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Preliminary Characterization of Enzyme Activities in Malpighian Tubules Involved in the Breakdown of Adipokinetic Hormones

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Adipokinetic hormones (AKH) from different insect species, crustacean red pigment-concentrating hormone (RPCH), and synthetic substrates were used to characterize enzyme activities present in the Malpighian tubules (MT) of the desert locust, *Schistocerca gregaria*, which are involved in the degradation of AKH.

When peptides containing proline (position 6) were incubated with MT homogenate they were cleaved by a post-proline cleaving enzyme (PPCE). The presence of such an enzyme was confirmed by the breakdown of a synthetic substrate for PPCE. Peptides which do not contain proline were broken down by a post-phenylalanine cleaving enzyme (PFCE) which could be chymotrypsin or chymotryptic. This PFCE activity(ies) seem(s) to be inactive on the proline-containing peptides or their fragments or digests these at a slow rate. The C-terminal chymotrypsin fragments of the AKHs were broken down by MT homogenates with no accumulation of new intermediate products. It is not clear whether another endopeptidase, PPCE, or leucine aminopeptidase (LAP) is responsible.

The MTs contain LAP activity; however, this enzyme(s) may be different from its vertebrate counterpart(s). Homogenates of MTs break down equimolar amounts of Pro-7AMC and Leu-7AMC at approximately the same rate, while porcine kidney LAP (cytosol) cleaved Pro-7AMC much slower than Leu-7AMC.

The demonstration of carboxypeptidase (CP) A and B activity in the MTs was not possible using conventional substrates such as hippuryl derivatives of amino acids. When CPA from porcine pancreas was added to MT homogenates hippuryl-phenylalanine was digested proving that the conditions were appropriate for CPA activity to occur. The treatment of a N-terminally blocked peptide fragment with MT homogenate led to the breakdown of the peptide giving evidence that the MT CP requires a substrate with a somewhat longer length of amino acid residues.

Key words: enzymatic degradation of peptides, leucine amino peptidases, carboxypeptidases A and B, post-proline cleaving enzyme, post phenylalanine cleaving enzyme

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INTRODUCTION

In *in vitro* incubations only the MT from the American cockroach, *Periplaneta americana*, removed neurohormone D from the bathing medium, other tissues were inactive [1]. Homogenates of the MTs* from the desert locust, *Schistocerca gregaria*, break down AKHI and AKHII-S ([2]; Siegert and Mordue [unreported results]). These insect neuropeptides are blocked by a pyroglutamate residue at the N-terminus and by an amide at the C-terminus. These modifications are believed to protect the peptides against breakdown by exopeptidases in the hemolymph. The fate of AKH in *in vitro* incubations can be influenced greatly by the choice of conditions. In a phosphate buffer at pH 8, major peptide fragments accumulate and can be characterized. From these fragments the sites of endopeptidic attack can be deduced (Siegert and Mordue, unreported results). The initial attack on AKHI is performed by a PPCE, while AKHII-S is broken down by a PFCE. Since AKHI also contains phenylalanine it is of interest to find out why the endopeptidase attack is different for these two peptides. Once the peptides are cleaved by the endopeptidases the now available unblocked N- and C-termini of the two fragments can be attacked by exopeptidases: LAP(s) and CP(s).

In the present paper we describe preliminary data characterizing PPCE, PFCE, as well as LAP and CPs from *S. gregaria* MTs.

MATERIALS AND METHODS

Insects

Desert locusts, *S. gregaria*, were kept as described by Morgan and Mordue [3].

Chemicals

Pro-7AMC, Leu-7AMC, H-Arg, H-Phe, BOC-VLGR, Z-GGP, CPA (type I, PMSF-treated; bovine pancreas), LAP (porcine kidney, cytosol), and α-chymotrypsin (type VII, TLCK-treated; bovine pancreas) were purchased from Sigma. The AKH from *S. gregaria* (AKHI and AKHII-S) and the cockroach MI were from Peninsula. The HTH from the cockroach, *Blaberus discoidalis*, was a gift from Dr. Timothy Hayes (A&M University, Department of Entomology, College Station, TX) and the AKH-M from the tobacco hornworm, *Manduca sexta*, and HTHII from the corn earworm, *Heliothis zea*, were gifts from Dr. Stuart E. Reynolds (University of Bath, School of Biological Sciences, England). The RPCH

*Abbreviations used: AKH = adipokinetic hormone; AKHI = adipokinetic hormone I from *L. migratoria* and *S. gregaria*; AKHII-S = adipokinetic hormone II from *S. gregaria*; AKH-M = adipokinetic hormone from *M. sexta*; BOC-VLGR = N-t-BOC-Val-Leu-Gly-Arg; CPA and CPB = carboxypeptidase A and B; H-Arg = hippuryl arginine; Hip = hippuric acid; H-Phe = hippuryl phenylalanine; HTH = hypertrehalosemic hormone from *B. discoidalis*; HTHII = hypertrehalosemic hormone II from *H. zea*; LAP = leucine aminopeptidase; Leu-7AMC = leucine 7-amido-4-methyl-coumarin; MI = myotropic peptide I from *P. americana*; MT = Malpighian tubule; PCAH = proline-containing adipokinetic hormones; PFAH = proline-free adipokinetic hormones; PFCE = post-phenylalanine cleaving enzyme; PMSF = phenylmethanesulfonyl fluoride; PPCE = post-proline cleaving enzyme; Pro-7AMC = proline 7-amido-4-methyl-coumarin; PTC = phenylthiocarbamyl; PTH = phenylthiohydantoin; RPCH = red pigment-concentrating hormone; RT = retention time; TFA = trifluoroacetic acid; TLCK = Nα-p-Tosyl-L-lysine chloromethyl ketone; Z-GGP = N-CBZ-Gly-Gly-Pro.

from the shrimp, *Pandalus borealis*, was a gift from Dr. Colin Wheeler (University of London, Birkbeck College, England).

