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# Bee venom hyaluronidase is homologous to a membrane protein of mammalian sperm

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**ABSTRACT** **The venom of honeybees, *Apis mellifera*, contains several biologically active peptides and two enzymes, one of which is a hyaluronidase. By using degenerate oligonucleotides derived from the amino-terminal sequence of this hyaluronidase reported by others, clones encoding the precursor for this enzyme could be isolated from a cDNA library prepared from venom glands of worker bees. The deduced amino acid sequence showed that bee venom hyaluronidase is a polypeptide composed of 349 amino acids containing four cysteines and three potential sites for N-glycosylation. The sequence of the precursor also indicated that the conversion of the pro-enzyme to the end product must involve cleavage of a Thr-Pro bond, a most unusual processing reaction. The mRNA encoding hyaluronidase could also be detected in testes from drones. Expression of the cloned cDNA in *Escherichia coli* yielded a 41-kDa polypeptide that had hyaluronidase activity. Interestingly, the hyaluronidase from bee venom glands exhibited significant homology to PH-20, a membrane protein of guinea pig sperm involved in sperm–egg adhesion. These structural data support the long-held view that hyaluronidases play a role in fertilization.**

Different types of hyaluronidases hydrolyze the long chains of alternating units of *N*-acetylglucosamine and D-glucuronic acid present in hyaluronan (hyaluronic acid), the most abundant mucopolysaccharide of vertebrate connective tissue. These enzymes are widely distributed in nature; they have been isolated from such diverse sources as mammalian testes, salivary glands of leeches, and different bacteria (1). At present, little is known about the structure of these enzymes apart from the sequence of a hyaluronidase encoded by a bacteriophage isolated from a strain of *Streptococcus pyogenes* (2).

Hyaluronidases are also present in venoms of snakes, scorpions, bees, and wasps. The enzyme from honeybee venom, originally described as a "spreading factor" that facilitates the diffusion of other venom constituents (3), has been purified and shown to be a glycoprotein with a molecular mass of about 43 kDa (4). Recently, the amino-terminal sequence of bee venom hyaluronidase was determined (5). As described in this paper, by using this information we were able to isolate two clones that encode the hyaluronidase precursor from a cDNA library prepared from bee venom glands. The sequence of this insect enzyme* shows homology to a protein present in guinea pig sperm (6).

## MATERIALS AND METHODS

**RNA and DNA Isolation.** Various honey bee tissues (venom glands, pupae, testes) were homogenized in a buffer containing 4.2 M guanidinium isothiocyanate, 100 mM NaOAc (pH 7.5), 2 mM EDTA, 2% mercaptoethanol, and 2% 1-butanol (7). After extraction with phenol/chloroform/isoamyl alcohol, 12:24:1 (vol/vol), the water phase was acidified with 2 M NaOAc (pH 4.4), extracted with phenol/chloroform, 3:1 (vol/vol), and precipitated with isopropyl alcohol. The pellet was dissolved in 6 M urea/100 mM Hepes, pH 7.5, and precipitated by adding 0.5 volumes of 9 M LiCl. Poly(A)-rich RNA was isolated by chromatography on oligo(dT)-cellulose. Genomic DNA from pupae of drones was prepared as described (8).

**cDNA Cloning.** cDNA was synthesized from poly(A)-rich RNA (2.5 $\mu$g) by using the oligo(dT) adaptor [TGATCAGGATCCATCGA$(T)_{17}$] as primer and reverse transcriptase (Superscript; GIBCO/BRL) as recommended by the supplier. The second strand was synthesized with DNA polymerase I (New England Biolabs) in the presence of DNA ligase and RNase H (Boehringer Mannheim) following the above protocol. Double-stranded cDNA was treated with phage T4 DNA polymerase (Bio Labs) to create blunt ends. After methylation and the addition of *Eco*RI linkers, the cDNA was ligated into the Bluescript vector (Stratagene). Competent *Escherichia coli* TG1 cells were transformed, and a library containing about one million independent colonies was obtained.

**Probe Preparation and Screening of the cDNA Library.** A sense oligonucleotide containing the codons for the peptide Phe-Asn-Val-Tyr-Trp-Asn-Val and an anti-sense oligonucleotide to the sequence Phe-Met-Cys-His-Lys-Tyr-Gly (taken from ref. 5) were synthesized. About 25 pmol of each of these oligonucleotides were used for PCR with genomic bee DNA as template. The reaction mixture contained 67 mM Tris·HCl (pH 8.8), 6.7 mM $MgCl_2$, 16.6 mM ammonium sulfate, 170 $\mu$g of bovine serum albumin per ml, 10% dimethyl sulfoxide, 1.5 mM each deoxynucleotide, and 2 units of *Taq* polymerase (United States Biochemical). After 35 cycles (40 sec at 92°C, 1 min at 45°C, and 30 sec at 72°C), the resulting PCR fragment was sequenced. It was found to contain the coding information for the above peptides plus two central codons for the dipeptide Pro-Thr in complete agreement with the published amino acid sequence data (5). The 47-bp fragment was then labeled in the presence of [$\alpha$-$^{32}$P]dATP and [$\alpha$-$^{32}$P]dCTP (NEN) by using both of the above primers and Klenow polymerase (Boehringer Mannheim). The cDNA library was screened with this labeled probe (9). From about 200,000 colonies, three positive clones were isolated and purified. Clones Hya-2 and Hya-3 contained the same 1.45-kb insert, and clone Hya-1 contained a 1.25-kb insert.

