Structural diversity of trypsin from different mosquito species feeding on vertebrate blood

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Summary. Mosquito trypsin was purified using a combination of ion exchange and affinity chromatography with the ligand soybean trypsin inhibitor. Three *Aedes* and three *Anopheles* species were tested, all of which are specialized in the digestion of vertebrate blood. Amino-terminal sequences of HPLC-purified trypsins from *Aedes aegypti* and *Anopheles quadrimaculatus* revealed homologies of 30–40% with vertebrate and other invertebrate proteases previously identified as serine-proteases. The purified mosquito trypsins have molecular masses between 25 kDa and 36 kDa, as determined by denaturing polyacrylamide electrophoresis, and are heterogeneous in size and number in the various species. The number of SDS-bands varies between 3 and 6 in *Aedes* and between 1 and 3 in *Anopheles*. The specific activities, determined with the substrate TAME, range from 240 U/mg in *Aedes aegypti* to 1065 U/mg in *Anopheles quadrimaculatus*. All mosquito trypsins tested have acidic isoelectric points between pH 3.5 and pH 5.4. No alkaline proteases were detected. Polyclonal antisera against *Aedes aegypti* and *Anopheles albimanus* trypsin do not cross-react with bovine trypsin. Cross-reactivity of the two sera with trypsin from six mosquito species suggests the presence of at least 2 enzyme families.

Key words. Trypsin; mosquito; amino acid sequence; *Aedes; Anopheles.*

Culicine mosquitoes represent a group of dipteran insects specialized in using exclusively vertebrate blood as a nutritional source for their reproduction. The blood is stored in the midgut, the site of both digestion and absorption of nutrients. Digestive enzymes are secreted into the midgut lumen within a few hours after feeding. Peak activity of proteolytic enzymes in the yellow fever mosquito, *Aedes aegypti*, is reached after 20–30 h [1–4]. In other species the dynamics of protease synthesis are similar [5, 6]. In *Ae. aegypti* one class of digestive enzymes, the serine proteases, has been studied in detail. Trypsin-like and chymotrypsin-like proteases were characterized by Huang [7] and others [8–10], and are going to be referred to as trypsin and chymotrypsin. Trypsin is synthesized in the larval gut as well as in the imaginal midgut, while chymotrypsin is present only in the larval gut [10]. After induction of synthesis, the amount of trypsin secreted depends not only on the amount of blood engorged [4] but also on endocrine factors from the head and ovaries [4, 11]. Insect serine proteases from various orders appear to be a more heterogeneous group of enzymes with respect to physico-chemical characteristics than the vertebrate enzymes. Most reports focussing on insect proteases of intestinal origin are restricted to physiological characterizations and to effects of pH, temperature and inhibitors on protease activity. Molecular masses of serine proteases have been found to be between 21 000 Da [7] and 60 000 Da [12], and the isoelectric points lie between pH 4 and pH 8 [13, 14].

Purification of insect proteases has been reported several times [8, 9, 14–17]. Because of the small size of insects, further characterization such as partial or total sequencing has been reported for enzymes from only a few species: *Hypoderma* trypsin [17], *Hypoderma* collagenase [18] and *Vespa* chymotrypsin [19], which originate in the gut, and *Antheraea* cocoonase [20], which is a salivary enzyme. In addition, the amino acid sequence of *Drosophila* trypsin has been deduced from a cloned DNA sequence [21].

In the mosquito *Aedes aegypti*, several attempts at purifying trypsin led to somewhat differing results; molecular masses varying from 21 000 Da [7] to 23 000 Da [9] have been reported, and we found values of 26–32 kDa [22]. The numbers of isozymes reported also differ, this variation is probably due to autolysis as well as to the methods of detection employed. Since a genetic basis for determination of isozymes is not at hand yet, we shall refer to trypsin forms instead.

We have set out to compare structural parameters of trypsin from several obligatory blood-feeding mosquito species and vertebrates. Immunological techniques [23] in combination with amino-terminal sequencing have allowed us to determine heterogeneities not only between insect and vertebrate trypsin but also within two genera of the mosquito family.

Material and methods

Mosquitoes *Aedes aegypti* (strain UGAL), *Ae. epactius, Ae. triseriatus, Ae. atropalpus, Anopheles albimanus, An. stephensi, An. quadrimaculatus*, and *Culex pipiens* were reared on a standard larval diet [4]. The adults were generally maintained at 27 °C, 85% relative humidity, with access to 10% (*Aedes* and *Culex*) or 20% (*Anopheles*) sugar ad libitum. Prior to purification of trypsin, 2–3-day-old females were allowed to feed for 15 min on a guinea pig and the mosquitoes were sacrificed after 24 h.