Preparation and Incubation of MT Homogenates With AKH Peptides and Synthetic Substrates

Under insect saline (128 mM NaCl, 5 mM KCl), MTs were removed from the digestive system and sonicated in buffer 1 (90 mM KH_2PO_4, 20 mM Na_2EDTA, pH 8; used for PPCE and PFCE), buffer 2 (100 mM TEA·HCl, 5 mM $MgCl_2 \cdot 6H_2O$, pH 8; used for LAP), buffer 3 (20 mM TEA·HCl, 200 mM NaCl, pH 7.5; used for CPA), or buffer 4 (200 mM citric acid, 150 mM NaCl, pH 7; used for CPB). After centrifugation (10 min, 12,000*g*), the supernatant was mixed with AKH peptides or other substrates dissolved in distilled water (final concentrations 1–5 μg per 400 μl). Aliquots were withdrawn immediately and after the times indicated in the text or figures at 30°C and 0.1% TFA added to bring the volume to 500 μl. Samples were then centrifuged (5 min, 12,000*g*) and stored on ice until further use.

Reverse-Phase High-Performance Liquid Chromatography

The equipment described by Siegert et al. [4] was used. The reaction mixture plus 0.1% TFA (see above) was injected onto an Aquapore RP-300 column and eluted with 0.1% TFA (pump A) and acetonitrile (pump B) as eluants at a flow rate of 1 ml min^{-1}. Two gradients were employed; gradient 1 started at 0%B and increased at a rate of 2%B min^{-1}; gradient 2 started at 16%B with an acetonitrile gradient of 0.3% min^{-1}.

Amino Acid Analysis

Analyses were carried out on an Applied Biosystems 420A Analyser. PTC derivatives of amino acids were identified using a 130A separation system with a C-18 reverse-phase narrow bore cartridge and β-lactoglobulin (A & B Variants) as standard.

Gas-Phase Sequencing

An Applied Biosystems sequencer was used and the PTH amino acid derivatives were analysed using a Waters HPLC system as outlined by Siegert and Mordue [5].

RESULTS

Breakdown of Adipokinetic Hormones by Endopeptidases: Post-Proline and Post-Phenylalanine Cleaving Enzymes

The presence of PPCE and PFCE in *S. gregaria* MTs has been established through the analysis of peptide fragments arising from enzymatic breakdown of AKHI and AKHII-S as well as through the use of synthetic substrates for the above enzymes (Siegert and Mordue, unreported data). The pathways of degradation were investigated for different naturally occurring AKH peptides.

The amino acid sequence of AKHI at the phenylalanine residue is slightly different from AKHII-S (see Table 1); PFCE may recognize specifically the AKHII-S sequence Asn-Phe-Ser (in AKHI this part is Asn-Phe-Thr). The HTH

TABLE 1. Peptide Sequences*

Proline-containing peptides	
Locust AKHI	pGlu-Leu-Asn-Phe-Thr-Pro-Asn-Trp-Gly-ThrNH_2
Cockroach HTH	pGlu-Val-Asn-Phe-Ser-Pro-Gly-Trp-Gly-ThrNH_2
Cockroach MI	pGlu-Val-Asn-Phe-Ser-Pro-Asn-TrpNH_2
Shrimp RPCH	pGlu-Leu-Asn-Phe-Ser-Pro-Thr-TrpNH_2
Proline-free peptides	
Locust AKHII-S	pGlu-Leu-Asn-Phe-Ser-Thr-Gly-TrpNH_2
Manduca AKH	pGlu-Leu-Thr-Phe-Thr-Ser-Ser-Trp-GlyNH_2
Heliothis HTHII	pGlu-Leu-Thr-Phe-Ser-Ser-Gly-Trp-Gly-AsnNH_2

*Sequences of the proline-free and proline-containing AKH peptides used in the present study.

from *B. discoidalis* is identical in positions 3–5 with AKHII-S but contains proline in position 6. When this peptide was incubated with MT homogenate (buffer 1), two major peaks were produced (Fig. 1): HTH-1 (RT: 20.5 min) and HTH-2 (RT: 15.6 min). The intact peptide eluted after 22.8 min. Peak HTH-1 did not absorb at 280 nm (not shown) and thus did not contain tryptophan. Amino acid analysis (data shown in legend to Fig. 1) revealed that this fragment represented the N-terminal part of HTH from the pyroglutamate to the proline residue (positions 1–6). The peaks eluting after 15.6 (HTH-2), 15.2 (HTH-3), and 14.7 min (HTH-4) all absorb at 280 nm (not shown). Tryptophan eluted on this gradient after 15.6 min. The fragment HTH-3 may contain Gly-Trp-Gly-ThrNH_2, while HTH-4 contained Trp-Gly-ThrNH_2. The sequence Asn-Phe-Ser does not seem to determine that a peptide is cleaved by PFCE.