**Amplification of the 3′ End of the cDNA.** To get the complete 3′ end of the hyaluronidase cDNA, we used the RACE (rapid amplification of cDNA ends) protocol (10). Starting with poly(A)-rich RNA (2.5 $\mu$g) from venom glands of worker bees, the first strand of cDNA was synthesized as described above, diluted to 500 $\mu$l with TE buffer (10 mM Tris/1 mM EDTA, pH 8), and used as a cDNA source in the

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Abbreviation: RACE, rapid amplification of cDNA ends.

*The sequences reported in this paper have been deposited in the GenBank data base [accession nos. L10710 (Hya-1) and L10711 (Hya-2)].



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amplification reaction. PCR was performed in 10 mM Tris·HCl, pH 9.0/50 mM KCl/0.01% gelatin/1.5 mM $MgCl_2$/0.1% Triton X-100 in the presence of 0.2 mM dNTPs (Pharmacia) and 0.5 units of Hi-*Taq* DNA polymerase (Vienna Laboratories, Vienna). The diluted cDNA (5 $\mu$l), 25 pmol of primer h-race (ACTGACGGTGGACGTGA) corresponding to nucleotides 1134–1150 of the cDNA, and 25 pmol of the adaptor (TGATCAGGATCCTATCG) were used in the amplification reaction. Conditions for the PCR were as follows: one cycle of 2 min at 92°C, 10 min at 55°C, and 40 min at 72°C; followed by 50 cycles of 40 sec at 92°C; 1 min at 55°C; and 2 min at 72°C; with a final extension of 20 min at 72°C. The reaction product was purified, subcloned into the Bluescript vector, and sequenced on both strands by using the Sequenase kit (United States Biochemical).

**RNA Blot Hybridization (Northern Blot).** Poly(A)-rich RNA was separated on 1% agarose gels containing 0.74 M formaldehyde and transferred to Nytran-membranes (Schleicher & Schuell). The 750-bp *Eco*RI/*Nae* I fragment of clone Hya-1 was labeled by using the random-primed DNA labeling kit from Boehringer Mannheim. Hybridization was performed overnight at 65°C. The filters were washed with decreasing salt concentrations and, after two final washes with 0.015 M NaCl/0.0015 M sodium citrate, pH 7/0.1% SDS at 65°C, used for autoradiography.

**DNA Sequencing and Sequence Analysis.** Nested deletions of clones Hya-1 and Hya-2 were generated by using exonuclease III and S1 nuclease (11). Deletions were selected according to size and sequenced by the enzymatic method (12) with the Sequenase 2.0 sequencing kit (United States Biochemical). DNA and protein data bases (EMBL release 31, Swiss-Prot release 22) were searched for homologous sequences with the FASTA program (13).

**Expression of the Cloned cDNA in *E. coli*.** Using synthetic oligonucleotides, a truncated fragment of the clone Hya-1 encoding the mature enzyme was amplified and inserted into the expression vector pMW172 (14). The vector was cleaved at the *Hin*dIII site and filled in with T4 DNA polymerase to generate blunt ends. Two clones, pMWH9 and pMWH7, were isolated that contained the inserted cDNA in opposite directions. Plasmid DNA was prepared and used to transform *E. coli* BL21(DE3) (ref. 14). Cultures were induced by adding 0.5 mM isopropyl $\beta$-D-thiogalactoside, grown for an additional 4 h, and harvested. Inclusion bodies were prepared (9) and dissolved in 5 M guanidinium hydrochloride in buffer (50 mM Tris·HCl, pH 8.0/1 mM EDTA/1 mM dithiothreitol). The solution was then dialyzed overnight at 4°C against distilled water. Precipitated material was removed by centrifugation, and the supernatant was lyophilized. The residue was dissolved in water, and hyaluronidase activity was measured by using hyaluronic acid from umbilical cords (Boehringer Mannheim) as substrate (15). The enzymatic activity of purified bee venom hyaluronidase was assayed under the same conditions.