Enzymatic assay. Esterolytic activity towards tosyl arginine methylester (TAME) (Sigma) was measured at 25 °C [24]. The procedure was modified for a 0.5-ml assay

volume, containing 400 µl Tris/HCl buffer (0.05 M, pH 8.4), 50 µl TAME (10 mM) and 50 µl of sample or buffer. Based on a molar extinction coefficient of 409 at 247 nm, trypsin activities were expressed in units as the conversion of 1 µmole TAME per minute. For protein determinations either the Biorad reagent[25] or a modified Lowry[26] procedure was used with a mixture of bovine serum albumin, ovalbumin and immunoglobulin G as standard protein.

Chromatography. For preparative chromatography, 200–500 dissected midguts were sonicated in 2 ml Tris/HCl buffer (0.05 M, pH 8.4). Alternatively, 1–2 g of whole mosquitoes were homogenized in the same buffer on ice in a glass homogenizer and centrifuged for 10 min at 3500 × g. Either homogenate was then applied to a diethylaminoethyl cellulose anion exchange chromatography column (DE-52, Whatman) equilibrated with Tris/HCl buffer, and eluted with 300 mM NaCl in Tris/HCl buffer[22]. Soybean trypsin inhibitor was used for affinity purification of the various trypsins, which was performed as described earlier[22] with the modification that the pH of the elution buffer for the affinity column was 2.4 for all experiments, except for *An. albimanus* trypsin which was eluted at pH 2.6. Elution of trypsin was most efficient at low pH in the presence of 10 mM TAME. Removal of TAME and its hydrolyzed products is described below. The final yield of activity after two columns was 41% for *Ae. epactius*, 30% for *Ae. triseriatus*, 40% for *Ae. aegypti*, 60% for *An. albimanus*, 59% for *An. stephensi*, and 54% for *An. quadrimaculatus*.

Analysis of molecular masses with size exclusion chromatography was conducted at 4 °C on a Sephadex G-200 superfine column (95 cm × 2 cm) equilibrated with Tris/HCl buffer (0.05 M, pH 8.4) and calibrated with bovine serum albumin, ovalbumin, myoglobin and blue dextran. To follow purification, aliquots of homogenates or enzyme fractions were incubated with tritium-labelled 1,3-^{3}H-di-isopropyl phosphorofluoridate (DFP, Amersham) for 4–6 h at room temperature and analyzed by electrophoresis.

Purification by high pressure liquid chromatography (HPLC). Affinity-purified *Aedes aegypti* trypsin was concentrated on a small DEAE-column and eluted at 300 mM NaCl in Tris-buffer. The sample was diluted 1:10 in 0.1% trifluoroacetic acid (TFA) prepared in double-glass-distilled water. The protein was applied to a reversed phase VYDAC C-4 column (25 × 0.46 cm, 5 µm particle size, 300 A pore size) which was controlled by an ANACOMP 220 computer (Kontron, Switzerland). The column was washed with 0.1% TFA and the protein was eluted with an increasing gradient of acetonitrile and TFA formed by mixing in buffer B (0.85% TFA, 95% acetonitrile). The shapes of individual gradients are indicated in figures 2 and 3. Peaks absorbing at 280 nm were collected manually at a flow rate of 1 ml/min. Three main peaks were separated and rechromatographed. Peaks A and C (fig. 2 top) were diluted with 30 mM phosphoric acid and applied in separate runs to the C-4 column. The gradient was formed by adding buffer B (30 mM phosphoric acid, 95% acetonitrile). Finally, the purified peaks were exchanged into the volatile TFA buffer system by rechromatography under the same conditions as the first HPLC-purification step. Peak B (table 2) was treated similarly in a separate set of purification experiments.

Optimal HPLC separation conditions for affinity-purified trypsin were worked out in pilot experiments. Trypsin, covalently labelled with ^{3}H-DFP was identified by counting individual fractions. The greater part of the radiolabel was found in unreacted and hydrolyzed DFP, which eluted between 0 and 10 min. ^{3}H-DIP-trypsin appeared between 20 and 40 min. The manually collected peak A contained 122,000 cpm while the neighboring fractions contained 7800 and 20,000 cpm, respectively. Peak C contained 570,000 cpm, compared with 6700 and 16,000 cpm in the neighboring fractions (compare fig. 2, middle and lower panels).

An. quadrimaculatus trypsin was purified according to an abbreviated protocol: the affinity-purified concentrated protein was taken directly into the phosphoric acid buffer and run as above (fig. 3). The single most prominent peak was diluted with TFA-buffer and rechromatographed on the reversed phase column. In a pilot run the manually collected peak Q contained 550,000 cpm DIP-trypsin, the neighboring fractions 95,000 and 134,000 cpm; all the other fractions contained less than 10,000 cpm (compare fig. 3, lower panel).