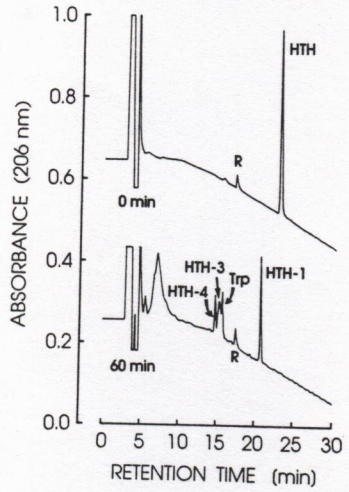

Fig. 1. Incubation of HTH from *B. discoidalis* with MT homogenate (buffer 1). **Top:** MT homogenate plus HTH, t = 0 min. **Bottom:** t = 60 min. Gradient 1. Retention times: HTH 22.8 min; HTH-1 20.5 min pGlu-Val-Asn-Phe-Ser-Pro (amino acid analysis: Glx 6,875 pmol [1X], Val 5,881 pmol [1X], Asx 5,844 pmol [1X], Phe 6,359 pmol [1X], Ser 5,618 pmol [1X], Pro 6,218 pmol [1X]; other amino acids were present at levels below 600 pmol). The failure of CPA (buffer 3) to cleave this fragment indicate the presence of a C-terminal Pro (not shown); Trp (HTH-2) 15.6 min; HTH-3 15.2 min; Gly-Trp-Gly-ThrNH_2 (Gly 10,549 pmol [2X], Trp not protected, but HTH-4 absorbs at 280 nm (1X), Thr 6,866 pmol [1X]); HTH-4 14.7 min; Trp-Gly-ThrNH_2 (Trp 2,846 pmol [1X], Gly 5,633 pmol [1X], Thr 5,194 pmol [1X]). A riboflavin compound (R) eluted after 17.4 min.

The occurrence of the C-terminal tetrapeptide HTH-3 also confirmed the presence of PPCE. The corresponding fragment for AKHI could not be identified [2].

Since proline introduces a change of direction into the peptide backbone, its presence may be of importance. The cockroach peptide MI (Table 1) was cleaved by MT homogenates producing a fragment with identical RT to HTH-1 indicating that MI was also cleaved behind the proline residue in position 6 (data not shown).

A natural analogue to AKHII-S is RPCH; these two peptides only differ in position 6 where RPCH contains proline but in AKHII-S threonine is present. To obtain an insight as to how this affects the degradation pathway, AKHII-S and RPCH were incubated together with MT homogenate. If both were cleaved by PFCE, only one fragment corresponding to pGlu-Leu-Asn-Phe should elute after 22.8 min (Siegert and Mordue, unreported data). Figure 2 clearly reveals that two peaks eluted: RPCH-1 after 22.4 min and AKHII-S-1 after 22.8 min. The retention time for the former fragment was confirmed when MT homogenate was incubated only with RPCH; other peaks arising from RPCH were RPCH-2 (Trp, RT: 15.6 min), RPCH-3 (probably Gly-TrpNH_2, RT: 14.9 min),and RPCH-4 (pGlu-Leu-Asn, RT: 11.7 min; amino acid compositions are detailed in legend to Fig. 2).

It was of interest whether proline-containing AKH peptides can give rise to PFCE fragments when incubated with MT homogenates. Since there were no peaks in the HPLC traces which were obvious candidates, *Blaberus* HTH was treated with bovine pancreas chymotrypsin, the breakdown products isolated

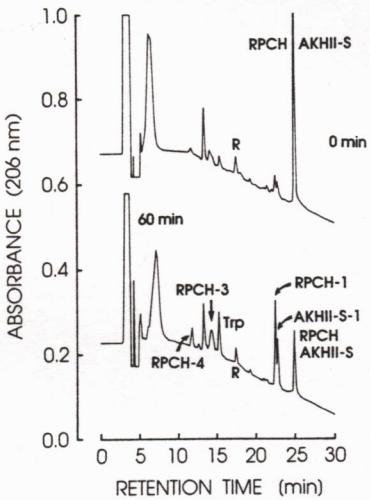

Fig. 2. Simultaneous incubation of *P. borealis* RPCH and AKHII-S with MT homogenate (buffer 1). **Top:** MT homogenate plus peptides, t = 0 min. **Bottom:** t = 60 min. Gradient 1. RT: RPCH and AKHII-S both eluted at 24.8 min when chromatographed separately; RPCH-1 22.4 min; pGlu-Leu-Asn-Phe-Ser-Pro (Glx 3,181 pmol (1X), Leu 3,013 pmol (1X), Asx 3,151 pmol (1X), Phe 3,255 pmol (1X), Ser 2,578 pmol (1X), Pro 2,835 pmol (1X)). Other peaks arising from RPCH were RPCH-2 15.6 min: Trp; RPCH-3 15.2 min. Since these two latter peaks eluted so closely they were gas-phase sequenced together. The first sequencing cycle contained Gly 597 pmol and Trp 547 pmol, the second Trp 400 pmol and Gly 9 pmol, suggesting that RPCH-3 represents the fragment Gly-TrpNH_2 while peak RPCH-2 contained Trp. RPCH-4 11.7 min pGlu-Leu-Asn (Glx 1,576 pmol [1X], Leu 1,615 pmol [1X], Asx 1,113 pmol [1X]). R: 17.4 min (see above).

