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PBAN selective antagonists: inhibition of PBAN induced cuticular melanization and sex pheromone biosynthesis in moths ☆

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Abstract

A D-Phe scan (sequential D-Phe replacement) library of linear peptides, synthesized on the basis of a slightly modified active sequence of PBAN (YFSPRL-amide) was employed to detect potential inhibitors of cuticular melanization in *Spodoptera littoralis* larvae and to compare their stimulatory and inhibitory melanization activity with their pheromonotropic agonistic and antagonistic activities. A quantitative melanotropic assay was used to monitor the extent of cuticular melanization elicited by Hez-PBAN1-33NH_2 in *S. littoralis* larvae in the presence and absence of the D-Phe peptides. The data revealed the presence of two partial melanotropic antagonists, and disclosed the presence of selective pure melanotropic agonists and pure pheromonotropic antagonists indicating differences in the inhibitory and stimulatory patterns of the library with respect to both activities. The differences between the pheromonotropic and melanotropic inhibitory patterns of the peptides hints at the possibility that sex pheromone biosynthesis in the pheromone gland of *Heliothis peltigera* females and induction of cuticular melanization in *S. littoralis* may be mediated by different receptors (that may result either from presence of different receptor sub-types or may reflect species differences in receptor structure and/or properties) despite the fact that they are induced by the same peptide (PBAN1-33NH_2).

Keywords: PBAN; Melanization; Neuropeptide antagonists; *Spodoptera littoralis*; Insect neuropeptide

1. Introduction

Insects display a wide variety of coloration and are capable of altering their pigmentation in response to external and internal factors by the synthesis of pigments that may be located in the epidermal cells or the cuticle (Raabe, 1989). Currently, it is well established that pigmentation in insects (whether cuticular or epithelial) is controlled by endocrine and neuroendocrine factors, and many studies have revealed that the source and nature of these factors differ from one species to another. Most studies on insect pigmentation were carried out in Lepidopteran insects and various grasshoppers (mainly locusts). In locusts and many other grasshopper species (Acrididae), pigmentation and color polymorphism has been found to be controlled by a variety of neuroendocrine factors (for review see Tanaka, 2001). A dark color-inducing neurohormone (DCIN, originally isolated from the CC of *Schitocerca gregaria* and *Locusta migratoria*) (Tawfik et al., 1999) which is identical to the [His^7] corazonin, isolated from the CC of *Schistocerca americana* (Veenstra, 1991) was found to induce gregarious black pigmentation in the albino *L. migratoria* mutant (that lacks the DCIN gene, Baggermann et al., 2001) and the nymph of the desert locust (*S. gregaria*) (Tawfik et al., 1999; Hoste et al., 2002). The neuropeptide was also found to control homochromy in the non locust grasshopper *Oedipoda miniata* (Yerushalmi and Pener, 2001) and other acridid nymphs (Tanaka, 2000). [Arg^7] corazonin (originally

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isolated from the CC of the cockroach, *Periplaneta americana*) (Veenstra, 1989) was reported to be a darkening inducer in the *L. migratoria* albino mutant and its activity was reported to be indistinguishable from that of the [His^7] corazonin (Shalev et al., 2003). Adipokinetic hormone II of *S. gregaria* (Scg-AKH) and AKH of *Gryllus bimaculatus* (Grb-AKH) were also found to elicit a moderate darkening response in albino locust nymphs because of their similar lipophilic nature and sequence homology with the active core of DCIN (Yerushalmi and Pener, 2001; Yerushalmi et al., 2002).

In noctuid moths cuticular pigmentation is controlled by the pyrokinin (PK)/pheromone biosynthesis activating neuropeptide (PBAN) family. The first indication of the possible involvement of this family in the control of larval cuticular melanization was demonstrated in the common army worm, *Leucania separata,* by Ogura and co-workers (Ogura and Saito, 1972; Ogura, 1975; Suzuki et al., 1976). The hormone, which was termed melanization and reddish coloration hormone (MRCH), was later found to initiate the melanization of the integument of other moth larvae such as *Leucania loreyi* (Matsumoto et al., 1981), *Spodoptera litura* (Morita et al., 1988) and *Mamestra brassicae* (Hiruma et al., 1984). Several MRCH peptides were partially purified from *Bombyx mori* (Suzuki et al., 1976; Matsumoto et al., 1981, 1984, 1986, 1988), and the primary structure of one of them, MRCH-I, was revealed by Matsumoto et al. in 1990, by using head extracts of adult insects. MRCH-I was found to be an amidated peptide consisting of 33 amino acids and identical to PBAN of *B. mori* (Bom-PBAN-I, Kitamura et al., 1989). The neuropeptide was also found to share 80% homology with the primary structure of Hez-PBAN isolated from *Helicoverpa zea* (Raina and Klun, 1984; Raina et al., 1989). Both natural and synthetic Bom-PBAN-I/MRCH-I were found to induce cuticular melanization in larvae of *L. separata*, *S. Litura*, and *S. littoralis* and to stimulate sex pheromone production in adults of *B. Mori* and *S. litura* (Matsumoto et al., 1990; Altstein et al., 1996). The c-DNA of *B. mori* PBAN/MRCH has been cloned by Kawano et al. (1992) as were many other peptides of this family neuropeptides, all of which share a common C-terminal sequence of Phe–Xxx–Pro–Arg–Leu–NH_2 (X = Ser, Thr, Val) (for reviews see Rafaeli and Jurenka, 2003; Altstein, 2004).

