

Arginine Kinase from the Cellar Spider (*Holocnemus pluchei*): A New Asthma-Causing Allergen

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Established Facts

- Allergy to spiders is rare, seldom reported and limited to cutaneous manifestations.
- Spiders represent an important but as yet unexplored source of indoor allergens, especially in agricultural environments.

Novel Insights

- We describe the first case of asthma caused by exposure to spiders.
- An arginine kinase (AK) was identified as an allergen in the cellar spider *Holocnemus pluchei*, also described in arthropods and silkworms. The sensitization to AK was confirmed by positive specific bronchial provocation tests.
- Given their rather common indoor presence, sensitization to spiders should be investigated in cases of persistent asthma due to either occupational or nonoccupational exposure.

Key Words

Arginine kinase • Asthma • Cellar spider • *Holocnemus pluchei* • Occupational asthma • Spider allergy

Abstract

Background: We report a 31-year-old farmer whose work consists in handling cereal and vegetables, who consulted our clinic because of asthma symptoms after inhalation of dust during manipulation of the deposited material, usually

inside the warehouse. **Methods and Results:** Skin prick tests and specific immunoglobulin E (IgE) determinations were negative with common aeroallergens. The patient noted the presence of many spiders in the warehouse, which were identified as the cellar spider *Holocnemus pluchei* and the common house spider, *Tegenaria domestica*. Extracts of spider bodies brought in by the patient were obtained and used to perform in vivo and in vitro studies. Molecular characterization of IgE-binding bands was performed by mass spectrometry. We obtained positive prick tests to the ex-

tracts of the bodies of both spiders. Immunoblotting displayed different bands in both spider extracts, in a range of 20–70 kDa. All were hemocyanins, except for a 17-kDa protein of *Holocnemus* identified as an arginine kinase (AK). Bronchial challenge was positive with the extract of the cellar spider and with the AK, but was negative with the domestic house spider. **Conclusion:** We present the first case of respiratory allergy due to sensitization to AK from a common spider, confirmed by bronchial provocation tests.

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Introduction

Up to 38,000 species of spiders have been described to date, but only a dozen are potentially dangerous to humans [1]. Also, while the medical literature abounds with cases of occupational diseases due to allergy to cockroaches, butterflies, grasshoppers, moths and mites [2, 3] in agricultural and laboratory workers, allergy to spiders is seldom reported [4] and is limited to cutaneous manifestations, such as an allergic airborne contact dermatitis in a farmer with positive skin tests to the common house spider *Tegenaria domestica* [5], immunoglobulin E (IgE)-mediated contact urticaria [6] and a case of anaphylaxis after contact with a spider belonging to the Sparassidae family [7].

The purpose of this article is to describe the first case of asthma caused by primary sensitization to a common spider, the cellar spider (*Holocnemus pluchei*), confirmed by immunologic and bronchial provocation tests.

Patient and Methods

Case Report

We report a 31-year-old man who, at the time of presentation, had been working on a farm in central Spain for the previous 10 years. His work consists mainly of handling cereals (barley, wheat) and legumes (peas) and participating in their sowing, harvesting and storage. Sometimes he also feeds the pigs on the farm with the leftovers from the grains. He presented at our clinic because in the previous 7–8 years he had noticed coughing, shortness of breath and wheezing in the late hours of the workday. These symptoms usually appear during the sowing of cereal (November–December) and peas (February) and also when harvesting both (June–July), while preparing the warehouses (download and storage) and especially when moving the grain and peas (he attributed his symptoms to inhalation of the loose powder generated in this process). The rest of the year he only experiences asthmatic symptoms 2–3 h after being on the pig farm. The patient is asymptomatic when on holiday (August) and when he does not handle cereal or peas nor goes to the pig farm. The patient's baseline