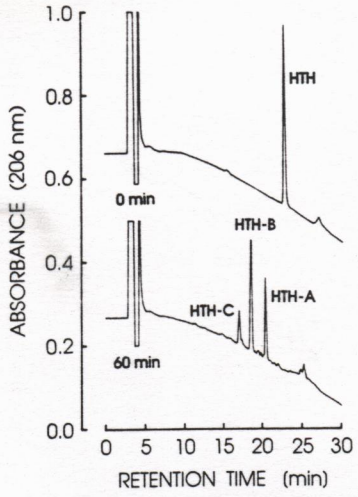

Fig. 3. Digestion of HTH with chymotrypsin (buffer 1). **Top:** t = 0 min. **Bottom:** t = 60 min (AKHI was treated in the same way—no chromatogram shown; Siegert and Mordue [unreported data]). Gradient 1. RT: HTH 22.8 min; HTH-A 20.5 min, pGlu-Val-Asn-Phe (Glx 5,375 pmol [1X], Val 4,640 pmol [1X], Asx 4,940 pmol [1X], Phe 4,936 pmol [1X]); HTH-B 18.6 min, Ser-Pro-Gly-Trp-Gly-ThrNH_2 (Ser 1,592 pmol [1X], Pro 1,915 pmol [1X], Gly 3,651 pmol [2X], Trp not protected, HTH-B absorbed at 280 nm [1X], Gly [see above, 1X], Thr 1,955 pmol [1X]). HTH-C 17.0 min (not analyzed). Not shown: AKHI 24.2 min, AKHI-A 22.4 min (1–4), AKHI-B 17.4 min (5–10).

and their amino acid compositions determined. The *Blaberus* HTH was cleaved into two major peaks: HTH-A (RT: 20.5 min; residues 1–4) and HTH-B (RT: 18.6 min; amino acids 5–10), respectively (details in legend to Fig. 3). A considerably smaller peak HTH-C eluted after 17.1 min (not analyzed). The first two peaks run very close to the pigment and the HTH-1 peaks, respectively, of the above chromatogram (Fig. 1). If there were two peptides eluting on top of one another after 20.5 min, the amino acid analysis data of the fragments obtained from treatment of HTH with MT homogenate should show higher amounts for the first four residues than for serine and proline (positions 5 and 6). This, however, was not found (e.g., see legend to Fig. 1). The peak was collected from several HPLC runs but no indications for more than one peak were ever observed. It can be concluded therefore that only the PPCE fragment was present in the reaction mixture, a view which is supported by results with AKHI and AKHII-S. The fragment pGlu-Leu-Asn-Phe eluted in the above gradient after 22.9 min, but no major peak could be observed in HPLC traces when AKHI was incubated with MT homogenate.

Since AKHI-1 and AKHI-A eluted in the present gradient with very similar retention times, 23.2 and 22.9 min, respectively, it was necessary to design a different gradient capable of unequivocally separating the two peptides (gradient 2). An MT homogenate was prepared as above (in buffer 1) and incubated with AKHI; aliquots were then withdrawn after t = 0, 15, 30, and 60 min. On gradient 2, AKHI eluted after 29.1 min, while AKHI-1 eluted after 18.6 min and AKHI-A after 16.9 min. During a 60-min-incubation ~75% AKHI was broken down (Fig. 4). After 15 min, only a small amount of AKHI-A was visible, but AKHI-1 was a prominent peak. After 60 min, the peak area of AKHI-A

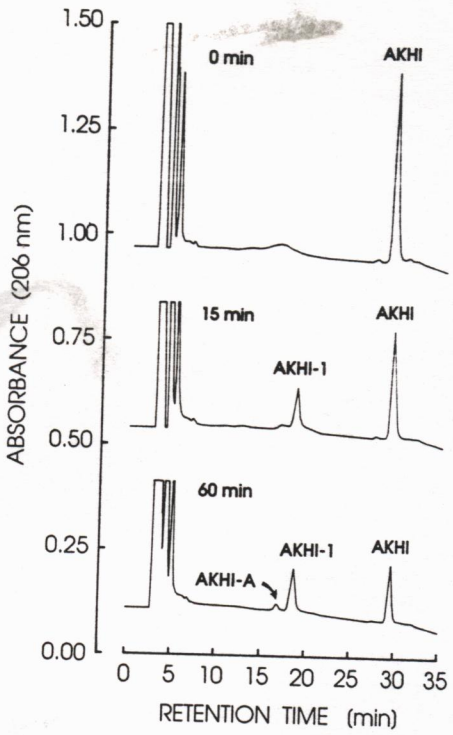

Fig. 4. Incubation of AKHI with MT homogenate (buffer 1). **Top:** MT homogenate plus AKHI, t = 0 min. **Middle:** t = 15 min. **Bottom:** t = 60 min. Gradient 2. RT: AKHI 29.1 min; AKHI-1 18.6 min; AKHI-A 16.9 min.

was approximately 8% of the one for AKHI-1, showing that AKHI-1 is the major primary intermediary breakdown product from which presumably AKHI-A arises.