Cuticular melanization in moth larvae has also been found to be induced by other members of this family including: Pss-pheromonotropin (Pss-PT, also termed Pss-MRCH), an 18 amino acid neuropeptide isolated from larval heads of *Pseudaletia* (= *Leucania*) *separata* (Matsumoto et al., 1992a, b), by a pheromonotropic melanazing peptide (PMP) isolated from *H. zea*, (which bears 83% sequence homology with Pss-PT) (Raina et al., 2003), by *L. migratoria* myotropin-I and II (Lom-MT-I and Lom-MT-II) and *Leucophaea maderae* PK (LPK) (Matsumoto et al., 1993). An unidentified factor, extracted from the nerve cord system, which differs from Bom-PBAN-I/MRCH-I or Hez-PBAN, was reported to be involved in cuticular melanization of *Manduca sexta* larvae (Hiruma et al., 1993). Bursicon, a 40 kDa neurosecretory protein, has also been reported to induce melanization. This neuropeptide is produced by neurons in *M. sexta* and its presence has been detected in a variety of different insects. Bursicon triggers sclerotization and melanization of newly formed cuticles (Kaltenhauser et al., 1995).

Studies performed in many laboratories including ours indicate that the PK/PBAN family (which currently comprises of over 30 peptides) is a multifunctional family of peptides and that in addition to their ability to stimulate cuticular melanization in moths, those peptides mediate key functions associated with feeding (gut muscle contractions) (Nachman et al., 1986; Schoofs et al., 1991), development (pupariation and diapause) (Imai et al., 1991; Nachman et al., 1993, 1997) and mating behavior (sex pheromone biosynthesis) (Raina and Klun, 1984; Altstein, 2004) in a variety of insects (moths, cockroaches, locusts and flies). These studies have shown that all of the above functions can be stimulated by more than one peptide, and that the peptides do not exhibit species specificity (for a detailed review see Gäde, 1997; Rafaeli and Jurenka, 2003; Altstein, 2004). The functional diversity of the PK/PBAN family raises many questions with respect to the mechanisms of actions by which these neuropeptides exert their effects and the nature of their receptors. Currently, our understanding of the mode of action of the various melanotropic peptides, the possible existence of multiple receptors for each function and/or for each neuropeptide, and their interactions with other components of the neuroendocrine or endocrine system is very limited, and requires further investigation. Antagonists, especially selective ones can shed light on some of these questions.

In the past few years we have developed a strategy for generation of antagonists on the basis of an agonistic neuropeptide. The strategy (termed Insect Neuropeptide Antagonist—INA) was applied to the PK/PBAN family and resulted in the discovery of several highly potent pheromonotropic antagonists. In the course of that study we synthesized a few linear and backbone cyclic conformational libraries and determined their agonistic and antagonistic pheromonotropic activity in *Heliothis peltigera* (Altstein et al., 1999, 2000; Zeltser et al., 2000, 2001; Altstein, 2001, 2003, 2004).

In the present study we tested the agonistic and antagonistic melanotropic activity of one such library (a D-Phe scan library of linear peptides) (Zeltser et al., 2000) by using a modified quantitative bioassay that was previously developed in our laboratory with *S. littoralis* larvae (Altstein et al., 1996) for the detection of

melanotropic antagonists and discovery of selective and non-selective melanotropic and pheromonotropic compounds.

2. Materials and methods

2.1. Insects

S. littoralis larvae were kept in groups of 100–200 insects in plastic containers (40 × 30 × 20 cm). Sawdust was placed at the bottom of the container and the top was covered with cheesecloth. Larvae were fed on castor bean leaves and kept in a thermostatically regulated room at 25 ± 2 °C with a light:dark regime of 14:10 h and 60% relative humidity.

H. peltigera moths were reared on an artificial diet as described previously (Dunkelblum and Kehat, 1989). Pupae were sexed and females and males were placed in separate rooms with a dark/light regime of 10:14 h, at 25 ± 2 °C and 60–70% relative humidity. Adult moths were kept in screen cages and supplied with a 10% sugar solution. Moth populations were refreshed every year with males caught from the wild by means of pheromone traps, as described previously (Dunkelblum and Kehat, 1989). All females used in this study were 3.5 days old.