## RESULTS

**Sequence of Hyaluronidase.** The amino acid sequence of the first 30 residues of bee venom hyaluronidase has been determined (5). A degenerate pair of sense and antisense oligonucleotides derived from this sequence (see Fig. 1) were synthesized and used for the PCR. From genomic DNA isolated from pupae of drones, which are haploid, a 47-bp fragment with the expected sequence could be amplified. This fragment was labeled and used to screen a venom gland cDNA library. Of three positive clones, two were investigated further. Fig. 2 shows the nucleotide sequence of the insert present in clone Hya-1. Starting with the first ATG, this sequence contains an open reading frame that can be translated into a polypeptide comprising 382 amino acids. The deduced amino acid sequence begins with a typical signal peptide, which is followed by a short proenzyme portion. The most likely signal peptidase cleavage sites are after Gly-28 or Ala-24 (16). The mature protein starts with the published sequence (5) except for one difference—namely, asparagine instead of aspartic acid in position 3. As deduced from the cloned cDNA, the bee venom hyaluronidase contains 349 amino acids including four cysteine residues. Three sites for N-glycosylation are present, all having the sequence Asn-Leu-Thr.

The second clone designated Hya-2 contained a larger insert than Hya-1. At the 5′ end, it started with an unknown sequence of 199 nucleotides, most likely representing an intron (data not shown), which terminated prior to the C

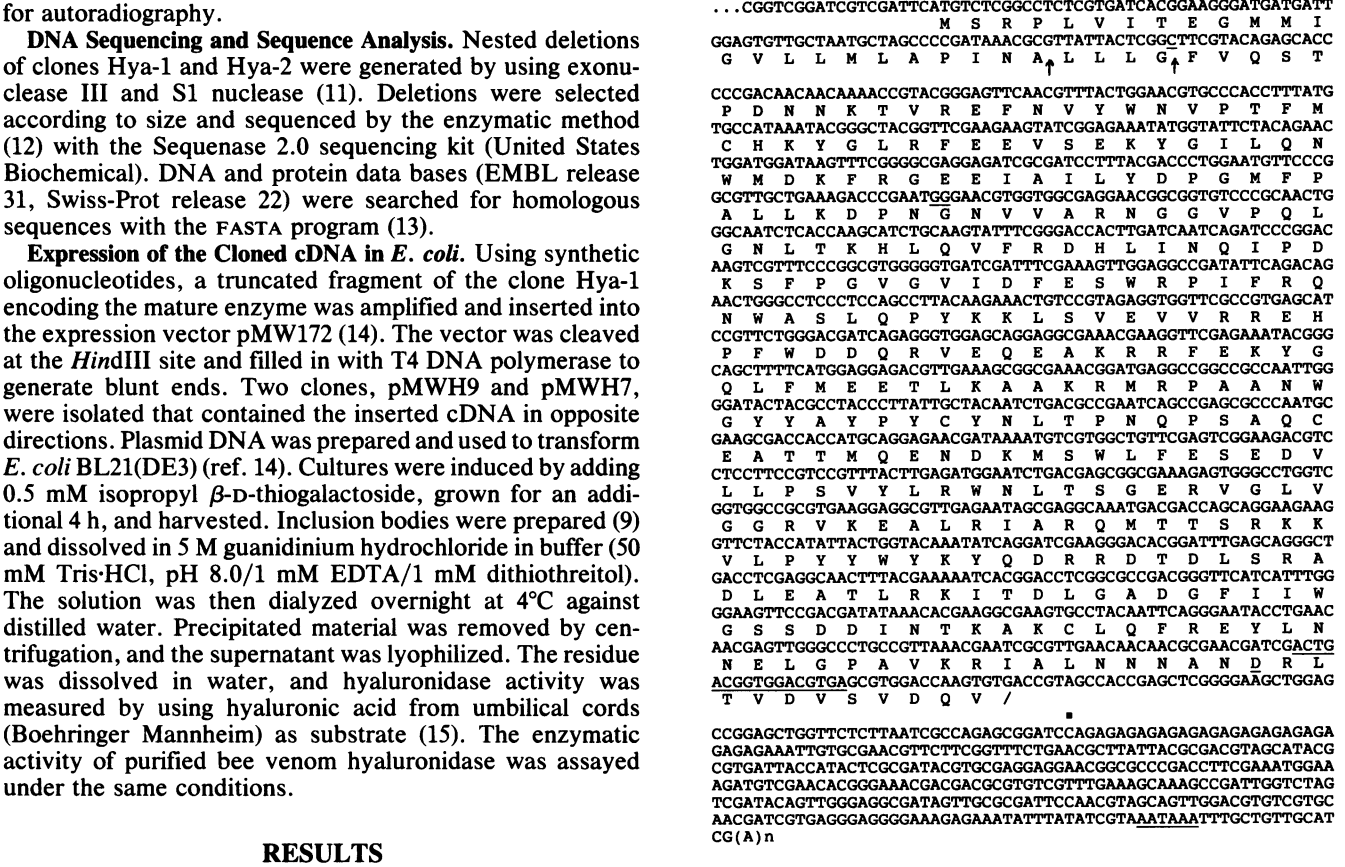



FIG. 1. Amino-terminal sequence in the amino acid single-letter code of bee venom hyaluronidase (5). The two parts of this sequence which were used for the synthesis of a degenerate pair of sense and antisense oligonucleotides are marked by arrows. X denotes an unknown residue.