Amino acid sequence analysis. Sequence analysis of proteins isolated by reversed phase HPLC were performed on a 470 A Gas/liquid phase microsequenator (Applied Biosystems). Phenylthiohydantoin derivatives of amino acids (PTH amino acids) were identified by reversed phase HPLC using a Model 120 A on-line PTH amino acid analyzer (Applied Biosystems). Experimental protocols for both procedures were as supplied by the instrument manufacturer.

Electrophoresis. A discontinuous polyacrylamide gel electrophoresis system (PAGE) employing 5% polyacrylamide in the stacking gel and 12.5% in the separating gel was used to prepare SDS gels[27]. Proteins were stained with Coomassie brilliant blue (Serva, 0.2% in 30% methanol, 10% acetic acid) for 1 h with slow shaking, then destained in the same solution omitting the dye. Marker proteins (Biorad) were used to estimate molecular masses. For preparation of Western blot screening strips, a 12.5% SDS-polyacrylamide gel was poured without placing a comb in the stacking gel, and the sample was evenly distributed along the whole width. Isoelectric focussing gels (IEF) were prepared according to the manufacturer's manual (Pharmacia). An IEP marker kit (Pharmacia) allowed determination of isoelectric points after Coomassie blue staining (0.02% in 30% methanol, 10% acetic acid). Trypsin was localized on IEF gels by incubation with benzoyl-arginine-naphthylamide

(BANA, Sigma, 22 mg dissolved in 1 ml methanol, then diluted in 50 ml Tris/HCl buffer 0.05 M, pH 8.4) at 37 °C for 20 Min. The released naphthylamide was visualized with Fast GBC (Sigma, 1 mg/ml H_2O).

Immunization procedure. Injection of *An. albimanus* trypsin into a New Zealand White rabbit followed essentially the protocol described earlier[23]. Initial immunization was induced by injecting 100 µg trypsin (in Freund's complete adjuvans), followed three weeks later by a 50-µg booster, both applied intracutaneously at multiple sites into the back. Subsequent boosters consisted of 70 µg and 75 µg i.p. (in Freund's incomplete adjuvans) and finally 55 µg i.v. in phosphate buffered saline (PBS), all at weekly intervals. For terminal bleeding the rabbit was anesthetized by intramuscular injection of Narketan (Chassot, 0.8 ml) plus Rompun (Bayer, 0.8 ml). The carotid artery was severed for the collection of 120 ml blood. The serum was prepared by overnight coagulation at 4 °C and thrice-repeated centrifugation at 1500 × g before it was stored frozen at − 25 °C. Western blots[28] were blocked with bovine serum (20% in PBS) and incubated overnight with the antisera (in PBS containing 10% bovine serum) at 4 °C. Goat/anti rabbit peroxidase coupled IgG (Biorad) in PBS (10% bovine serum) was then applied prior to staining with o-chloronaphthol (18 mg dissolved in 6 ml methanol, diluted with 60 ml PBS containing 30 µl H_2O_2).