2.2. Peptide synthesis

2.2.1. Chemicals

Protected amino acids, Rink amide methylbenzhydrylamine (MBHA) resin and coupling reagents were purchased from Novabiochem (Laufelfingen, Switzerland). Other chemicals were purchased from Sigma, St. Louis, MO, USA or Merck, Darmstadt, Germany. Solvents and reagents for peptide synthesis were purchased from Baker (Phillipsburg, NJ, USA).

2.2.2. Synthesis of PBAN 1-33NH_2

Hez-PBAN (Raina et al., 1989) was synthesized on an ABI 433A automatic peptide synthesizer on Rink amide MBHA resin by means of the FastMoc™ chemistry as described previously (Zeltser et al., 2000).

2.2.3. Synthesis of the D-Phe peptide library

Peptides (Table 1) were synthesized by the Simultaneous Multiple Peptide Synthesis (SMPS) "tea bags" methodology on Rink amide MBHA resin as previously described (Zeltser et al., 2000).

2.2.4. Purification and characterization of peptides

The purity of all peptides (PBAN and D-Phe substituted peptides) was assessed by analytical reverse-phase high-performance liquid chromatography (RP-HPLC) (Altstein et al., 1999; Zeltser et al., 2000) and was found to be in the range of 90–95%. Purified peptides were characterized by time-of-flight mass spectrometry (TOF-MS) and amino acid analysis of hydrolyzates. The analytical methods and data of the D-Phe peptides were described by Zeltser et al. (2000).

2.3. Melanotropic bioassay

The melanotropic bioassay was performed essentially according to Altstein et al. (1996), except that the analysis of scanned images was performed with a much more advanced software which enabled a detailed and a more precise evaluation of the extent of melanization under the various treatments. Fifth-instar larvae, at head capsule slippage, were injected in the thorax with 10 µl 50 mM HEPES (*N*-[2-Hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid]), pH 7.6, or the tested compounds dissolved in the same buffer. After injection, larvae were ligated between the second and the third abdominal segments, the part of the body anterior to the ligature was cut off and the posterior part of the larvae were kept at 25 °C. After 18–20 h, the dorsal part of the exoskeleton was placed on Whatman paper and scanned with an Astra 1220S scanner (Umax, Germany). Scanned images were evaluated by computerized image analysis using TINA (version 2.10 g) software (Raytest, Germany). Each experiment involved analysis of the intensity of the melanized area in untreated, ligated, and ligated plus buffer-, PBAN- or test peptide-injected larvae (Fig. 1). The cuticular melanization was quantified as the ratio between the optical-density and the scanned cuticular area (in millimeters) and was compared between control and experimental animals (Fig. 2). All experiments were performed with a minimum of nine or ten larvae per treatment. The only experiments considered were those in which the extent of melanization in buffer-injected larvae did not differ significantly from that in ligated animals and did differ significantly from that of those injected with PBAN1-33NH_2 (5 pmol).

Melanotropic agonistic activity of the D-Phe peptides was determined by an evaluation of the ability of the injected peptides (at 1 nmol) to induce cuticular melanization in larvae (in the absence of PBAN1-33NH_2). Larvae injected with 5 pmol PBAN1-33NH_2 served as a reference for agonistic activity. Antagonistic activity was determined by monitoring the ability of the D-Phe peptides (at 1 nmol), injected together with the elicitor PBAN1-33NH_2 (at 5 pmol) to inhibit the cuticular melanization. Larvae injected with 5 pmol PBAN1-33NH_2 served as a reference for maximal stimulation and those injected with 50 mM HEPES, pH 7.6 served to determine the basal cuticular melanization of the ligated insects in both the agonistic and antagonistic activity experiments.