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...CGGTCGGATCGTCGATTCATGTCTCGGCCTCTCGTGATCACGGAAGGGATGATGATT
                              M  S  R  P  L  V  I  T  E  G  M  M  I
GGAGTGTTGCTAATGCTAGCCCCGATAAACGCGTTATTACTCGGCTTCGTACAGAGCACC
 G  V  L  L  M  L  A  P  I  N  A  L  L  L  G  F  V  Q  S  T

CCCGACAACAACAAAACCGTACGGGAGTTCAACGTTTACTGGAACGTGCCCACCTTTATG
 P  D  N  N  K  T  V  R  E  F  N  V  Y  W  N  V  P  T  F  M
TGCCATAAATACGGGCTACGGTTCGAAGAAGTATCGGAGAAATATGGTATTCTACAGAAC
 C  H  K  Y  G  L  R  F  E  E  V  S  E  K  Y  G  I  L  Q  N
TGGATGGATAAGTTTCGGGGCGAGGAGATCGCGATCCTTTACGACCCTGGAATGTTCCCG
 W  M  D  K  F  R  G  E  E  I  A  I  L  Y  D  P  G  M  F  P
GCGTTGCTGAAAGACCCGAATGGGAACGTGGTGGCGAGGAACGGCGGTGTCCCGCAACTG
 A  L  L  K  D  P  N  G  N  V  V  A  R  N  G  G  V  P  Q  L
GGCAATCTCACCAAGCATCTGCAAGTATTTCGGGACCACTTGATCAATCAGATCCCGGAC
 G  N  L  T  K  H  L  Q  V  F  R  D  H  L  I  N  Q  I  P  D
AAGTCGTTTCCCGGCGTGGGGGTGATCGATTTCGAAAGTTGGAGGCCGATATTCAGACAG
 K  S  F  P  G  V  G  V  I  D  F  E  S  W  R  P  I  F  R  Q
AACTGGGCCTCCCTCCAGCCTTACAAGAAACTGTCCGTAGAGGTGGTTCGCCGTGAGCAT
 N  W  A  S  L  Q  P  Y  K  K  L  S  V  E  V  V  R  R  E  H
CCGTTCTGGGACGATCAGAGGGTGGAGCAGGAGGCGAAACGAAGGTTCGAGAAATACGGG
 P  F  W  D  D  Q  R  V  E  Q  E  A  K  R  R  F  E  K  Y  G
CAGCTTTTCATGGAGGAGACGTTGAAAGCGGCGAAACGGATGAGGCCGGCCGCCAATTGG
 Q  L  F  M  E  E  T  L  K  A  A  K  R  M  R  P  A  A  N  W
GGATACTACGCCTACCCTTATTGCTACAATCTGACGCCGAATCAGCCGAGCGCCCAATGC
 G  Y  Y  A  Y  P  Y  C  Y  N  L  T  P  N  Q  P  S  A  Q  C
GAAGCGACCACCATGCAGGAGAACGATAAAATGTCGTGGCTGTTCGAGTCGGAAGACGTC
 E  A  T  T  M  Q  E  N  D  K  M  S  W  L  F  E  S  E  D  V
CTCCTTCCGTCCGTTTACTTGAGATGGAATCTGACGAGCGGCGAAAGAGTGGGCCTGGTC
 L  L  P  S  V  Y  L  R  W  N  L  T  S  G  E  R  V  G  L  V
GGTGGCCGCGTGAAGGAGGCGTTGAGAATAGCGAGGCAAATGACGACCAGCAGGAAGAAG
 G  G  R  V  K  E  A  L  R  I  A  R  Q  M  T  T  S  R  K  K
GTTCTACCATATTACTGGTACAAATATCAGGATCGAAGGGACACGGATTTGAGCAGGGCT
 V  L  P  Y  Y  W  Y  K  Y  Q  D  R  R  D  T  D  L  S  R  A
GACCTCGAGGCAACTTTACGAAAAATCACGGACCTCGGCGCCGACGGGTTCATCATTTGG
 D  L  E  A  T  L  R  K  I  T  D  L  G  A  D  G  F  I  I  W
GGAAGTTCCGACGATATAAACACGAAGGCGAAGTGCCTACAATTCAGGGAATACCTGAAC
 G  S  S  D  D  I  N  T  K  A  K  C  L  Q  F  R  E  Y  L  N
AACGAGTTGGGCCCTGCCGTTAAACGAATCGCGTTGAACAACAACGCGAACGATCGACTG
 N  E  L  G  P  A  V  K  R  I  A  L  N  N  N  A  N  D  R  L
ACGGTGGACGTGAGCGTGGACCAAGTGTGACCGTAGCCACCGAGCTCGGGGAAGCTGGAG
 T  V  D  V  S  V  D  Q  V  /

