNAALYVR
T21	(284–289)	VQEAIR
T22	(293–307)	IASVESPLPV FVYAR
T23	(377–391)	MCSQVLCHNE GVCTR
T24	(392–416)	KHWNSSDYLH LNPMNFAIQT GEGGK
T25	(417–430)	YTVPGTVTLE DLQK
T26	(431–446)	FSDTFYCSCY ANIHCK
T27	(449–452)	VDIK
T28	(453–472)	NVHSVNVCMA EDICIDSPVK
T29	(473–478)	LQPSDH
E1	(106–122)	KTGKTVFGGI PQLGNLK
E2	(127–146)	KAKNDIAYYI PNDSVGLAVI
E3	(150–166)	NWRPTWARNW KPKDVYR
E4	(172–184)	LVLQKNPQLS FPE
E5	(204–233)	TLKLGKLLRP NHLWGYYLFP DCYNHNYNQP
E6	(252–256)	WLWKE
E7	(257–286)	STALFPSVYL NIRLKSTQNA ALYVRNRVQE
E8	(287–306)	AIRLSKIASV ESPLPVFVYA
C1	(36–70)	LDFRAPPLIS NTSFLWAWNA PVERCVNRRF QLPPD
C2	(202–210)	QETLKLGKL
C3	(341–349)	WGSLNLSLS
C4	(355–376)	NLGTYLNTTL NPYIINVTLA AK
C5	(378–404)	CSQVLCHNEG VCTRKHWNSS DYLHLNP
C6	(406–435)	NFAIQTGEGG KYTVPGTVTL EDLQKFSDTF
C7	(462–476)	AEDICIDSPV KLQPS

T, peptides obtained after hydrolysis with trypsin; E, peptides after hydrolysis with endoproteinase Glu-C; C, peptides generated by treatment with cyanogen bromide. Numbers in brackets refer to the sequence of the PH-20 precursor deduced from the cDNA sequence. At the E residues in T19 and T21 (underlined), D and I, respectively, were detected as well.

The bovine PH-20 enzyme has six potential N-glycosylation sites. The relevant peptides were sequenced in their native and enzymatically de-glycosylated forms. Five Asn residues, marked (■) in Fig. 1, could only be detected after de-glycosylation, while one (marked * in Fig. 1) could be detected in the native peptide. This indicates that five of the six sites are indeed glycosylated. An unusual result was obtained with peptide T28 which was present in two forms in the tryptic digest. On sequencing, one gave no signal at Asn-458 (marked + in

Fig. 1) suggesting N-glycosylation at an atypical N-X-C consensus sequence. However, the peptide could not be de-glycosylated with peptide N glycosidase and the side chain present on this asparagine thus remains to be established.

4. Discussion

Hyaluronidases have been partially characterized from mammalian testes [1,19–22]. These preparations contained soluble polypeptides with molecular masses of 60–75 kDa as well as several smaller forms. It is not known whether these differ in their amino acid sequence or their carbohydrate moieties. The only sequence data on any of these enzymes have been presented in a letter to the Biochemical Journal, where it was reported that the amino-terminal sequence of ovine hyaluronidase was related to that of mammalian PH-20 proteins [20]. Moreover, it was recently shown that the soluble hyaluronidase from guinea pig testes reacts with an antiserum raised against the sperm PH-20 protein [18].

Here we show that the main hyaluronidase present in a commercial extract from bovine testes is a 60-kDa polypeptide comprised of 443 amino acids. As compared to the PH-20 polypeptide deduced from cloned cDNAs from the same source, it lacks the signal peptide and 56 residues from the carboxyl end. PH-20 is a glycoprotein with a molecular mass of about 65 kDa which is bound to the head of spermatozoa via a GPI-anchor [7]. It seems likely that the soluble forms are generated by proteolysis. At present, it is not clear whether these soluble enzymes have distinct functions or are largely degradation products formed e.g. during the aging of spermatozoa.

In a recent study, it has been shown that during the acrosome reaction of macaque PH-20, soluble forms of 64 kDa and 53 kDa are formed [23]. The former may represent the intact glycoprotein which only lacks the GPI-anchor. Interestingly, the hyaluronidase activity of the 64-kDa species is highest at neutral pH, while the soluble 53-kDa form, which may be generated through the action of glycosidases present in the acrosome [22], has an activity peak at pH 4. The commercial bovine enzyme used in this study is active over a broad pH-range with a maximum at about 4.

It has previously been shown that a single gene for PH-20 is present in the genome of mammals [7,23], which is normally only expressed in testes. However, the mRNA encoding PH-20 was recently also detected in several human tumors [24]. In all instances, only a single PH20 mRNA species could be detected. This fact and the evidence presented in this communication support the notion that the hyaluronidases present in extracts from mammalian testes are all encoded by the PH-20 gene. Soluble forms of this enzyme are apparently generated through cleavage of the GPI anchor and further processing by proteases and/or glycosidases. In addition, the larger PH-20 hyaluronidase present in peak A of the bovine testes extract (see Fig. 2A) may represent yet another variant.

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