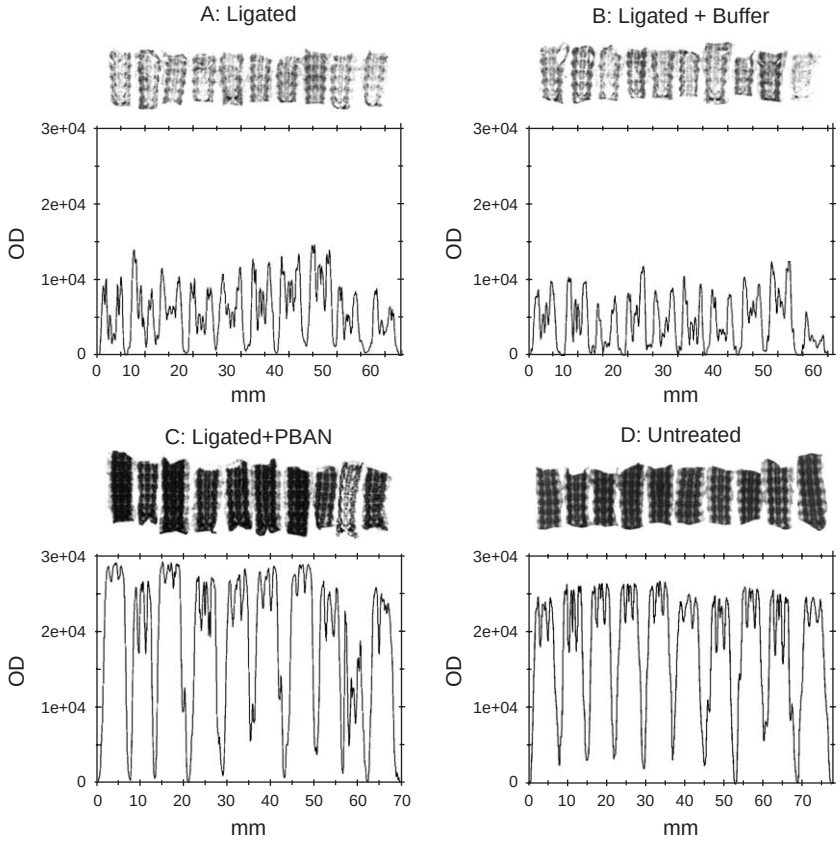

Fig. 1. Melanization pattern and scanning profile of *S. littoralis* cuticles (dorsal region) obtained from untreated larvae, ligated larvae, larvae that have been ligated and injected with buffer or with PBAN. The amount of injected PBAN1-33NH_2 was 5 pmol per larva.

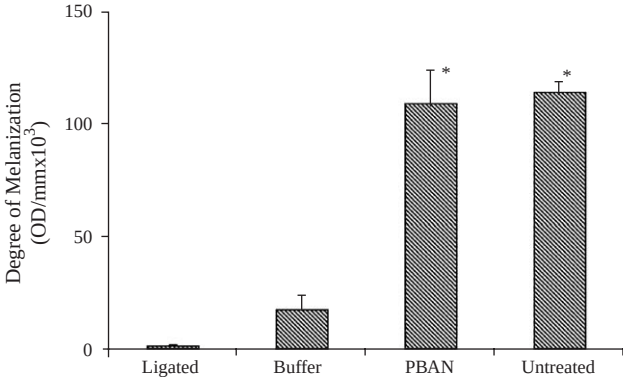

Fig. 2. Quantitative representation of the melanization assay. Degree of melanization is expressed as optical density per mm^2 of cuticle. Measurements were performed on individual *S. littoralis* cuticles and the values in each treatment represent the mean degree of melanization ± SEM of 9 or 10 samples minus the mean value obtained with the ligated larvae. The amount of PBAN1-33NH_2 injected was 5 pmol per larva. Statistical analysis compared the degree of melanization in the different treatments with that of ligated larvae. An asterisk (*) indicates a significant difference in activity at $P<0.05$.

2.4. Pheromonotropic bioassay

The pheromonotropic bioassay was performed with *H. peltigera* as described previously (Altstein et al., 1995). Agonistic activity of the D-Phe peptides was determined by monitoring the ability of the injected peptides (at 100 pmol) to induce sex pheromone biosynthesis in females (in the absence of PBAN1-33NH_2). Females injected with 1 pmol PBAN1-33NH_2 served as a reference for agonistic activity. Antagonistic activity was determined by monitoring the ability of the D-Phe peptides (at 100 pmol) to inhibit sex pheromone biosynthesis elicited by 0.3 pmol exogenously injected PBAN1-33NH_2. Females injected with 0.3 pmol PBAN1-33NH_2 served as a reference for maximal stimulation and those injected with 100 mM phosphate buffer served to determine the basal pheromone biosynthesis at photophase in both the agonistic and antagonistic activity experiments. The pheromone content in buffer-injected moths did not exceed 20 ng/female. Pheromone glands were excised 2 h post injection and sex pheromone was extracted and quantified by capillary gas chromatography as described previously (Altstein et al., 1995). All experiments were performed with a minimum of 10 females per treatment.

2.5. Statistical analysis

The results were subjected to one-way ANOVA. The significance of differences among means was evaluated

with the Tukey-Kramer HSD (honestly significant difference) test at $P<0.05$.