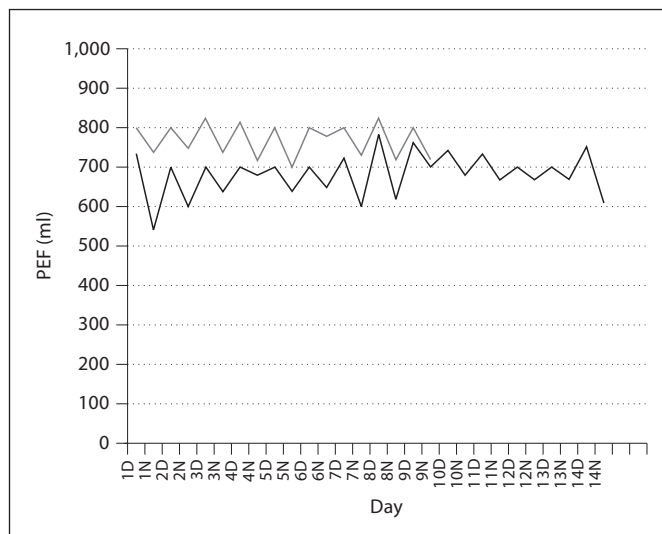


Fig. 1. PEF readings while the patient was working (upper curve) and off work (lower curve). D = Daytime measurements; N = nighttime measurements.

spirometric parameters were normal, but the fractional exhaled nitric oxide (FeNO) concentration was high (203 ppb). The methacholine test, performed in accordance with the 5-breath dosimeter method [8], yielded a positive result, with a PC₂₀ of 6.20 mg/ml (normal value >16 mg/ml). The patient was instructed to perform peak expiratory flow (PEF) measurements while working (4 measurements daily, 2 of them during working hours) and on medical leave. The visual analysis of the 2 PEF registers showed a clear improvement in the PEF readings while the patient was off work (fig. 1). On the basis of these results, the patient was diagnosed with occupational asthma, and then we carried out an allergological study in search of the causative allergen.

Both skin prick tests (SPTs; ALK-Abelló, Madrid, Spain) and specific IgE determinations (ImmunoCAP, Phadia, Uppsala, Sweden) were negative with common inhalant allergens, as well as with storage mites, molds, animal epithelia, flours, additives and legumes. Specific IgE determinations were also negative (<0.35 kU/l) with tropomyosin (rPen a 1), latex and classic contaminants of stored grain [miller beetle (*Tenebrio molitor*), destroyer beetle (*Trogoderma angustum*), grain weevil (*Sitophilus granarius*) and flour butterfly (*Ephesia kuehniella*)].

The patient brought in some of the grain and pea dust to which he had attributed his symptoms. A SPT with an in-house 1/10 w/v extract of the pea dust was positive (4-mm wheal diameter). Since the SPTs with the commercial extracts of pea and other vegetables, as well as with the usual stored grain contaminants, had been negative, SPTs were performed with other probable contaminants of pea dust, giving negative results with insects (flea, fly, mosquito and cockroach) and a strongly positive result (17 mm) with the spider species *T. domestica*.

When asked, the patient affirmed the presence of numerous spiders in the warehouses and on the pig farm, so we asked him to bring us spiders and pictures from his workplace. The most

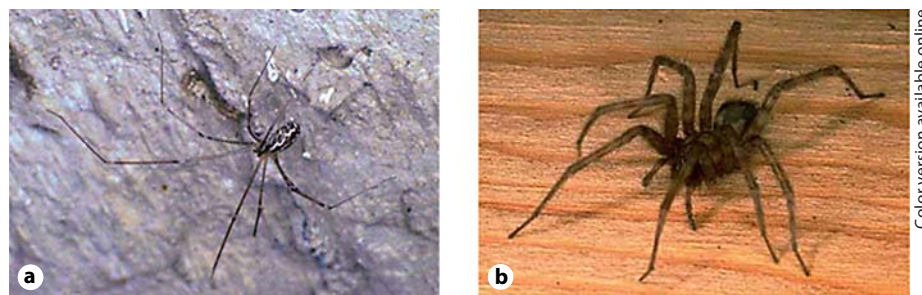


Fig. 2. **a** *H. pluchei*. **b** *T. domestica*. **c** SDS-PAGE and IgE immunoblotting with the patient's serum. MW = Molecular weight in kilodaltons. Lane 1: SDS-PAGE with *Holocnemus* extract (75 µg); lane 2: IgE immunoblotting with *Holocnemus* extract (50 µg); lane 3: SDS-PAGE with AK (10 µg); lane 4: IgE immunoblotting with AK (5 µg); lane 5: SDS-PAGE with *Tegenaria* extract (75 µg); lane 6: IgE immunoblotting with *Tegenaria* extract (50 µg).