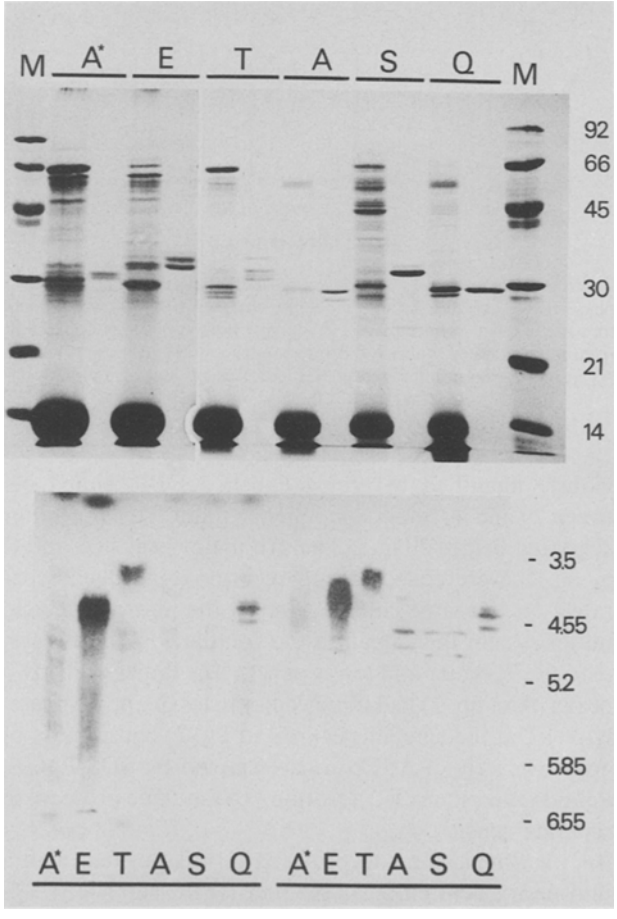

Figure 1. Comparison of mosquito trypsin from 6 different species. Upper panel: raw homogenate or purified proteins were run on an SDS-PAGE and stained with Coomassie dye; each double track was loaded with 80 µg midgut homogenate protein (left) and 3 µg purified trypsin (right). M, marker proteins, size of markers given in kDa at right. A*: *Ae. aegypti*; E: *Ae. epactius*; T: *Ae. triseriatus*; A: *An. albimanus*; S: *An. stephensi*; Q: *An. quadrimaculatus*. Lower panel: isoelectric points of trypsin from the same six species. Raw homogenates of 0.6 midgut equivalents or 2 µg purified trypsin were run on an isoelectric focusing gel with a gradient from pH 2.5 to pH 8. Raw homogenate (left half) and purified trypsin (right half) were incubated with a substrate specific for trypsin (BANA) followed by a color reaction (Fast GBC). Photograph taken from a dried gel to enhance intensity. Abbreviations as above.

Results

Purification of trypsin from different mosquito species. Purification of trypsin from six different mosquito species by DEAE-anion exchange chromatography and soybean trypsin-inhibitor affinity-chromatography yielded protein fractions which consisted of one to six bands as determined by SDS-PAGE (fig. 1). Determination of the molecular masses of the purified proteins is summarized in table 1. All of the observed SDS-bands were DFP-labelled (not shown). Mosquito trypsins from all species investigated had molecular masses in the range 25,000–36,000 Da. To confirm the molecular masses, we applied *An. quadrimaculatus* trypsin to a size exclusion column, which gave a molecular mass of 28,000 + 1000, a good correspondence with the electrophoretically determined mass of 29,900. Assessment of purity was obtained by estimation of the specific activities determined with the substrate TAME and two different protein assays. Specific activities were above those of bovine trypsin (184 U/mg): *Ae. aegypti*, 242 U/mg; *Ae. triseriatus*, 326 U/mg; *Ae. epactius*, 521 U/mg; *An. albimanus*, 372 U/mg; *An. stephensi*, 581 U/mg; *An. quadrimaculatus*, 1065 U/mg. The relative specific activities varied considerably within a genus and reached an almost 5-fold

Table 1. Molecular masses and isoelectric points of trypsin from 6 mosquito species. Molecular masses of purified proteins in figure 1 are given in Daltons as determined by SDS-PAGE, while the same samples run on an isoelectric focussing (IEF) gel were Coomassie-stained and compared with an IEP-standard. To identify trypsin forms, an IEF run was stained with a trypsin-specific substrate. For comparison, corresponding data for *Ae. aegypti* are taken from our earlier report[22]. Note that IEPs and molecular masses do not correspond.

Ae. aegypti	Da	32,000 31,000 30,700 28,700 26,700
	pH	5.4/5.2/4.9/4.4/4.2
Ae. triseriatus	Da	35,900 33,300 32,700 31,600 30,800 28,800
	pH	4.4/4.2/3.9/3.8/3.7/3.6
Ae. epactius	Da	35,500 34,100 33,000 29,000
	pH	4.85/4.75/4.4/4.3/4.25/4.05/3.95/3.9
An. albimanus	Da	29,800 28,600
	pH	4.8/4.65
An. stephensi	Da	33,900 32,700 25,100
	pH	4.85/4.65
An. quadrimaculatus	Da	29,900
	pH	4.65/4.4/4.3