3. Results

The antagonistic melanotropic activities of the D-Phe peptides are shown in Table 1. Two peptides (LA-1 and LA-5) out of the seven library members, showed high melanotropic antagonistic activity at 1 nmol (i.e., they inhibited PBAN elicited melanization by 50%). Two other peptides (LA-3 and LA-4) exhibited lower antagonistic activity, inhibiting melanization by 27% and 36%, respectively. Since all of the peptides tested in the present study were derived from the putative active site of PBAN, they were tested for their agonistic activity at the same concentration and under the same conditions as had been used to assess their antagonistic activity (1 nmol). As indicated in Table 1, a few peptides (LA-2, LA-3 and LA-7) exhibited high agonistic activity that did not differ significantly from that of PBAN 1-33NH_2, the other peptides were also active but to a somewhat lesser extent. Comparison of the agonistic activity of the D-Phe peptides (at 1 nmol) with that of the parent peptide Arg-PBAN28-33NH_2 (RYFSPRL-NH_2) at the same concentration revealed that the peptide exhibits a high agonistic activity which does not differ significantly from that of PBAN1-33NH_2 at 5 pmol (Table 1) and 1 nmol (data not shown). The agonistic activity of the D-Phe peptides and that of PBAN28-33NH_2 (YFSPRL-NH_2) and Arg-PBAN28-33NH_2 was also tested at 10 pmol and was compared with that of PBAN1-33NH_2. Only two peptides (LA-5 and LA-7) exhibited some activity (13% and 31%, respectively) compared with that obtained by 5 pmol PBAN1-33NH_2. All other peptides were inactive at this concentration (data not shown).

Table 1
Melanotropic agonistic and antagonistic activities of the D-Phe scan library in *S. littoralis*

Peptide	Activity (%)			
	Antagonistic		Agonistic	
dFYFSPRL-NH_2 (LA-1)	50±13	($n=10$)	76±9*	($n=10$)
R**dF**FSPRL-NH_2 (LA-2)	1±1	($n=10$)	96±8	($n=10$)
RY**dF**SPRL-NH_2 (LA-3)	27±15	($n=9$)	88±6	($n=10$)
RYF**dF**PRL-NH_2 (LA-4)	36±9	($n=19$)	60±12*	($n=10$)
RYFS**dF**RL-NH_2 (LA-5)	51±10	($n=19$)	68±11*	($n=10$)
RYFSP**dF**L-NH_2 (LA-6)	1±1	($n=10$)	61±14*	($n=10$)
RYFSPR**dF**-NH_2 (LA-7)	1±1	($n=9$)	89±8	($n=10$)
YFSPRL-NH_2	n.t.		35±7*	(n = 9)
RYFSPRL-NH_2	n.t.		85±7	(n = 9)

Antagonistic activity is expressed as 100 minus the ratio (as a percentage) between the extents of melanization elicited by the injection of PBAN1-33NH_2 (5 pmol) and by each of the tested D-Phe peptides (at 1 nmol). Agonistic activity is expressed as the ratio (as a percentage) between the extents of melanization elicited by the injection of each of the D-Phe peptides, at 1 nmol, and by 5 pmol PBAN1-33NH_2. Each value represents the mean±SEM. Statistical analysis of the antagonistic activity compared the degree of melanization obtained with PBAN-1-33NH_2 in the presence and absence of the D-Phe peptides. Statistical analysis of agonistic activity compared the degree of melanization obtained with each of the D-Phe peptides with that obtained with 5 pmol PBAN1-33NH_2. An asterisk (*) indicates significant differences in activity at $P<0.05$. n.t.: not tested.

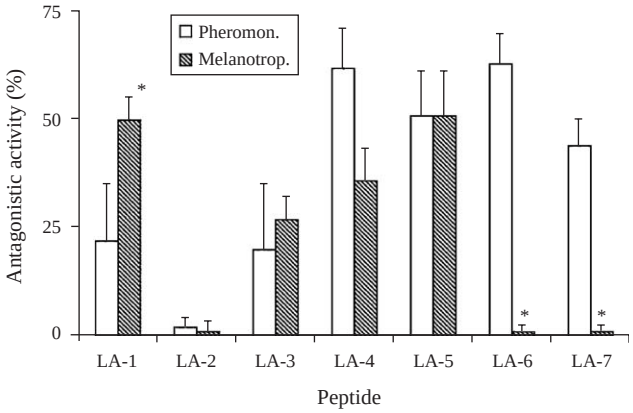

Fig. 3. Comparison of the melanotropic and pheromonotropic antagonistic activities of the D-Phe peptides. Inhibitory activity is expressed as 100 minus the ratio (as a percentage) between the activities elicited by the injection of PBAN1-33NH_2 in the presence and absence of the tested D-Phe peptides±SEM of 9–19 samples. D-Phe peptides were tested at 100 pmol together with 0.3 pmol PBAN1-33NH_2 (in the *H. peltigera* pheromonotropic bioassay) or at 1 nmol together with 5 pmol PBAN1-33NH_2 (in the *S. littoralis* melanotropic bioassay). Statistical analysis compared differences between melanotropic and pheromonotropic antagonistic activities obtained with the same peptide. An asterisk (*) indicates a significant difference in activity at $P<0.05$ (pheromonotropic antagonistic data are taken from Zeltser et al., 2000).

