ISOLATION AND PARTIAL CHARACTERIZATION OF A PROTEASE WITH KALLIKREIN-LIKE ACTIVITY FROM THE EGG-NESTS OF *Hylesia metabus* (CRAMMER 1775) (LEPIDOPTERA: SATURNIDAE), PRELIMINARY COMMUNICATION

Aislamiento y Caracterización Parcial de una Proteasa con Actividad Semejante a la Callikreina de las Posturas de *Hylesia metabus* (Crammer 1775) (Lepidoptera: Saturnidae), Comunicacion Preliminar

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RESUMEN

Hylesia metabus es una mariposa nocturna distribuida principalmente en el nor-oeste de Venezuela. Las hembras adultas usan sus vellos abdominales para cubrir y proteger los huevecillos de parásitos y depredadores. Estos vellos tienen propiedades urticantes, causando una dermatitis severa entre la población humana, cuyos síntomas se acentúan al usar jabón y se mejoran al aplicar soluciones de un pH ligeramente bajo tal como el vinagre. Sin embargo los vellos de los machos adultos no causan síntoma alguna. En el presente estudio se aisló y caracterizó en forma parcial una proteasa con propiedades parecidas a la calicreina de los vellos presentes en el abdomen de las hembras adultas. El material protéico de las posturas, constituidas por los vellos abdominales de la hembra, fue extraído en tampón Tris-Salino pH 8.5. El extracto fue centrifugado y luego cromatografiado en una columna de exclusión molecular (Sephadex G-75). La actividad biológica de los picos obtenidos fue determinada midiendo la actividad amidolítica con el sustrato cromogénico S-2288 (diseñado para serina-proteasas de amplio espectro) y S-2302 (diseñado para calicreina). Las fracciones con actividad en sustrato S-2302 fueron concentradas mediante ultrafiltración y usadas para otros experimentos. La medición de especificidad a substratos cromogénicos mostró una preferencia para el substrato S-2302 seguido por S-2288. La actividad enzimática tuvo su máximo a pH 9, mientras que por debajo de pH 5 no hubo actividad. Los resultados del presente estudio apoyan la idea de que esta sustancia puede tener importancia en las lesiones cutáneas observadas en personas expuestas a las posturas o las hembras adultas.

Palabras clave: *Hylesia metabus*, calicreina, proteasa.

ABSTRACT

Hylesia metabus is a species of moth, distributed principally in North Eastern Venezuela. Adult females use their abdominal hairs to cover and protect the eggs from predators and parasites. These hairs have urticating properties, causing a severe dermatitis in humans, whose symptoms worsen using soap and are alleviated by slightly acid substances e.g. vinegar. The hairs from male moths however do not produce any symptoms. In the present study we have isolated and partially characterized a protease with kallikrein-like activity from the female abdominal hairs of this species. Egg-nests (consisting mainly of female abdominal hairs) were collected from the twigs of mangrove-trees in affected areas after hatching. The proteic substances were extracted into Tris-buffered saline solution at pH 8.5, centrifuged, and chromatographed by size-exclusion chromatography (Sephadex G-75). Biological activity in the peaks was determined by amidolytic activity in the chromogenic substrates S-2288 (Broad Spectrum Serine Protease Substrate) and S-2302 (Kallikrein substrate). The eluates showing biological activity were concentrated by ultrafiltration and used for further analyses. The specificity in chromogenic substrates showed a preference for the kallikrein substrate S-2302 followed by the broad-spectrum serine protease substrate S-2288. In addition the enzyme showed a pH optimum at

Recibido: 14 / 05 / 2001. Aceptado: 14 / 01 / 2002.

pH 9, with no activity below pH 5. Thus the results of the present study support the hypothesis that this substance may be of importance in the lesions observed in individuals exposed to adult females or the egg-nests.

Key words: *Hylesia metabus*, kallikrein, protease

INTRODUCTION

Urticating hairs or setae are found in at least 15 families belonging to the five superfamilies in the Lepidoptera [14,19]. In most cases it is the larvae that have the urticating setae. They are found among adults, i.e. in the *Anaphae* genus in the Thaumetopoeidae and in the *Hylesia* genus in the Saturnidae. Moths of the *Hylesia* genus are widely distributed in Central and South America. In Venezuela the local variety has been identified as *Hylesia metabus*, commonly known as “Palometa Peluda” among the population. Entire towns are invaded periodically by millions of swarming moths, attracted at night-time by the street-lights shedding copious amounts of their urticating hairs in the air [4, 5].