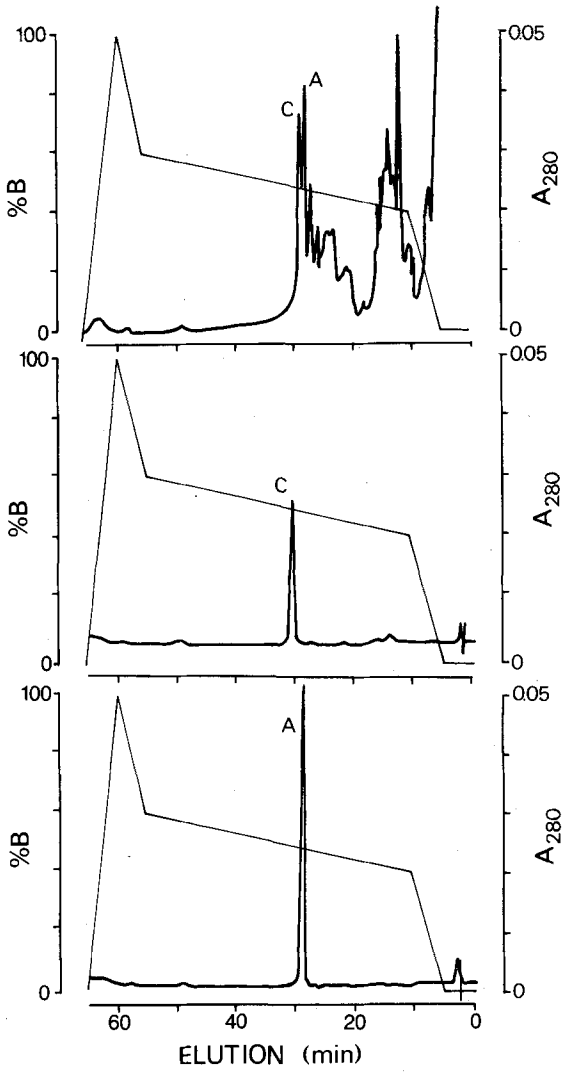

Figure 2. Purification of *Ae. aegypti* trypsin forms with a reversed phase HPLC column. Starting material (upper panel) was an affinity eluate which was run with a TFA/acetonitrile gradient at 1 ml/min. The collected peaks were run on a phosphate/acetonitrile gradient, followed by a TFA/acetonitrile gradient. The resulting peaks are shown in the middle panel (peptide C) and lower panel (peptide A), respectively. A_{280}: optical density at 280 nm, %B indicates amount of buffer B. Peptide B, purified in another run (not shown), eluted at 27 min.

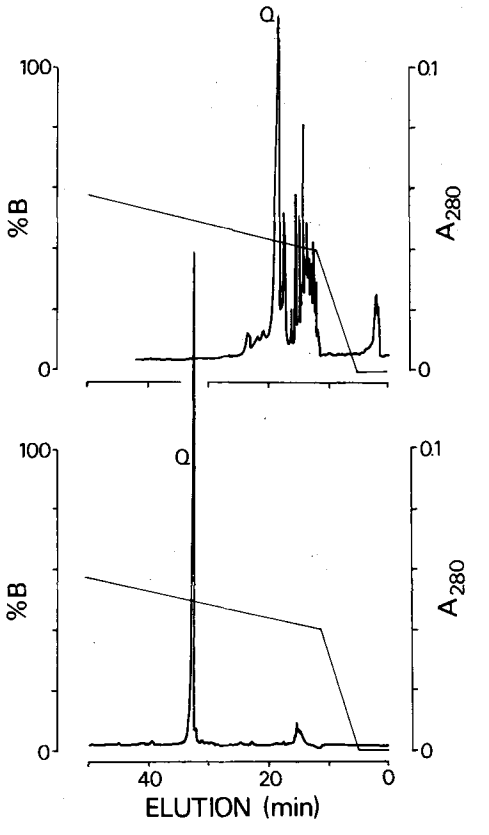

Figure 3. Purification of *An. quadrimaculatus* trypsin with a reversed phase HPLC column. An affinity eluate was run with a phosphate/acetonitrile gradient (upper panel), the largest peak (Q) was collected and rechromatographed with a TFA/acetonitrile gradient (lower panel). A_{280}: optical density at 280 nm, %B indicates amount of buffer B.

difference between *An. quadrimaculatus* and *Ae. aegypti* trypsin. Isoelectric points determined by IEF-PAGE ranged between pH 3.5 and 5.4 and are summarized in table 1. To detect artifactual pH shifts during the purification procedure (fig. 1, lower panel), both midgut homogenate (left) and purified fractions (right) of each species were analyzed, and showed a satisfactory agreement between their respective IEPs, with minor differences in the *Aedes* group.

Affinity eluates of *Ae. aegypti* trypsin were rechromatographed on an anion-exchange column to remove TAME-products and to concentrate the sample. Reversed phase HPLC chromatography separated trypsin into several distinct peaks (A, B, C, fig. 2). Trypsin, previously identified by tritium-labelled DFP, eluted between 20 and 40 min. Components of the Tris/HCl buffer appeared before 20 min. The two major peaks (A and C in fig. 2) were collected and rechromatographed separately on the same column, first in the phosphoric acid buffer system then again in the volatile TFA buffer system (fig. 2, center and lower panel). The larger of the two peaks (A in fig. 2) had a molecular mass of approximately 30 kDa, the smaller peak (C in fig. 2) had a mass of approximately 29.5 kDa as determined by SDS-PAGE. Peptide B was purified according to the same protocol in separate runs.