Comparison of the antagonistic melanotropic activity of the D-Phe peptides in *S. littoralis* with that of their pheromonotropic activity in *H. peltigera* revealed three selective antagonists (peptides whose pheromonotropic and melanotropic activities differ significantly, Fig. 3): LA-1, of which the melanotropic inhibitory activity was significantly higher than its pheromonotropic inhibitory activity; and LA-6 and LA-7, which are selective pheromonotropic antagonists. All other peptides were either non-selective (LA-3, LA-4, LA-5) or inactive (LA-2). Similar results were obtained when the agonistic activities of the peptides were tested on both functions (Fig. 4). Peptides LA-1, LA-4, LA-6 and LA-7 were found to be selective melanotropic agonists. All other peptides were non-selective. A summary of the selective and non-selective agonistic and antagonistic properties of the linear D-Phe scan peptides is depicted in Table 2.

4. Discussion

In the present study a D-Phe scan (sequential D-Phe replacement) library of linear peptides, synthesized on

the basis of a slightly modified active sequence of PBAN (YFSPRL-amide) was employed to detect potential inhibitors of cuticular melanization in *S. littoralis* larvae and to compare their stimulatory and inhibitory melanization activities with their pheromonotropic agonistic and antagonistic activities.

The use of D-amino acids for the discovery of antagonists has previously been applied to mammalian peptides such as luteinizing hormone-releasing hormone(LHRH) (Rees et al., 1974; Vale et al., 1972), substance P (SP) and other neurokinins (Folkers et al., 1984; Piercey et al., 1981; Rosell et al., 1983), vasopressin (Sawyer et al., 1981), bombesin (Heinz-Erian et al., 1987), bradykinin (Rhaleb et al., 1991; Vevrek and Stewart, 1985), endothelin (Cody et al., 1995), enkephalin (Collins et al., 1996) and parathyroid hormone (Maretto et al., 1998). In a previous study, we extended the D-amino acid substitution approach and applied it to the insect PBAN for the discovery of pheromonotropic antagonists. We exploited our knowledge of the pheromonotropic structure activity relationship (SAR) of PBAN which revealed that, under certain conditions (e.g. short injection times), the six amino acids of the carboxyl terminus (YFSPRL-amide) are as active as the full-length peptide (Altstein et al., 1995), to generate a D-Phe scan library in which each amino acid within a slightly modified sequence (to which an Arg was added at the N-terminus to increase solubility) was substituted sequentially with a D-Phe. Analysis of the pheromonotropic antagonistic activity of the peptides in the library revealed the presence of three potent antagonists capable of effectively inhibiting (by over 50%) sex pheromone production in *H. peltigera* females at a concentration of 100 pmol (Zeltser et al., 2000). In the present study this library was used to explore melanization antagonists. A similar approach has recently been applied to the DCIN peptide in locusts in search for antagonists. The 11-amino acid peptide was sequentially substituted with D-Phe and the ability of the peptides to antagonize dark coloration induced by the parent molecule was examined at 100 pmol. No antagonistic activity of any of the D-Phe substituted peptides could be detected, despite the low agonistic activity of some of the analogs (Grach et al., 2003). Substitution of D-Phe was also applied to the insect neuropeptide proctolin and resulted in the successful discovery of a few peptides with antagonistic activity (Kuczer et al., 1999).

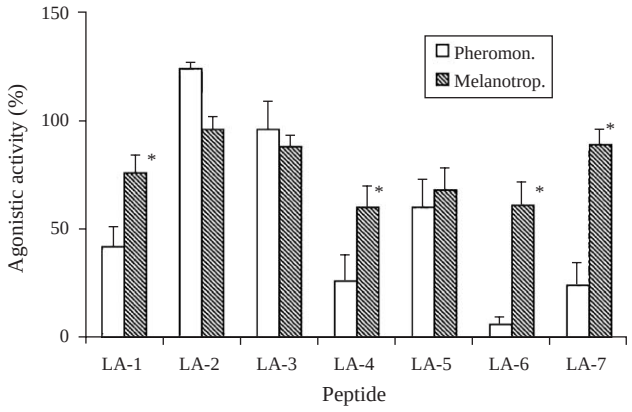

Fig. 4. Comparison of the melanotropic and pheromonotropic agonistic activities of the D-Phe peptides. Activity is expressed as the ratio (as a percentage) between the extents of melanization or pheromone biosynthesis elicited by the injection of each of the D-Phe peptides (1 nmol and 100 pmol in the melanotropic and pheromonotropic assays, respectively) and by PBAN1-33NH_2 (at 5 and 0.3 pmol, respectively) ± SEM of 9 or 10 samples. Statistical analysis compared differences between melanotropic and pheromonotropic agonistic activities obtained with the same peptide. An asterisk (*) indicates a significant difference in activity at $P<0.05$ (pheromonotropic agonistic data are taken from Zeltser et al., 2000).