CCGGAGCTGGTTCTCTTAATCGCCAGAGCGGATCCAGAGAGAGAGAGAGAGAGAGAGA
GAGAGAAATTGTGCGAACGTTCTTCGGTTTCTGAACGCTTATTACGCGACGTAGCATACG
CGTGATTACCATACTCGCGATACGTGCGAGGAGGAACGGCGCCCGACCTTCGAAATGGAA
AGATGTCGAACACGGGAAACGACGACGCGTGTCGTTTGAAAGCAAAGCCGATTGGTCTAG
TCGATACAGTTGGGAGGCGATAGTTGCGCGATTCCAACGTAGCAGTTGGACGTGTCGTGC
AACGATCGTGAGGGAGGGGAAAGAGAAATATTTATATCGTAAATAAATTTGCTGTTGCAT
CG(A)n
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FIG. 2. Nucleotide sequence of cloned hyaluronidase cDNAs and the deduced amino acid sequence (single-letter code) of the preproenzyme. The sequence shown is from clone Hya-1, which terminated at the cytidine marked by a black square, and the overlapping RACE product of the protocol to obtain the complete 3′ end. The sequence of the synthetic oligonucleotide used for the RACE protocol and the AATAAA polyadenylylation signal are underlined. The two most likely bonds hydrolyzed by signal peptidase are marked by arrows. The insert present in clone Hya-2 started with an unrelated, probable intron sequence of 199 nucleotides prior to the underlined cytidine (C in second line). Its sequence was interrupted by another intron of 127 nucleotides inserted between two guanosine residues (see GG in line 6). The GAT codon for aspartic acid close to the carboxyl terminus (see underlined D) was replaced by AGT encoding serine in Hya-2.

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underlined in Fig. 2. A second intron comprising 127 bp that started with G-T and ended with A-G (sequence not shown) was present between the two guanosine residues marked by underlining, see GG in Fig. 2. The common sequence present in clones Hya-1 and Hya-2 differs by only four point mutations, three in the coding region and one in the 3′ untranslated end. The predicted polypeptides differ by one amino acid, serine instead of aspartic acid close to the carboxyl terminus (see Fig. 2).

Since both clones did not terminate with a poly(A) stretch, the RACE protocol of Frohman *et al.* (10) was used to obtain the complete 3′ end of the mRNA encoding the bee venom hyaluronidase. A synthetic oligonucleotide corresponding to nucleotides 1134–1150 of Hya-1 (see Fig. 2) was used for the amplification reaction. The reaction product was ligated into the Bluescript vector and sequenced on both strands.

The mRNA encoding bee venom hyaluronidase thus has a minimal length of 1559 nucleotides excluding the poly(A) part. In the nontranslated 3′ end, a segment is present comprising 15 repeats of the dinucleotide A-G.

**Northern Blots.** Using the cloned cDNA present in Hya-1, we performed Northern blots to test the possible expression of the hyaluronidase gene in other bee tissues. The mRNA encoding this enzyme could also be detected in testes from drones but not in queen-bee venom glands or in pupae (Fig. 3). The control mRNA from worker bee venom glands gave a strong positive signal. The second band with a higher molecular mass present in the latter mRNA possibly corresponds to pre-mRNAs like the one found in clone Hya-2.

**Expression of the Cloned cDNA in *E. coli*.** By using synthetic oligonucleotides, a fragment of Hya-1 containing the coding sequence of the mature enzyme was amplified and inserted into the *Hin*dIII site of the expression vector pMW172 (14) downstream of the T7 promoter. Plasmid DNA was used to transform *E. coli* BL21(DE3), which contains the gene for the phage T7 RNA polymerase inserted after the *E. coli lac* promoter. Two clones that were tested further contained the hyaluronidase cDNA in opposite directions. After induction with isopropyl β-D-thiogalactoside, an additional protein was found in cells transformed with plasmid pMWH9 (see Fig. 4, lanes 2 and 3) but not in those transformed with plasmid pMWH7 (lane 1 of Fig. 4). This protein could be purified from inclusion bodies. These were dissolved in 5 M guanidinium hydrochloride under reducing conditions, and the proteins were then allowed to refold by dialysis against water. In the supernatant of the dialysate, a single protein with an apparent molecular mass of 41 kDa was present (see Fig. 4, lane 4). Native bee venom hyaluronidase is a glycoprotein with an apparent molecular mass of about 43 kDa (Fig. 4, lane 5). By using the test described by Dorfman (15), the recombinant protein was found to have hyaluronidase activity. Compared to the specific activity of the native bee venom hyaluronidase, the recombinant enzyme was about 80% as active (data not shown). In control cells transformed with pMWH7, no inclusion bodies could be isolated, and no hyaluronidase activity could be detected after this purification procedure.