An. quadrimaculatus trypsin was HPLC-purified with a phosphoric acid buffer in the first HPLC-run (fig. 3, upper panel) followed by the TFA-buffer in the second run (fig. 3, lower panel). This purified peak comigrated with the original affinity-purified trypsin on SDS-PAGE (not shown).

Determination of amino terminal sequences. The amino-terminal sequences of the three peptides of *Ae. aegypti* trypsin (A, B, C) and of that of *An. quadrimaculatus* (Q) are shown in table 2. All three *Ae. aegypti* peptides show identical sequences in the stretches of homologous amino acids (table 3). Trypsin B appears to be a truncated form

Table 2. Amino terminal sequences of *Aedes aegypti* trypsin (A, B, C) and of *Anopheles quadrimaculatus* trypsin (Q). Initial yield was consistently around 50%; repetitive yields were 93.8% for A, 92.7% for B, 90.2% for C and 93.7% for Q. X indicates that no PTH amino acid was identified in this cycle. The sequences of peptides A and Q were determined twice, and those of peptides B and C were determined once.

Cycle	Phenylthiohydantoin amino acid (pmol)			
	A	B	C	Q
1	Val 206	X* –	Val 220	Val > 291
2	Val 202	Val 39	Val 190	Val > 291
3	Asn 199	Glu 35	Asn 150	Gly > 456
4	Gly 153	Leu 26	Gly 135	Gly > 456
5	Gln 175	Ser 12	Gln 115	Phe > 308
6	Thr 80	Gln 32	Thr 77	Glu > 342
7	Ala 159	Gly 27	Ala 99	Ile > 380
8	Thr 62	Arg 7	Thr 62	Asp > 258
9	Leu 120	Ala 36	Leu 67	Val > 291
10	Gly 93	Leu 19	Gly 88	Ser 283
11	Gln 112	X –	Gln 74	Glu 248
12	Phe 99	Gly 21	Phe 60	Ala 372
13	Pro 78	Gly 26	Pro 60	Pro 228
14	Phe 68	Ser 6	Phe 39	Tyr 246
15	Gln 59	Leu 9	Gln 36	Gln 197
16	Val 59	Leu 11	Val 32	Val 244
17	Leu 56	Ser 5	Leu 28	Ser 148
18	Leu 73	Asp 4	Leu 34	Leu 211
19	Lys 37	X	Lys –	Gln 139
20	Val 51	Asp 3	Val 21	Tyr 150
21	Glu 32	X	Glu 17	Tyr 217
22	Leu 46	X	Leu 22	Asn 109
23	Ser 14	Thr 2	Ser 23	Arg 145
24	Gln 27	Val 6	Gln 13	His
25	Gly 34		Gly 34	Asn 90
26	Arg 29		Arg 5	–
27	Ala 32		Ala 16	Gly 123
28	Leu 34		Leu 17	Gly 141
29	X			Asp 32
30	Gly 32			Ser 62
31	Gly 37			Leu 80
32	Ser 9			Asn 49

* This residue was not identified due to contamination present in the first cycle derivative.

of trypsin A or C. Homologies of the amino terminal sequences of the mosquito trypsins with vertebrate trypsin suggest that they belong to the family of serine proteases. This conclusion is based on the finding that several stretches of highly conserved sequences overlapped with corresponding segments in serine proteases of other organisms (table 3). The overall homology between the mosquito and vertebrate trypsin N-terminal regions is only 30–40%, and the homology between the two mosquito species compared does not exceed 40% (table 3, lower segment).

Immunological cross-reactivities. To evaluate relationships between the various mosquito trypsins, immunological methods were employed. In addition to the *Ae. aegypti* trypsin antiserum[23] a polyclonal antiserum against *An. albimanus* trypsin was produced. Two proteins were recognized on a Western blot loaded with *An. albimanus* raw homogenate (not shown). The specificity of the antiserum was tested by preincubation with excess trypsin, which resulted in the removal of all cross-reactivity.