The habitat of the moth is the mangrove forests of the north-eastern part of the country. In this species the abdomen of the adult females is covered with loosely attached urticating setae. In the process of oviposition the female deposits her urticating abdominal hairs over the eggs in order to protect them from predators. Contact with the airborne urticating abdominal hairs or accidental contact with the egg-nests gives rise to an intense pruriginous papuloerythematous dermatitis with discreet vesicular degeneration which appears without previous sensitization [4, 16]. Papules may be small and isolated or continuous, spreading out from the initial point of contact. This is particularly the case when the hairs fall on sensitive areas such as the neck, upper arm or stomach. The pruritus and erythema is increased by scratching, possibly because the hairs get more embedded in the skin. Affected persons have also observed that the pruritus, is increased by using common soap and that it is alleviated by slightly acid solutions such as vinager (direct observation by the authors). Other authors have described frequent haemorragic elements in the pruriginous areas, characterized by fine purpuric lesions [13]. Necrotizing lesions have also been observed [4, 9]. The duration of the lesions is very variable and may last from three to 15 days [9].

The presence of one or more urticating substances in the setae has been suggested previously by several authors [4, 5, 9, 14]. In the present study, a substance with amidolytic activity in several chromogenic substrates from the female abdominal hairs used to form the egg-nests was identified and partially characterized The substance shows a preference for the kallikrein-specific substrate H.D.-Propyl-L-Phenylalanine-L-Arginine-p-nitroanilide-dihydrocholride (S-2302).

MATERIALS AND METHODS

Reagents

Unless otherwise specified all reagents were purchased from Sigma Chemical Co. St. Louis Mo. USA. The highest degree of purity available of the reagents was always used. Chromogenic substrates were purchased from Chromogenix AB, Mölndal, Sweden.

Preparation of Extract

Hylesia metabus egg-nests were collected from the twigs of the mangrove-trees, macroscopically cleaned from debris, cut into pieces of approximately 0.5 x 0.5 cm and extracted into 50 mM Tris buffer pH 8.5 supplemented with 0.15 M NaCl for 6 hours at 4.0°C with gentle agitation. Approximately 120 mg of egg-nest material was used. The extract was centrifuged at 16.000 g in a Sorvall RC-5B Centrifuge using a HB4 rotor (DuPont Instruments) and concentrated ten-fold by ultrafiltration in an Amicon model 52 Apparatus using YM2 ultrafiltration membranes (Amicon Corporation, Danvers MA. 01923, USA). Determination of protein concentration was performed as described by Lowry et al. [11]. Bovine Serum Albumin (BSA) was used as a standard. Amidolytic activity was evaluated using chromogenic substrates S-2288 (broad spectrum serine protease substrate) and S-2302 (plasma kallikrein substrate) according to the instructions of the manufacturer. 120 mg of male wings were cut into pieces of 0.5 x 0.5 cm, extracted and prepared in the same way as the egg-nest material.

Gel filtration chromatography

Concentrated crude egg-nest extract was applied to a Sephadex G-75 column (1.5 x 80 cm). Elution was performed using 50 mM Tris buffer supplemented with 150 mM NaCl at a flow-rate of 0.1 mL/min. Fractions of 1.5 mL were taken using a Frac-100 Fraction Collector (Pharmacia Fine Chemicals, Uppsala, Sweden). Absorbance at 280 nm was monitored using a UV1 UV detector (Pharmacia Fine Chemicals, Uppsala, Sweden). Fractions showing amidolytic activity in chromogenic substrate S-2302 were pooled and concentrated by ultrafiltration as described above. Molecular Weight determinations were done by comparing the retention volume of the peak of interest to the retention volumes of proteins with known molecular weights.