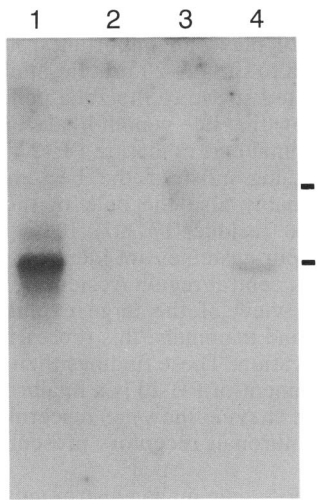



FIG. 3. Northern blot analysis. Onto each lane, 5 μg of poly(A)-rich RNA were loaded. Filters were hybridized with a 750-bp fragment of clone Hya-1 (see *Materials and Methods*). Lanes: 1, worker-bee venom gland; 2, queen-bee venom gland; 3, drone pupae, 1–2 days before emergence; 4, testes from drones. Bars mark the position of 28S and 18S ribosomal RNAs. The film was exposed for 2 days.

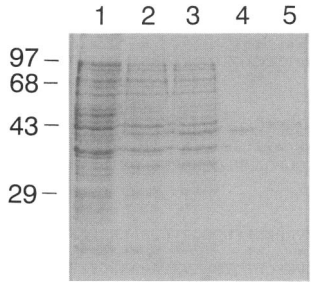



FIG. 4. Expression of the cloned cDNA in *E. coli* BL21(DE3). SDS/PAGE in a 15% gel of cell extracts (about 20 μl of cell culture) is shown along with the purified recombinant protein. Lanes: 1, *E. coli* transformed with plasmid pMWH7, which contains the cDNA in the antisense direction; 2 and 3, cells transformed with pMWH9 before (lane 2) and after (lane 3) induction with isopropyl β-D-thiogalactoside 4, about 1 μg of recombinant bee venom hyaluronidase purified from inclusion bodies; 5, about 1 μg of native bee venom hyaluronidase. Numbers on the left refer to the molecular mass (in kDa) of reference proteins.

**Homology Search.** The deduced amino acid sequence of bee venom hyaluronidase shows no discernible similarity to the hyaluronidase from bacteriophage H4489A of *S. pyogenes* (2). A search in the EMBL data bank (13) revealed that bee venom hyaluronidase is related to PH-20, a protein located on the plasma membrane and the acrosomal membrane of guinea pig sperm (6). The two polypeptides share 36% identity in a region encompassing amino acids 14–303 of the bee enzyme and 17–307 of PH-20 (Fig. 5). In this region, the corresponding nucleotide sequences show 51% identity (data not shown). The similarity is greatest in the central region (amino acids 75–190 of the bee enzyme), where it shows 44% identity to PH-20. Several clusters of even greater homology, including two identical pentapeptides and two tetrapeptide sequences, are present in these polypeptides. Moreover, the spacing of the four cysteines in hyaluronidase and in the amino-terminal domain of PH-20 is quite similar (see Fig. 5). It has also been noted that PH-20 has amino acid sequence homologies to α and β subunits of leukocyte integrins (5, 17)—i.e., proteins that mediate the adhesion of cells to extracellular matrices. In a segment encompassing 34 amino acids, where PH-20 and the $\beta_2$-integrin chain have 11 residues in common (17), 7 are also present in the bee enzyme (see Fig. 5).

## DISCUSSION

In this communication, we present the amino acid sequence of bee venom hyaluronidase as deduced from two cloned cDNAs. The two cDNAs are very similar in their coding regions but differ by the presence of two introns in clone Hya-2. Another cDNA isolated from the same library encoding the precursor of apamin was also found to contain an intron (unpublished results).

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FIG. 5. Comparison of the amino acid sequence of bee venom hyaluronidase (upper sequence) and PH-20 from guinea pig sperm (lower sequence). The alignment starts with residues 1 and 4 of the two polypeptides, respectively. Identities between the two sequences are marked by a black square, and the cysteines are emphasized by asterisks. Gaps introduced to maximize homology are indicated by dashes. Residues also present in a region considered to form part of the ligand-binding pocket in $\beta_2$-integrin (17) are indicated by a plus sign. The end of the bee sequence is marked by a slash at 349; PH-20 contains a total of 495 residues (5).