The polyclonal antiserum against *Ae. aegypti* trypsin was applied to a Western blot containing midgut homogenates from seven species. To minimize biased interpretation, we loaded an average of 0.2–0.4 µg trypsin per band, with the exception of *An. stephensi* where only about 0.06 µg could be loaded because the activity present in this homogenate was much lower. Raw homogenate was used to ensure that the combination of different trypsin forms was not distorted during isolation procedures. A strong reaction of the *Ae. aegypti* trypsin antiserum with the original antigen and a somewhat weaker cross-reactivity with *Ae. epactius* and *Ae. triseriatus* trypsins was observed, while *An. quadrimaculatus* trypsin was only faintly recognized. *Culex pipiens* trypsin also showed cross-reactivity with this antiserum (fig. 4 upper panel). In contrast, when an antiserum against *An. albimanus* trypsin was used on a similar blot (fig. 4, lower panel) *Ae. aegypti* trypsin was not stained, neither were the 30 kDa trypsins of both *Ae. epactius* and *Ae. triseriatus* (compare triangles at 30 and 33 kDa), while *An. quadrimaculatus* trypsin was stained much more with this antiserum. From this, we conclude that there are at least two immunogenically different sets of mosquito trypsins.

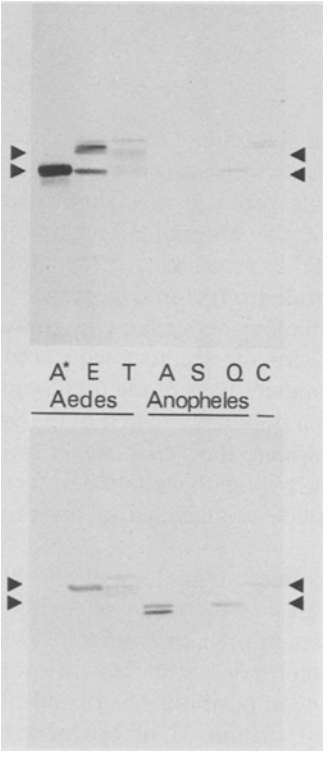

Figure 4. Immunological cross-reaction of *Ae. aegypti* (top) and *An. albimanus* (bottom) trypsin antisera with trypsin of other species. Proteins from an SDS-PAGE, loaded with midgut homogenates, were transferred electrophoretically to a nitrocellulose membrane. Above, the immunoblot incubated with the *Ae. aegypti* antiserum at a dilution of 1:1000, followed by the peroxidase coupled secondary antibody at 1:500; the blot was then developed with the peroxidase reaction. Below, the blot incubated with *An. albimanus* trypsin antiserum at a dilution of 1:2000. Bovine trypsin (not shown) was not recognized by either antiserum. Triangles point to 33 and 30 kDa, respectively. Abbreviations: C for *Culex pipiens*, others as in figure 1.

Table 3. Amino terminal sequences of *Aedes aegypti* trypsin (A, B, C) and of *Anopheles quadrimaculatus* trypsin (Q). Sequences of known vertebrate and invertebrate proteases were manually aligned to fit. Amino acids are given in the one letter code. Arbitrary numbering starts with 1 at the mature N-terminus. The lower segment gives the abbreviations for the various sources and the extent of homologies with the 2 mosquito species are expressed as percentages. The X at position 29 is assumed to represent cysteine. To calculate % homology the sequences of peptide A & B were combined to give a hypothetical peptide of 42 amino acids. X = unknown amino acids. There were not counted except for position 29.

TryA	V V N G Q T A T L G	Q F P F Q V L L K V	E L S - Q G R A L X	G G S	
TryB		X V	E L S - Q G R A L X	G G S L L S D X D X	X T V
TryC	V V N G Q T A T L G	Q F P F Q V L L X V	E L S - Q G X A L		
TryQ	V V G G F E I D V S	E A P Y Q V S L Q Y	- - Y N - R H N - X	G G X L N	
BovTry	I V G G Y T C G A N	T V P Y Q V S L - -	- N S - G Y H F - C	G G S L I N S Q W V	V S A
BovChy	I V N G E E A V P G	S W P W Q V S L Q D	- K T - G F H F - C	G G S L I N E N W V	V T A
DroTry	I V G G S A T T I S	S F P W Q I S L Q -	- R S - G S H S - C	G G S I T S A N I I	V T A
CraTry	I V G G T D A V L G	E F P Y Q L S F Q -	E T F L G F H F - C	G A S I Y N E N Y A	I T A
CrbCol	I V G G V E A V P N	S W P H Q A A L - -	- F I D D M Y F - C	G G S L I P S - W I	L T A
CrbCII	I V G G Q D A T P G	Q F P Y Q L S F Q D			
HorChy	I V G G T N A P R G	K Y P Y Q - S L - -	- - R A P K H F - C	G G S - I S K R Y V	L T A
HypCol	I I N G Y E A Y T G	L F P Y Q A G L D I	T L Q D Q R R V W C	G G S L I D N K W I	L T A
HypTry	I V G G V E M K I E	X F P W E I X L Q X	P		
AntCoc	I V G G Y T I G I D	T V P Y			