Currently, only one other melanization-inhibiting factor has been reported: the pupal melanization reducing factor (PMRF). PMRF, found in various parts of the nervous system of several Lepidopteran species, was reported to reduce background-induced melanization in pupae of *Inachis io* and of other moth species (Koch et al., 1990). The factor has not been sequenced but seems to be a peptide with a molecular weight between 1000 and 5000 (Bückmann and Maisch, 1987). The mechanism by which the peptide reduces melanization is not clear at present, but is most unlikely to be

Table 2
Summary of selective agonistic and antagonistic activities of the D-Phe scan library of linear peptides

Peptide	Selectivity	Biological activity	Mode of action
LA-1	Selective agonist	Melanotropic	Partial agonist
	Selective antagonist	Melanotropic	Partial antagonist
LA-4	Selective agonist	Melanotropic	Partial agonist
LA-6	Selective agonist	Melanotropic	Pure agonist
	Selective antagonist	Pheromonotropic	Pure antagonist
LA-7	Selective agonist	Melanotropic	Pure agonist
	Selective antagonist	Pheromonotropic	Partial antagonist

Results are based on Table 1 and Figs. 3 and 4.

mediated via the MRCH/PK/PBAN family of peptides, since attempts to induce melanization in pre-pupae of *I. io* by partially purified MRCH did not activate melanization of the pupal cuticle (Starnecker et al., 1994). It may very well be, as suggested by the authors, that melanization reduction in these experiments may result from enhancement of the carotenoid content of the cuticle and not from the inhibition of melanization (Koch et al., 1990).

Examination of the melanotropic activity of the peptides in the D-Phe library reveals that some of them exhibited mixed activities (i.e. high antagonistic and high agonistic activity or high agonistic and low antagonistic activity). Partial agonistic or antagonistic activity, especially among linear peptides in which the antagonist is based on the structure of an agonist, is a well known phenomenon and has been widely documented (for review see Hruby, 1992). A possible explanation for the phenomenon is that the peptide binds to its receptor both in the agonistic conformation that partially activates the receptor, and also in the "non-activating" antagonistic conformation, that does not enable further activation of secondary messengers (Hruby, 1992).

It is interesting to note that in the present study some of the peptides (LA-2, LA-3 and LA-7) exhibited high agonistic activities (at 1 nmol) which did not differ significantly from that of PBAN1-33NH_2. All other peptides also exhibited agonistic activity although lower than that of the full-length peptide. The melanotropic agonistic activity of the parent molecule Arg^{27}PBAN28-33NH_2 also did not differ significantly from that of PBAN1-33NH_2. The C-terminal hexapeptide PBAN28-33NH_2 was significantly less active (Table 1). Thus, addition of an Arg at the N-terminus of the peptides and substitution of Tyr^{28}, Phe^{29} and Leu^{33} with D-Phe resulted in a stereo-conformational change that conferred on the molecule a higher affinity to the receptor. Similar results were obtained when the library of peptides was tested for its pheromonotropic agonistic activity; peptides (LA-2, LA-3 and LA-5) exhibited high agonistic activities that did not differ significantly from that of PBAN1-33NH_2 under conditions where C-terminal derived peptides (PBAN28-33NH_2 and Arg^{27} PBAN28-33NH_2) usually have a very low activity (e.g. 2 h post injection) (Altstein et al., 1995). Increase in activity due to D-Phe substitution was also obtained by Raina and Kempe (1992) with PBAN 28-33NH_2, where replacement of Phe^{29} with D-Phe increased the pheromonotropic activity, and with several mammalian neuropeptides (e.g., LHRH, enkephalin and somatostatin), where replacement of L-amino acid residues with D-amino acid residues at appropriate sites caused a considerable increase in potency (for review see Morley, 1980). Contrary to the above, substitution of amino acids with D-Phe in the locust darkening hormone DICN resulted in a marked loss in agonistic activity (Grach et al., 2003). Substitutions with other D-amino acids in PBAN28-33NH_2 (e.g., D-Tyr^{28}, D-Tyr^{29}, D-Arg^{32} and D-Leu^{33}) (Raina and Kempe, 1992) and D-Ala^{31} or D-Pro^{31} (Kuniyoshi et al., 1991) resulted in a complete loss of pheromonotropic agonistic activity.