Two more naturally occurring AKH peptides were tested: AKH-M from *M. sexta*, and HTH-II from *H. zea* (Table 1). The four N-terminal amino acids are identical in these two peptides but AKH-M contains a threonine in position 5 and HTHII contains a serine; both peptides are proline-free. Incubation of these peptides with MT homogenate produced fragments with identical RT of 23.5 min (gradient 1) which do not absorb at 280 nm and are thus tryptophan-free (no chromatogram shown). Amino acid analysis showed that the fragment contained the first four amino acids of the hormone (Glx 1,485 pmol (1×), Leu 1,234 pmol (1×), Thr 1,311 pmol (1×), Phe 1,209 pmol (1×)).

The results of these experiments with different natural AKHs indicate that the presence of proline plays a paramount role in the selection of the degradation site. Peptides containing a proline in position 6 were cleaved between residue 6 and 7; proline-free peptides were cleaved after the phenylalanine residue (position 4). All the peptides listed in Table 1 were also treated with chymotrypsin, the digestion products collected and analysed. In all cases the peptides were cleaved between phenylalanine and the amino acid in position 5. None of the fragments generated by chymotrypsin treatment of proline-containing peptides were observed in substantial amounts when the peptides

were incubated with MT homogenates; the N-terminal tetrapeptides only accumulated when proline-free peptides were incubated.

Secondary Breakdown

When MTs were homogenized in buffer 2, the adipokinetic peptides were completely digested (no chromatogram shown). Only minor peaks of the above mentioned intermediary breakdown products were observed. One major peak, however, was found which corresponded to tryptophan. This results showed that apart from endopeptidases also exopeptidases such as LAP(s) and CP(s) must be present in MTs which cleave peptide fragments with free N- and/or C-termini.

LAP(s) successively removes amino acids from the unblocked N-terminus of peptides and proteins. Its activity can easily be demonstrated with amino acid amides such as TrpNH_2 (Siegert and Mordue, unreported data), since tryptophan absorbs strongly at 206 and 280 nm. Proline-amide does not absorb in the UV range and the amino acid must be coupled to a fluorescent compound such as 7AMC to make it and all the other amino acids available for HPLC analysis. The substrates Leu-7AMC and Pro-7AMC were used to show LAP activity in MT homogenate. When 5 μg of these two substrates were incubated with MT homogenate (buffer 2), both disappeared approximately at the same rate: some 75% Leu-7AMC and ~65% Pro-7AMC were cleaved within 60 min (Fig. 5). When the same amount of amino acid derivatives was incubated with 10 units porcine kidney LAP instead of MT homogenate only 17% of Pro-7AMC disappeared, while in the same reaction mixture Leu-7AMC was entirely cleaved (Fig. 6). This suggests that *S. gregaria* MTs either contain a LAP which is better adapted to the degradation of Pro-7AMC or they contain a special

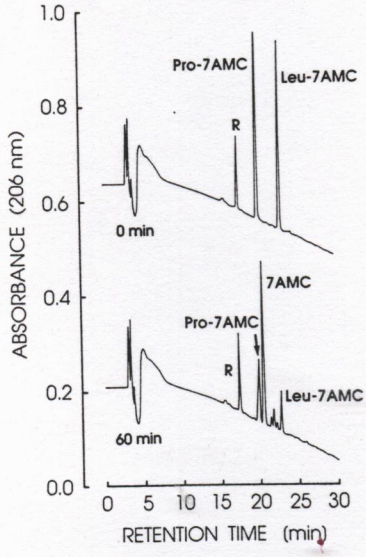

Fig. 5. Treatment of Pro-7AMC and Leu-7AMC with MT homogenate (buffer 2). **Top:** t = 0 min. **Bottom:** t = 60 min. Gradient 1; RT: Leu-7AMC 22.7 min, 7AMC 20.2 min, Pro-7AMC 19.6 min; R 17.4 min (see above).

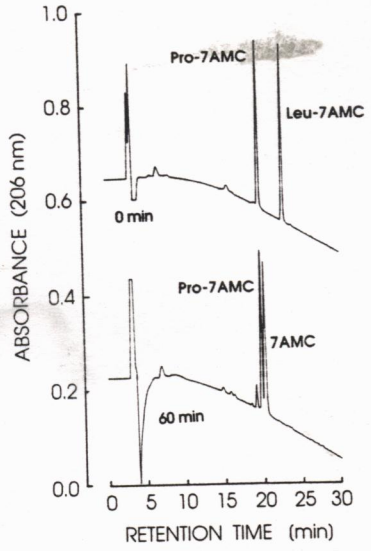

Fig. 6. Treatment of Pro-7AMC and Leu-7AMC with bovine pancreas LAP (buffer 2). **Top:** t = 0 min. **Bottom:** t = 60 min. Gradient 1; RT: Leu-7AMC 22.7 min, 7AMC 20.2 min, Pro-7AMC 19.6 min.

enzyme which cleaves N-terminal proline residues (PPCE from lamb kidneys does not cleave substrates like Pro-7AMC [7]).

To further explore the LAP activity in the MTs, the C-terminal part of AKHI containing residues 5–10 was produced through chymotrypsin digestion and then incubated with MT homogenate (buffer 2). It was found that this compound (eluting after 17.4 min) was easily broken down. It must be borne in mind, however, that two enzyme activities may have been attacking the peptide: LAP and PPCE. There was, however, no indication of a new intermediary product suggesting the action of PPCE on this fragment (no chromatogram shown).