Bee venom hyaluronidase is derived from a preproenzyme. A typical signal peptide is present at the amino end following the predicted initiating methionine. The signal peptide may comprise 24 or 28 residues. In any case, further processing of the resulting proenzyme to yield the mature enzyme must involve some unusual processing reaction(s). The two putative propeptides contain none of the characteristics found in other precursors of bee venom constituents. It has previously been shown that melittin is liberated from promelittin by sequential cleavage of dipeptides from the amino end (18). Two other precursors of bee venom constituents, those for secapin (19) and phospholipase $A_2$ (20), both contain prosegments terminating with single arginine residues. As judged from the deduced amino acid sequence of preprohyaluronidase, neither a dipeptidyl aminopeptidase nor an endoprotease hydrolyzing after arginine residues can be involved in the formation of the mature enzyme. Instead, cleavage at a Thr-Pro bond has to occur to generate the amino terminus of the mature enzyme. To our knowledge, no precedent for this type of precursor–product conversion exists. Moreover, the existence of an endoprotease with this type of specificity has not been reported. However, exoproteases termed iminopeptidase P have been described that hydrolyze Xaa-Pro bonds at the amino end of polypeptides. Thus, the activation of prohyaluronidase may well involve a novel type of processing enzyme.

As deduced from the sequence of the cloned cDNAs, mature bee venom hyaluronidase comprises 349 amino acids with a calculated molecular mass of 40,746 Da. It contains four cysteine residues present as disulfide bonds in the extracellular enzyme. Three potential N-glycosylation sites are present, all having the sequence Asn-Leu-Thr. The earlier studies on this enzyme showing that it is a glycoprotein with an apparent molecular mass of about 43 kDa agree with these findings (4).

From the cloned cDNAs, two enzymes differing by one residue (aspartic acid or serine) can be predicted. The amino-terminal sequence (5) also differs by one residue from the one deduced from the cDNA sequences. This indicates that several alleles of the hyaluronidase gene are present in bee populations.

Northern blots with RNA from male reproductive organs have demonstrated that the hyaluronidase gene is also expressed in this tissue. The presence of this enzyme in mammalian testes and also, as suggested by these Northern blot data, in insect testes indicates a rather general role of hyaluronidases in reproduction of animal species. Conversely, no signal could be obtained when using RNA from queen-bee venom glands. This is consistent with earlier results showing that the content of hyaluronidase in queen-bee venom is much lower than in worker-bee venom (21). Queen-bee venom also lacks phospholipase $A_2$ (20, 22) and, as we have shown recently, the mRNAs encoding the precursors of apamin and mast-cell degranulating peptide (unpublished results). Considerable differences thus exist in the composition of the venom of these two genetically identical castes.

A fragment of the cloned cDNA encoding the mature enzyme has been expressed in *E. coli*. From inclusion bodies, a protein with the expected molecular mass was purified. After refolding, this protein had about 80% of the hyaluronidase activity of the native-bee venom enzyme.

Since the bee venom enzyme is the first sequence of any eukaryotic hyaluronidase, it was of some interest to check whether similar sequences had been detected in other proteins. Interestingly, we found that significant homology exists between this insect enzyme and PH-20, a membrane protein isolated from guinea pig sperm. Moreover, genomic Southern blots have shown that homologues to PH-20 are also present in DNA from humans and several other mammalian species (6, 23). This membrane protein is first synthesized as a single polypeptide with an apparent molecular mass of 64 kDa. Subsequent posttranslational processing yields two domains, an amino-terminal region of 41–48 kDa linked via disulfide bonds to the 27-kDa carboxyl-terminal segment, which also contains the glycosyl phosphatidylinositol membrane anchor (6, 24). PH-20 was shown to be present on sperm heads as well as on the acrosomal membrane. In acrosome-reacted sperm, additional copies of PH-20 are exposed at the surface. Evidence has been presented that PH-20 plays an important role in sperm–egg adhesion; antisera to the PH-20 protein inhibit the adhesion of acrosome-reacted sperm to the zona pellucida (25).

Our results show that bee venom hyaluronidase is related to the amino-terminal part of mature PH-20 (see Fig. 5). In a region encompassing most of the bee enzyme (residues 14–303) and probably also the bulk of the 41- to 48-kDa fragment of PH-20 (residues 17–307), 104 of 290 amino acids (36%) including four cysteines are identical. Sequence identity is highest in a central region (residues 75–190), where it reaches 44%. In view of the large evolutionary distance between insects and mammals, this represents a rather high degree of conservation. These findings then suggest that the 41- to 48-kDa segment of PH-20 is a hyaluronidase. Neither PH-20 nor the bee enzyme show any discernible homology to two different hyaluronan receptors present on mammalian cells (26, 27).