Studies of the melanotropic activity of the PK/PBAN peptides revealed that cuticular melanization can be induced by several members of this family (Matsumoto et al., 1992a, b, 1993; Raina et al., 2003). In addition, our previous SAR melanotropic studies (Altstein et al., 1996) led to the conclusion that C-terminal part of PBAN, which contains the signature sequence of the PK/PBAN family, constitutes the active site associated with the melanotropic activity, by analogy with what was already known for the pheromonotropic activity of this peptide family. It was, therefore, not surprising that the peptides of the D-Phe scan library which were designed on the basis of the C-terminal part of PBAN, and have previously been shown to exhibit both agonistic and antagonistic pheromonotropic activity, also exhibited melanotropic agonistic and antagonistic activities. What is interesting, though, is the finding that the inhibitory and stimulatory patterns of the various peptides in the library differ with respect to the two activities. Comparison of the pheromonotropic and melanotropic antagonistic activities revealed three selective antagonists (Table 2 and Fig. 3). Two peptides (LA-6 and LA-7) that exhibited pheromonotropic antagonistic activity but no melanotropic antagonistic activity; and one peptide (LA-1) that exhibited a significantly higher antagonistic melanotropic activity while having some antagonistic pheromonotropic activity as well. Peptide LA-4 also exhibited a higher pheromonotropic activity although statistical analysis did not reveal a significant difference between the two (at $P<0.05$). Comparison of the agonistic activity revealed that the same four peptides (LA-1, LA-4, LA-6 and LA-7) are selective exhibiting significantly higher melanotropic agonistic activity than pheromonotropic stimulation.

It is interesting to note that all the selective compounds, whether agonistic or antagonistic, resulted from substitution of the D-Phe with the N-terminal Arg, Ser^{30}, Arg^{32} and Leu^{33}, which indicates the importance of these amino acids in selective activity. Substitution of Phe^{29} or Pro^{31} with D-Phe did not have any affect on selectivity (agonistic or antagonistic), and substitution of Tyr^{28} with D-Phe failed to convert the agonistic peptide into an antagonist (whether melanotropic or pheromonotropic). The finding that the pheromonotropic and the melanotropic inhibitory patterns of the peptides in the library differ hints at the possibility that sex pheromone biosynthesis in the pheromone gland of *H. peltigera* and induction of cuticular melanization in *S. littoralis* may be mediated by different receptors (or

receptor sub-classes), despite the fact that both functions are stimulated by the same peptide (PBAN1-33NH_2). Since the agonistic/antagonistic activity was determined in two different moth species and different developmental stages (adult *H. peltigera* moths and *S. littoralis* larvae) it is possible that the different activities are caused by species differences in receptor structure and/or properties. The possible presence of receptor sub-classes is currently being studied in our laboratory by a detailed examination of the antagonistic effects of the D-Phe scan library on different stimulatory peptides (Pss-PT, Lom-MT-I and LPK) in the same moth species (*H. peltigera*).

Availability of agonists or antagonists is of major importance in the study of neuropeptides in general, and of the PK/PBAN family of neuropeptides in particular. The PK/PBAN family of peptides is involved in the regulation of critical reproductive, developmental and digestive processes (e.g., sex pheromone biosynthesis, cuticular melanization, myotropic activity, oviposition, pupariation and diapause) in moths and other insects and it exhibits considerable functional, and inter- and intra-specific diversity (for reviews, see Rafaeli and Jurenka, 2003; Altstein, 2004). Despite the intensive studies of the PK/PBAN peptides, which provided most interesting information on the chemical and molecular nature of the peptides (such as their origin, localization, target organ, route of transport, etc.), many aspects, mainly those related to their mode of action are still unresolved, and much remains to be learned about the structural, chemical and cellular bases of their activity, downstream cellular events, species specificity, receptor heterogeneity and functional diversity. The availability of antagonists for this family of neuropeptides, together with the availability of bioassays that have been developed for each of the above-mentioned functions (Raina and Klun, 1984; Imai et al., 1991; Schoofs et al., 1993; Altstein et al., 1995, 1996; Nachman et al., 1997) open the way to a better understanding of the endogenous mechanisms of this peptide family in moths and other insects. The present study introduces an excellent example for such an application.

As indicated in the Introduction, pigmentation in insects is quite a complex mechanism which is controlled by endocrine and several neuroendocrine factors, some of which have not even been identified. The availability of the above antagonists can shed light on the nature of such factors and the similarity of their cellular mode of action in other Lepidopteran and non-Lepidopteran insects.

The discovery of linear selective and non selective antagonists can also lead to the design of additional improved agonistic and antagonistic compounds (e.g., conformationally constrained, highly selective and metabolically stable) for the further study of the above issues and serve as a basis for the design of further improved non-peptide mimetic compounds for agrochemical applications. Indeed, the LA-4 linear lead antagonist has already been used as a basis for the design and synthesis of two conformationally constrained libraries out of which metabolically stable backbone cyclic (BBC) antagonists, which inhibited sex pheromone biosynthesis in *H. peltigera* female moths evoked by exogenously administered PBAN 1-33NH_2 or by the endogenous mechanism were discovered (Altstein et al., 1999; Altstein, 2003). These compounds are currently being screened for their ability to stimulate or inhibit cuticular melanization in *S. littoralis* larvae.

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