The presence of CPs is usually demonstrated with synthetic substrates in which an amino acid is N-terminally linked to hippuric acid. Since there are different types of CPs, we used H-Phe for CPA and H-Arg for CPB. When MT homogenate (see legend to figures for buffers) was incubated with either of these substrates, neither was broken down in substantial amounts (not shown). The substrate H-Phe was digested after the addition of 5 units CPA, demonstrating that the conditions were suitable for CPA. To show the presence of CPs, MT homogenate (buffer 3) was incubated with H-Phe, H-Arg, and *Blaberus* HTH (Fig. 7). Within 60 min HTH completely disappeared while H-Phe and H-Arg were still present in their original amounts (Fig. 7, middle trace). In all experiments performed with HTH in MT homogenate prepared in buffer 1 a peak with RT 20.5 min appeared corresponding to the N-terminal hexapeptide. This peptide, however, was missing from the present chromatogram suggesting that a CP(s) had cleaved all free C-terminal amino acids successively. When 5 units CPA were present in the reaction mixture, H-Phe was also broken down (Fig. 7, lower trace). This showed that CPs were present in MT homogenates but either at very low activities or they could not break down hippuryl derivatives of amino acids.

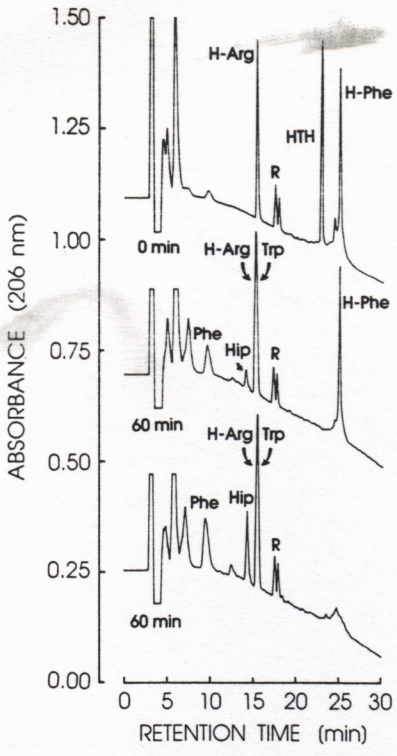

Fig. 7. Incubation of HTH, H-Arg, and H-Phe with MT homogenate and with 5 units CPA (buffer 3). **Top:** Homogenate, H-Arg, H-Phe, HTH, t = 0 min. **Middle:** t = 60 min. **Bottom:** Homogenate, H-Arg, H-Phe, HTH plus CPA, t = 60 min. RT: H-Phe 25.0 min, HTH 22.8 min, H-Arg 15.3 min, R 17.4 min (see above), Hip 14.3 min, Phe 9.4 min. Homogenates in buffer 1 always produced large amounts of HTH-1 (see Fig. 1). In buffer 2, which favours CPA activity this peak must have been cleaved, while both H-Arg and H-Phe were still present (middle trace); the addition of 5 units CPA led to the breakdown of H-Phe, H-Arg was still present (bottom trace).

More suitable substrates for the CPs from *S. gregaria* MTs therefore seem to be AKH fragments which carry C-terminal phenylalanine or arginine residues. Such a fragment with a C-terminal phenylalanine was produced by treating AKHI with chymotrypsin, AKHI-A (pGlu-Leu-Asn-Phe) was then isolated and incubated with MT homogenate and H-Phe (Fig. 8). While there was no breakdown of H-Phe, some 25% of the AKHI-A peak was cleaved demonstrating the presence of CPA in MTs.

To show the presence of CPB the peptide BOC-VLGR was chosen (Fig. 9), since no naturally occurring AKH is known which contains an arginine residue. During a 60-min-incubation period, 19% of the peptide were cleaved without the appearance of other major peaks. This suggests that the tetrapeptide was completely digested from the C-terminus. Since the Leu-Gly configuration may also be attacked by chymotrypsin the same amount of the tetrapeptide was incubated with 2 units of chymotrypsin for 60 min. Only 5% of the peptide was cleaved showing that the contribution of this endopeptidase may not explain the disappearance of the 20% peptide in the MT experiment.

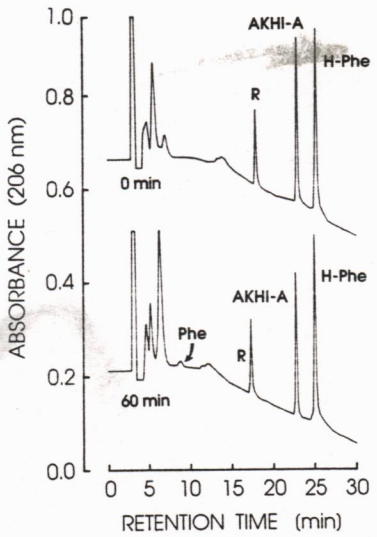

Fig. 8. Simultaneous incubation of AKHI-A and H-Phe with MT homogenate (buffer 3). **Top:** t = 0 min. **Bottom:** t = 60 min. Gradient 1. RT: H-Phe 25.1 min, AKHI-A 22.8 min, Phe 8.7 min, R 17.4 min.