Determination of kinetic parameters

The *kcat/Km* ratio was evaluated with chromogenic substrates: S-2288 (broad spectrum serine protease substrate) and S-2302 (Plasma Kallikrein Substrate). The incubation buffer was prepared according to the instructions of the manufacturer. For this purpose 0.1 M of the purified protein was incubated with 0.2 mM of chromogenic substrate at a final volume of 1 mL in 50 mM Tris buffer pH 8.5 suplemented with 0.15 M NaCl. The absorbance readings at 405 nm were recorded in a

Spectronic Genesys 2 spectrophotometer (Spectronic Instruments Inc., Rochester, NY, USA) at 5 second intervals. The substrate concentration at any given moment (S) was obtained by:

$$S = So - P$$

Where S is the substrate concentration at any given moment and *So* is the initial substrate concentration. P is the *p*-n-itroaniline (p-NA) concentration as determined by the absorbance at 405 nm. The natural logarithm for the substrate concentration at any given time (LnS) was calculated and plotted as a function of time. The slope of this curve (-Kobs) was used to determine Kcat by the following equation:

$$\frac{kcat}{Km} = \frac{kobs}{Et}$$

Where kobs is the first order rate constant and Et is enzyme concentration. The calculation of the kinetic parameters Km and Vmax was done using the substrate S-2302. The rate of 1/p-nitroanilide formation at different substrate concentrations was plotted as a function of 1/Substrate according to Lineweaver-Burk. Km and Vmax were then calculated from the linear equations obtained by the minimum square fit method using the “Linest” function in Microsoft Excel.

Determination of pH optimum

Determination of pH optimum was performed by incubating Chromogenic Substrate S-2302 with concentrated Sephadex G-75 purified extract (Peak 2 in FIG. 1.). Incubation medium consisted of 0.15 M NaCl supplemented with 50 mM of either Tris or sodium acetate buffer adjusted to cover a pH range of 3.0 to 11.0. Absorbance at 405 nm was determined after 10 min incubations at 37°C.

RESULTS

Extraction of egg-nest material

The concentrated crude egg-nest extract (1 mL) showed an activity of 27 International Kallikrein Units (1 International Kallikrein Unit hydrolyses 1 x 10^{-6} moles of chromogenic substrate S-2302 per minute). The protein concentration was 3.2 mg/mL. Thus the specific activity of the extract under these conditions was 8.4 IKU/mg as can be seen in TABLE I. It should be noted that the extract from the male wings had a protein concentration of 0.94 mg/mL but showed no amidolytical activity in the chromogenic substrates used.

Gel filtration chromatography

FIG. 1. shows the elution profile in Sephadex G-75 of the concentrated egg-nest extract. The material with activity in the chromogenic substrates S-2302 and S-2288 was confined to the second peak (hatched area). The specific activity of the concentrated fraction (1 mL) was 34 IKU/mg as shown in TABLE I. The molecular weight of the substance, as determined by comparison to standards of known molecular weight, was approximately 32 kD.

Kinetic studies

The results of the k3/kM determinations of the purified egg-nest material are shown in TABLE II. As can be seen, the plasma kallikrein substrate S-2302 showed the highest specificity as determined by K3/kM measurements, followed by the broad spectrum serine substrate S-2288. Kinetic analysis performed using S-2302 showed a kM of 7.8 micromoles and a Vmax of 85 M/sec.

pH dependence

FIG. 2. shows the pH dependence of the substance, purified from the egg-nest material, measured as rate of hydrolysis of the kallikrein substrate S-2302. As can be seen the pH optimum is close to pH 9.0 with practically no activity below pH 5.

DISCUSSION

A protein with urticating properties has been isolated from the caterpillar *Thaumetopoea pityocampa* [10], and urticating proteins have also been reported from the hairs of two species of caterpillar from the genus *Euproctis* [7].

The skin lesions described are probably caused by several urticating agents. One of the more common of these is histamine, known to be a mediator of the inflammatory reaction [17], and possibly the chronic allergic reaction [16]. Histamine has been found in various urticating Lepidopteran species including *Hylesia* moths [8]. Nonetheless, as Benaim-Pinto *et al.* point out [4], the effect of histamine subsides in a short time.

TABLE I

RECOVERY OF KALLIKREIN-LIKE ACTIVITY AT DIFFERENT STAGES OF PURIFICATION

Fraction	Activity (IU)	Protein Content(mg/mL)	Specific Activity(IU/mg)
Crude Extract	27.00	3.02	8.4
Sephadex G-75 (Fraction II)	25.34	0.85	30.0

Activity was measured in Cromogenic Substrate S-2302.