abundant spider present in the workplace was identified as the cellar spider *H. pluchei* (Scopoli, 1763; Pholcidae) (fig. 2a). The common house spider *T. domestica* (Clerk, 1757; Agelenidae) (fig. 2b) was found less abundantly. After obtaining written informed consent from the patient, the allergological workup was completed, as detailed below.

Preparation of Extracts

Using dead spiders provided by the patient, after crushing the bodies in a mortar, 1/10 w/v aqueous extracts in phosphate-buffered saline (PBS) were prepared. The extracts of the bodies were used for performing in vivo [SPTs, specific bronchial challenges (BCs)] and in vitro [SDS-PAGE, immunoblotting and identification of allergens by mass spectrometry (MS)] studies.

SPTs and Specific BCs

SPTs with extracts of both spiders (1/10 w/v) and with a subsequently purified protein (10 µg/ml) were performed on the patient and on 5 healthy nonexposed control subjects. Specific BCs with the spider extracts were carried out on the patient and 2 control patients with asthma. BC with the purified protein could not be performed in the control patients, as they refused the second challenge.

The BCs were performed while the patient was off work and asymptomatic from a respiratory standpoint, and they were separated by nearly a month.

BC was performed using an electronic dosimeter Spire Elektro (Respiratory Care Center, Hameelinna, Finland). The patient inhaled the aerosolized allergen in progressive concentrations ac-

cording to a 5-breath dosimeter protocol [9]. Increasing doses of the allergen solutions, starting with the concentration that had induced a 2-mm wheal on SPT [10], were inhaled every 10 min. The forced expiratory volume during the first second (FEV₁) and the forced vital capacity (FVC) were measured 5 and 10 min after each dose, according to the recommendations of the American Thoracic Society [11], using a SpiroAnalyzer ST-75 spirometer (Fukuda Sangyo, Nagareyama, Japan). The BC was interrupted if FEV₁ dropped by ≥20% compared to the postsaline FEV₁ or when the final dose had been reached. Then, PEF and FEV₁ were measured 1 and 2 h after the end of the BC. Afterwards, the patient measured his PEF with a portable peak flow meter every 2 h at home, except during sleep, until 24 h later, when a new spirometry was performed. A methacholine inhalation test and FeNO determinations before and 24 h after the BCs were also performed [12].

Immunoblotting Analysis

Protein bands separated by SDS-PAGE were transferred by semidry blotting onto nitrocellulose sheets by the method of Towbin et al. [13]. Membranes were blocked with PBS containing 0.1% Tween 20 (PBS-T), 3% skimmed milk powder and 3% bovine serum albumin for 2 h at room temperature and incubated overnight at 4°C with sera diluted 1:10 in 50 mM Tris-buffered saline, 150 mM NaCl, 5 mM EDTA and 0.05% triton X-100, pH 8.8. After washing 5 times with PBS-T, blots were incubated for 1 h at room temperature with goat antihuman IgE antibody conjugated with horseradish peroxidase (Nordik Cultek, Tilburg, The Netherlands) diluted 1:5,000 in PBS containing 0.05% Tween-20 and 1.5% skimmed milk powder. Finally, after washing 5 times with