Upon fertilization, mammalian sperm must first penetrate a layer of cumulus cells that surrounds the egg before reaching the zona pellucida. Subsequently, carbohydrate-mediated interaction between a sperm-head protein and ZP3 (28, 29), one of the main constituents of the zona pellucida, triggers the acrosome reaction whereby the protease acrosin and also additional amounts of PH-20 are exposed. The cumulus cells are embedded in a matrix containing hyaluronic

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acid which is formed prior to ovulation (30–32). This matrix which extends into the outer layer of the zona pellucida can be destroyed by hyaluronidases (33). Our results would then suggest that the sperm-head protein PH-20 aids in penetrating the layer of cumulus cells by digesting hyaluronic acid. The large increase in the amount of PH-20 exposed on acrosome-reacted sperm indicates that this protein also plays a role upon contact of sperm with the outer layer of the zona pellucida.

Finally, it has been known for a long time that testes are a rich source of hyaluronidase (1). In addition, this enzyme is present in the acrosome of mammalian spermatozoa. It is currently not known whether these soluble enzymes are related to the membrane protein PH-20 and to bee venom hyaluronidase.

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1. Meyer, K. (1971) in *The Enzymes*, ed. Boyer, P. D. (Academic, New York), 3rd Ed., Vol. 5, pp. 307–320.
2. Hynes, W. L. & Ferretti, J. J. (1989) *Infect. Immun.* **57**, 533–539.
3. Habermann, E. (1972) *Science* **177**, 314–322.
4. Kemeny, D. M., Dalton, N., Lawrence, A. J., Pearce, F. L. & Vernon, C. A. (1984) *Eur. J. Biochem.* **139**, 217–233.
5. Jacobson, R. S., Hoffman, D. R. & Kemeny, D. M. (1991) *J. Allergy Clin. Immunol.* **89**, 292 (abstr.).
6. Lathrop, W. F., Carmichael, E. P., Myles, D. G. & Primakoff, P. (1990) *J. Cell Biol.* **111**, 2939–2949.
7. Chirgwin, J. M., Przybyla, A. B., MacDonald, L. J. & Rutter, W. S. (1979) *Biochemistry* **18**, 5294–5299.
8. Blin, N. & Stafford, D. W. (1976) *Nucleic Acids Res.* **3**, 2303–2308.
9. Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab., Plain View, NY).
10. Frohman, M. A., Dush, M. K. & Martin, T. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 8998–2002.
11. Henikoff, S. (1984) *Gene* **28**, 351–359.
12. Sanger, F., Nicklen, S. & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 5463–5467.
13. Pearson, W. R. & Lipman, D. J. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 2444–2448.
14. May, W., Pope, B., Gooch, J., Hawkins, M. & Weeds, A. G. (1990) *EMBO J.* **12**, 4103–4109.
15. Dorfman, A. (1955) *Methods Enzymol.* **1**, 166–173.
16. von Heijne, G. (1986) *Nucleic Acids Res.* **14**, 4683–4690.
17. Giancotti, F. G. & Ruoslahti, E. (1991) *New Biol.* **3**, 525–526.
18. Kreil, G., Haiml, L. & Suchanek, G. (1980) *Eur. J. Biochem.* **111**, 49–58.
19. Vlasak, R. & Kreil, G. (1984) *Eur. J. Biochem.* **145**, 279–282.
20. Kuchler, K., Gmachl, M., Sippl, M. J. & Kreil, G. (1989) *Eur. J. Biochem.* **184**, 249–254.
21. Owen, M. D. (1979) *Toxicon* **17**, 94–98.
22. Marz, R., Mollay, C., Kreil, G. & Zelger, J. (1981) *Insect Biochem.* **11**, 685–690.
23. Primakoff, P. & Myles, D. G. (1983) *Dev. Biol.* **98**, 417–428.
24. Phelps, B. M., Primakoff, P., Koppel, D. E., Low, M. G. & Myles, D. G. (1988) *Science* **240**, 1780–1782.
25. Primakoff, P., Lathrop, W., Woolman, L., Cowan, A. & Myles, D. G. (1988) *Nature (London)* **335**, 543–546.
26. Aruffo, A., Stamenkovic, I., Meinick, M., Underhill, C. B. & Seed, B. (1990) *Cell* **61**, 1303–1313.
27. Hardwick, C., Hoare, K., Owens, R., Hohn, H. P., Hook, M., Moore, D., Crippa, V., Austen, L., Nance, D. M. & Turley, E. A. (1992) *J. Cell Biol.* **117**, 1343–1350.
28. Wassarman, P. M. (1990) *Development* **108**, 1–17.
29. Miller, D. J., Macek, M. B. & Shur, B. D. (1992) *Nature (London)* **357**, 589–593.
30. Eppig, J. J. (1979) *Nature (London)* **281**, 483–484.
31. Salustri, A., Yanagishita, M. & Hascall, V. C. (1989) *J. Biol. Chem.* **264**, 13840–13847.
32. Salustri, A., Yanagishita, M., Underhill, C. B., Laurent, T. C. & Hascall, V. C. (1992) *Dev. Biol.* **151**, 541–551.
33. Talbot, P. (1984) *J. Exp. Zool.* **229**, 309–316.

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