TABLE II

ETERMINATION OF *KCAT/KM* IN CHROMOGENIC SUBSTRATES S-2302 (PLASMA KALLIKREIN) AND S-2288 (BROAD SPECTRUM SERINE PORTEASE SUBSTRATES)

Chromogenic Substrate	K3/Km
S-2302	6.2 x 10^4
S-2288	4.8 x 10^4

Furthermore Dinehart et al. [8] mention that antihistamine therapy does not eradicate the symptoms, suggesting that other pathological mechanisms are involved. Nevertheless, histamine could be involved in the initial reaction, either directly or indirectly, although it is probable that other substances are responsible for the more persistent symptoms.

Benaim-Pinto et al. [5] found that closed patch tests with extract obtained from female abdominal hairs produced symptoms similar to those produced by direct skin contact (erythematous papules, sensitive to friction, which lasted for several hours). Passive transfer and direct RAST testing indicated that the material was highly antigenic and of an enzymatic nature [4]. They concluded that one or several enzymes with a proteolytic activity could cause the delayed papular and sometimes necrotic reactions resulting from contact with the urticating hairs. Bleumink et al. [6], undertook a study of the activities of proteases in the urticating hairs of two species of caterpillar in the genus *Euproctis*. These authors found urticating substances with activities similar to trypsin, urokinase, and kallikrein (amongst others). These authors suggested that the release of bradykinin could be an important pathogenic element and that contact with the urticating proteins found in *Euproctis* spp. may provoke allergic reactions due to the fact that human tissue is not capable of neutralizing these agents.

Plasma kallikrein is a serine protease produced by the proteolytic cleavage of the inactive zymogen, prekallikrein, by the serine protease Factor XIIa belonging the intrinsic coagulation pathway. Plasma kallikrein activates the inactive precursor of Factor XIIa: Factor XII or Hageman Factor by limited proteolytic cleavage. Kallikrein also releases the peptide bradykinin from the glucoprotein High Molecular Weight Kininogen [18]. Bradykinin is a potent mediator of the inflammatory reaction, giving rise to increased vascular permeability, pain, and smooth muscle contraction [15].Thus it seems likely that the cutaneous irritation, at least in part, may be explained by bradykinin released by the action of the venom, confirming the observations made by Bleumink et al. [6].

The venom from *Hylesia metabus* showed a preference for the plasma kallikrein substrate S-2302 (k3/kM = 6.2 x 10^4) as compared to the broad spectrum serine protease substrate S-2288 (k3/kM = 4.8 x 10^4). They may thus be considered as good substrates for the enzyme.

Chromogenic substrates simulate natural protein substrates. Although these substrates are not completely specific, the results of the enzymatic activity experiments indicate that the crude extract and purified enzyme of the egg-nests contain substances with broad-spectrum protease activities as seen by the activity in chromogenic substrate S-2288 as well as kallikrein-like activity as demonstrated by the activity in chromogenic substrate S-2302. The wings of the males, on the other hand, did not demonstrate any proteolytic activity. It is interesting to note that in *Lonomia acheolus* caterpillars, tests with chromogenic substrates suggest the presence of substances