To test the CP's capability to handle a C-terminal proline, HTH-1 (containing residues 1–6) was isolated from MT homogenates (in buffer 1) and incubated with MT homogenate (in buffer 3). Within a 60 min incubation, some 55% of the peptide disappeared without the occurrence of new peaks (not shown). When the same amount of the fragment was treated with 2 units chymotrypsin, only 25% of the peptide were broken down. Since HTH-1 is probably also subject to breakdown by PFCE, a different substrate carrying a C-terminal proline residue was chosen, i.e., Z-GGP. Figure 10 shows that MT homogenate (in buffer 4) cleaved approximately 10% of the peptide within a 60 min incubation period at 30°C.

DISCUSSION

The MTs of *S. gregaria* were found to contain all the enzymes required to break down completely doubly blocked neuropeptides such as the AKHs. There are at least two different pathways by which these neurohormones can be broken down depending on their amino acid sequence. We have tested eight neuropeptides so far (including the crustacean RPCH) and these peptides can be divided into two groups: the proline-containing and the proline-free adipokinetic hormones. The PCAHs give rise to N-terminal hexapeptides with a C-terminal proline. The PFAHs give rise to N-terminal tetrapeptides with a C-terminal phenylalanine. Both of these peptides then are degraded by CPs (see below). The C-terminal fragments of the native AKHs then are substrates for LAP activity present in the MTs.

The reaction conditions used allowed the collection of enough N-terminal fragments to make conclusions concerning the primary site of attack. From this it follows that the CPs must work rather slowly at these amino acids (phe-

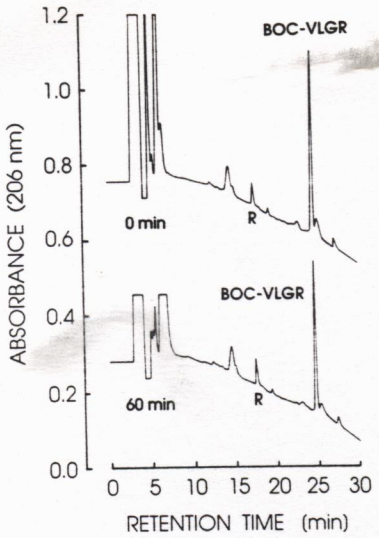

Fig. 9. Incubation of BOC-VLGR with MT homogenate (in buffer 4). **Top:** t = 0 min. **Bottom:** t = 60 min. Gradient 1. RT BOC-VLGR 24.5 min, R 17.4 min.

nylalanine and proline) to allow the accumulation of these peptides. At present it is impossible to say why the tetrapeptide pGlu-Leu-Asn-Phe for example accumulates in large amounts only from AKHII-S but only in small amounts from AKHI. It may be that the ratio of enzyme activities and their substrate specificities are such that the intermediates are broken down. In their analysis of the breakdown of MII in the cockroach, *P. americana*, Skinner et al. [6] found breakdown products for the corresponding hexa- and tetrapeptides. This may have been a consequence of their using a different homogenization/assay buffer, species and tissue from the present study. This result was also obtained when the HTH-1 (1–6) fragment alone was incubated with MT homogenate. From this it seems to follow that the presence of the proline residue is more important than the resulting β-bend. Siegert and Mordue [2] could not find the C-terminal part of the AKHI peptide Asn-Trp-Gly-ThrNH_2, which cast some doubt on the PPCE action. Apart from the use of one synthetic substrate to unequivocally demonstrate the presence of the enzyme, there are also indications for the corresponding C-terminal tetrapeptide Gly-Trp-Gly-ThrNH_2 from HTH and the dipeptide Gly-TrpNH_2 from RPCH.

The C-terminal tetrapeptides from PFAHs do not seem to accumulate at all. Their RTs are known from digestion with chymotrypsin and could have easily been identified if they accumulated in large amounts. These peptide fragments, however, seem to be under fierce attack from LAP. In AKHI and the *Blaberus* HTH the tryptophan residue (position 8) seems to slow down the LAP action leading to the accumulation of Trp-Gly-ThrNH_2.

The sequences in positions 5–7 of the PCAHs do not show great variation (Table 1); position 5 is threonine/serine, 6 is proline, and 7 is glycine/asparagine. Threonine and serine are both hydroxyl group carrying amino

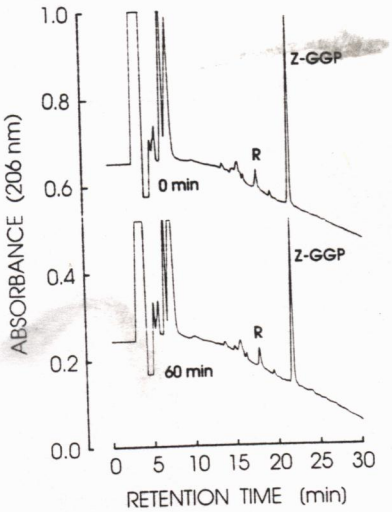

Fig. 10. Incubation of Z-GGP with MT homogenate (in buffer 4). **Top:** t = 0 min. **Bottom:** t = 60 min. Gradient 1. RT: Z-GG 21.7 min, R 17.4 min.

acids, while glycine and asparagine are both without charge or hydroxyl moiety. Koida and Walter [7] reported that lamb kidney PPCE preferred aromatic amino acids on the carboxyl side of proline. The above amino acids are not aromatic and carry no charge. The bond Pro-Gly was cleaved fairly rapidly [7], this may also be true for the MT enzyme since after a very short incubation of RPCH with MT homogenate already large quantities of the hexapeptide were observed (see Fig. 2). The requirements of the enzyme for the amino side of proline are yet to be investigated.