Peptide		*Ae. aegypti*	*An. quad.*	Ref.
BovTry	Bovine trypsin	34%	39%	(32)
BovChy	Bovine chymotrypsin A	35%	39%	(33)
DroTry	Drosophila trypsin	31%	34%	(21)
CraTry	Crayfish trypsin	32%	32%	(34)
CrbCol	Crab collagenase	32%	32%	(35)
CrbCII	Crab collagenase II	50%	40%	(29)
HorChy	Hornet chymotrypsin	35%	37%	(19)
HypCol	Hypoderma collagenase	43%	36%	(18)
HypTry	Hypoderma trypsin	24%	39%	(17)
AntCoc	Antheraea cocoonase	29%	43%	(20)

Discussion

Comparison of the amino terminal sequences of the 4 mosquito trypsins with sequences from both vertebrate and invertebrate proteases are summarized in table 3. Homologies of the N-terminal region of the various trypsins with other proteases exceeded 40% only with a very short peptide from crab collagenase [29]. Interestingly, even the homology between the two mosquito trypsins was only 38% for the segment sequenced, which comprises approximately 12% of the total sequence of either protein. When a homology search of the Swissprot database was performed, the *Aedes aegypti* peptide revealed significant similarity with coagulation factor X, and the *Anopheles* peptide was similar to the proteins elastase and kallikrein.

The overall sequence homology of 30–40% within the N-terminal region is the result of highly conserved sequence stretches in otherwise unrelated segments of the sequence. The conserved stretches are located at the amino terminus and at positions 11–19 and 29–32. Position 29 of trypsin (position 31 of bovine trypsinogen and position 42 of chymotrypsinogen A [30] is characterized by a cysteine residue; in the present sequence analysis of both species, no amino acid residue was identified in this position, which indicates that a cysteine at that position is likely. Thus, in a class of enzymes which is highly conserved among vertebrates, there is a remarkable variability between these insect species and genera.

The differential recognition of the two antisera employed suggested at least two enzyme families, on the basis that *Ae. aegypti* trypsin antiserum did not cross-react with trypsin from *An. albimanus* and vice versa. The cross-reactivities with other trypsins are complex but support our conclusion by the fact that the 33,000 Da trypsin of *Ae. epactius* and *Ae. triseriatus* are recognized by both antisera, while the 30,000 Da trypsin of these two species are recognized solely by *Ae. aegypti* antiserum. The existence of two trypsin families among six species could possibly be derived from two genetic loci which separated before the branching of the two genera *Anopheles* and *Aedes*. The absence of staining of *An. stephensi* trypsin with either antiserum suggests the presence of a third family. However, since we do not have an antiserum against *An. stephensi* trypsin at hand we cannot probe for cross-reactivities with our other trypsins.

Structural differences in these mosquito trypsins are also reflected in the number and the molecular size of the trypsin forms. Molecular mass determinations by SDS-PAGE are accurate only in the absence of sugar residues. For *Ae. aegypti* trypsin there is no evidence for glycosylation [22], and neither wheat-germ agglutinin binding to trypsin, nor molecular mass shifts of trypsins in tunicamycin-treated midguts were observed (not shown). Molecular masses estimated by SDS-PAGE in all species investigated were higher than the masses found for vertebrate trypsin [31]. This has been confirmed previously for *Ae. aegypti* trypsin [22] and in this report for *An. quadrimaculatus*, by size exclusion chromatography. These increased molecular masses of mosquito trypsin fit into the broad spectrum of values from other insect proteases [16].

Enzymatic staining of *Aedes* trypsin run on IEF-PAGE (fig. 1) appeared rather diffuse. When the purified trypsin was examined by Coomassie stain, however, many distinct bands were resolved at that particular pH-segment. We had described such a complex pattern previously for *Ae. aegypti*[22], and now it was also found in two additional *Aedes* species. A purification artifact can be excluded because homogenates (fig. 1, left) of freshly dissected midguts showed staining patterns similar to those of the purified trypsins, with minor differences (fig. 1, right). In contrast, with *Anopheles* trypsin, a few distinct bands were consistently observed. It is possible that in *Aedes* autolysis and degradation, leading to micro-heterogeneity, are physiological processes occurring during the digestive cycle. In *Anopheles*, however, trypsin might be more stable. The investigated mosquito species belong to one family, representing a small group of highly specialized insects which are adapted to the efficient digestion of a unique diet, i.e. vertebrate blood and yet there is a considerable structural diversity in their major proteolytic system. The specific activities of the pure trypsin, the isoelectric points and also the molecular masses vary significantly among these species. This is clearly reflected in the low homology between two selected amino terminal sequences of mosquito trypsin. Compared to those of vertebrates, this insect proteolytic system seems to have maintained a surprising plasticity throughout its evolution.

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