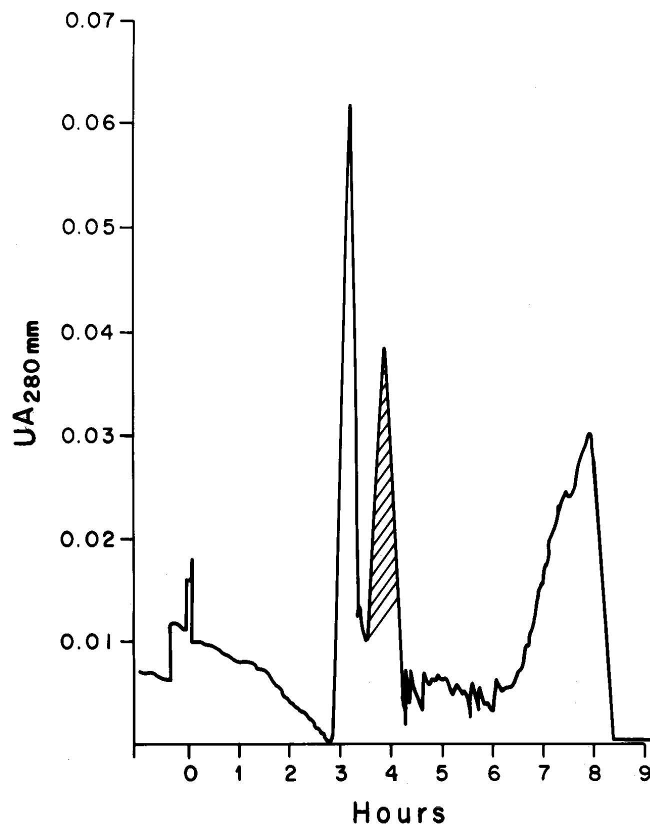

FIGURE 1. CHROMATOGRAM OBTAINED BY UV ABSORBANCE AT 280 nm FROM A TYPICAL RUN OF CONCENTRATED EGG-NEST CRUDE EXTRACT ON A SEPHADEX G-75 COLUMN (1.5 X 90 CM). FLOW-RATE: 0.1 mL/min. BIOLOGICAL ACTIVITY IN THE SEPARATED PEAKS WAS DETERMINED BY AMIDOLYSIS IN CHROMOGENIC SUBSTRATES S-2288 (BROAD SPECTRUM SERINE PROTEASE) AND S-2302 (PLAMA KALLIKREIN SUBSTRATE).

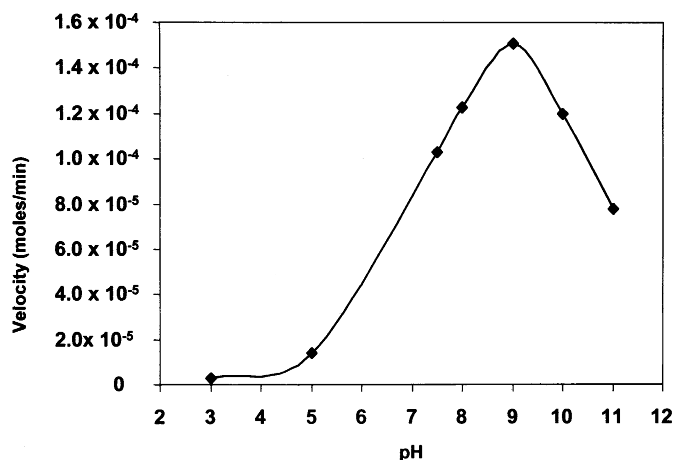

FIGURE 2. AMIDOLYTIC ACTIVITY OF SEPHADEX G-75 PURIFIED EGG NEST PROTEIN AS A FUNCTION OF pH. X-AXIS SHOWS pH UNITS AND Y-AXIS THE AMIDOLYTIC ACTIVITY IN CHROMOGENIC SUBSTRATE 2302, IN MOLES PER MINUTE.

with kallikrein-like activity, results that the authors interpreted as being related to pain and burning sensations at the site of contact [1,2,3].

The results from the experiments of pH dependence showed that this enzyme has a pH optimum around pH 8.8, with no activity below pH 5. This observation may explain why washing affected skin areas with common household soap worsens the rash, while application of vinegar or other slightly acidic solutions has a calming effect.

Thus in conclusion we have shown evidence for the presence of a kallikrein-like substance in the urticating hairs of female *Hylesia metabus* moths. This substance is likely to be at least partly responsible for the stinging sensation and skin lesions caused by contact with the hairs. The presence of other substances with properties similar to trypsin, urokinase and plasmin may also be implicated in the production of the skin lesions. Studies are at present being undertaken which include the further separation of the urticating fractions using ionic exchange chromatography and electrophoresis.

ACKNOWLEDGEMENTS

We would like to thank the habitants of the village of Yaguaraparo, estado Sucre, Venezuela for their patience and invaluable support in obtaining the egg-nest material. This project was financed in part by the Consejo de Investigación of the Universidad de Oriente No:CI-5-1901-0820/98 y Fundacite through the Fondo de Investigación Regional.

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