The sequences of the PFAHs also show two different amino acids on either side of pheynlalanine (Table 1). In position 3 it is asparagine/threonine, phenylalanine in 4 and serine/threonine in 5. If the enzyme was chymotrypsin, it would require Ca^{2+} as an activator. In our incubation medium (buffer 1) EDTA was present at a concentration of 20 mM. Both MT enzymes were active under these conditions, it therefore appears that this ion cannot be very important for the activity of the insect enzyme. On the other hand it remains to be seen how the enzyme performs after purification and under controlled ionic conditions with Ca^{2+}. Perhaps under these conditions it could also cleave PCAHs. The PFCE from MTs does not seem to cleave short peptide substrates containing phenylalanine as well as tyrosine-containing ones (unreported data). The verification of this statement, however, also awaits the isolation of the enzyme. The same is true when looking at the potency of MT PFCE to cleave proline-containing peptides. The data suggest that there is substantial activity present in the MTs but unless the purified enzyme is available the differential activity on PCAH and PFAH can be only speculated upon. Vertebrate chymotrypsin cleaves after phenylalanine, tyrosine, tryptophan, and leucine. We have found evidence for the enzymatic cleaving after phenylalanine and tyrosine, but not for tryptophan or leucine. In MT homogenates, the fragments containing these amino acids may be too susceptible to LAP and CPs to be collected for detailed analysis.

The demonstration of LAP activity was possible with TrpNH_2 (unreported data), Leu-7AMC and Pro-7AMC. The presence of this enzyme was already clear from the disappearance of the C-terminal fragments of AKH peptides after the initial endopeptidase incision. It was, however, surprising that Leu-7AMC and Pro-7AMC were cleaved at the same rate by MT homogenates. The vertebrate enzyme showed the expected behavior: Pro-7AMC was cleaved slower than Leu-7AMC. It remains unclear why the result was different with MT homogenates: either insect LAP is better adapted to cleave N-terminal proline residues or a special enzyme (proline aminopeptidase) is present at approximately the same activity as LAP.

When the C-terminal fragment of AKHI (produced by chymotrypsin treatment) was incubated with MT homogenate no clear-cut interpretation of the result was expected since the peptides are under attack by a variety of enzymes: LAP, PPCE and others still to be identified. Vertebrate LAP cannot digest peptides with a proline residue in position 2, but it is clear that this type of peptide can be cleaved by PPCE [7]. It will be of interest to see whether this peptide is cleaved by PPCE, LAP, and/or also by a specialized exopeptidase which acts on proline-containing substrates. This is of particular interest in the light of the discussion of the involvement of LAP in the breakdown of peptides with N-terminal proline residues.

The result that CPA and CPB could not be demonstrated with conventional substrates such as H-Phe and H-Arg was unexpected. The use of longer chain substrates allowed eventually the demonstration of these enzymes. The presence of CPA was shown using the AKHI/II-S fragment pGlu-Leu-Asn-Phe; CPB digested both BOC-VLGR and Z-GGP as was the isolated HTH fragment with proline as the C-terminal residue. The addition of commercial CPA showed that the conditions were favorable for these enzymes. On the other hand the added enzyme activity may have been very high in comparison with the endogenous activity present in the MT that apparent differences in substrate specificities may not really exist.

All these experiments have been performed with MT homogenates and not with isolated enzymes. Since different proteases/peptidases certainly overlap in their activities, the isolation of these enzymes is required to make them available for detailed analysis and comparison with their vertebrate counterparts. Perhaps insects do contain proteases and peptidases which are more adapted to the breakdown of endogenous insect peptides.

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LITERATURE CITED

1. Baumann E, Penzlin H: Inactivation of neurohormone D by Malpighian tubules in an insect, *Periplaneta americana*. **J Comp Physiol B** *157*, 511 (1987).

2. Siegert KJ, Mordue W: Breakdown of locust adipokinetic hormone I by Malpighian tubules of *Schistocerca gregaria*. **Insect Biochem** *17*, 705 (1987).

3. Morgan PJ, Mordue W: Separation and characteristics of diuretic hormone from the corpus cardiacum of *Locusta*. **Comp Biochem Physiol** [B] *75*, 75 (1983).

4. Siegert KJ, Morgan PJ, Mordue W: Primary structures of locust adipokinetic hormones II. **Biol Chem Hoppe Seyler** *366*, 723 (1985).

5. Siegert KJ, Mordue W: Elucidation of the primary structures of the cockroach hyperglycaemic hormones I and II using enzymatic techniques and gas-phase sequencing. **Physiol Entomol** *11*, 205 (1986).

6. Skinner WS, Quistad GB, Adams ME, Schooley DA: Metabolic degradation of the peptide periplanetin CC-2 in the cockroach, *Periplaneta americana*. **Insect Biochem** *17*, 433 (1987).

7. Koida M, Walter R: Post-proline cleaving enzyme. Purification of this endopeptidase by affinity chromatography. **J Biol Chem** *251*, 7593 (1976).