Table 1. Match between sequenced peptides and AKs from other arthropods

	Sequence	Identities, %	Positives, %
Sequenced peptide	E M E S K V S S T L S G L E G E L K		
Dro m 9	- - - - - - - - - - - - - - -	100	100
Bla g 9	- - - D - - - - - - - - - -	94	94
Lit v 2	- - - A - - - - - S - - - - -	88	88
Pen m 2	- - - A - - - - - S - - - - -	88	88
Per a 9	- - - D - - - - - - - - A - P -	83	83
Lim p 2	- - - E - - - - - S M - D - - -	77	83
Der p 20	- - - T - - K G Q - A T F - - - -	61	61
Sequenced peptide	T F L V W C N E E D H L R		
Dro m 9	- - - - - - - - - - - - - - -	100	100
Bla g 9	- - - - - - - - - - - - - - -	100	100
Per a 9	- - - - - - - - - - - - - - -	100	100
Lit v 2	- - - - - V - - - - - - - - -	92	92
Pen m 2	- - - - - V - - - - - - - - -	92	92
Lim p 2	- - - - - V - - - - - - - - -	92	92
Der p 20	- - - M - V - - - - - - - - -	84	84

PBS-T, protein bands were visualized by electrochemiluminescence (Amersham Bioscience, GE Healthcare, Milwaukee, Wisc., USA), following the instructions given by the manufacturer.

Protein Identification and Characterization by MS

Identification of proteins by MS and/or MS/MS (MS in tandem) was performed as previously described [14] at the proteomic service of the Complutense University of Madrid, a member of the ProteoRed Network. Briefly, selected bands were cut out from the gels with a sterile scalpel and subjected to in-gel trypsin digestion according to Shevchenko et al. [15], with some modifications. The in-gel digests were purified as described by Gobom et al. [16].

Protein Purification

The 17-kDa IgE-binding protein was purified by 2 different chromatography steps in an HPLC system (AKTA purifier, GE Healthcare). Briefly, spider extract, dialyzed in 20 mM Tris, pH 7.9, was loaded onto a Mono Q 5/50 GL column (GE Healthcare). The bound proteins were eluted with a linear salt gradient from 0 to 0.5 M NaCl, and we obtained a fraction containing a 17-kDa IgE-binding protein. This fraction, dialyzed in 20 mM Tris, pH 6.8, was loaded onto a Mono S 5/50 GL column (GE Healthcare). In this step, proteins were eluted with a linear salt gradient from 0 to 0.5 M NaCl, and we obtained a fraction containing purified 17-kDa IgE-binding protein.

Results

Identification of Proteins

SDS-PAGE of the extracts of both spiders showed multiple protein bands with an apparent molecular weight ranging from 6 to 200 kDa (fig. 2c, lanes 1 and 5). IgE im-

munoblotting with the patient's serum demonstrated various IgE-binding bands, of approximately 66, 55, 45, 43, 34, 31, 23 and 20 kDa for *T. domestica* (fig. 2c, lane 6) and 17, 33, 34, 64 and 66 kDa for *H. pluchei* (fig. 2c, lane 2).

To identify the proteins, we performed MS/MS and obtained the sequence of several internal peptides with the following sequences for the 17-kDa protein: EME-SKVSSTLSGLELKE, EVQQKLIDDHFLFK, TFLVWC-NEEDHLR and GTRGMHTEAEGGIYDISNK. Research conducted with protein databases identified the 17-kDa protein as an arginine kinase (AK). As shown in table 1, 2 of these protein peptide sequences yielded a strong match (>70%) with AKs from other arthropods defined as allergens, i.e. Der p 20 (house dust mites), Per a 9 and Bla g 9 (American and European-type cockroaches), Lit v 2, Pen m 2 and Hom q 2 (shrimp), and Dro m 9 (fruit fly *Drosophila melanogaster*) (see also www.allergome.org). SDS-PAGE and IgE immunoblotting of the purified 17-kDa AK from the cellar spider are presented in figure 2c (lanes 3, 4).

The other IgE-binding proteins from the *H. pluchei* extract (of 33, 34, 64 and 66 kDa), as well as all the IgE-binding proteins from the *T. domestica* extract, were identified by MS as different forms of hemocyanins.

Results of the in vivo Studies

SPTs with the extracts from both species of spider, *Holocnemus* and *Tegenaria*, were positive in our patient at

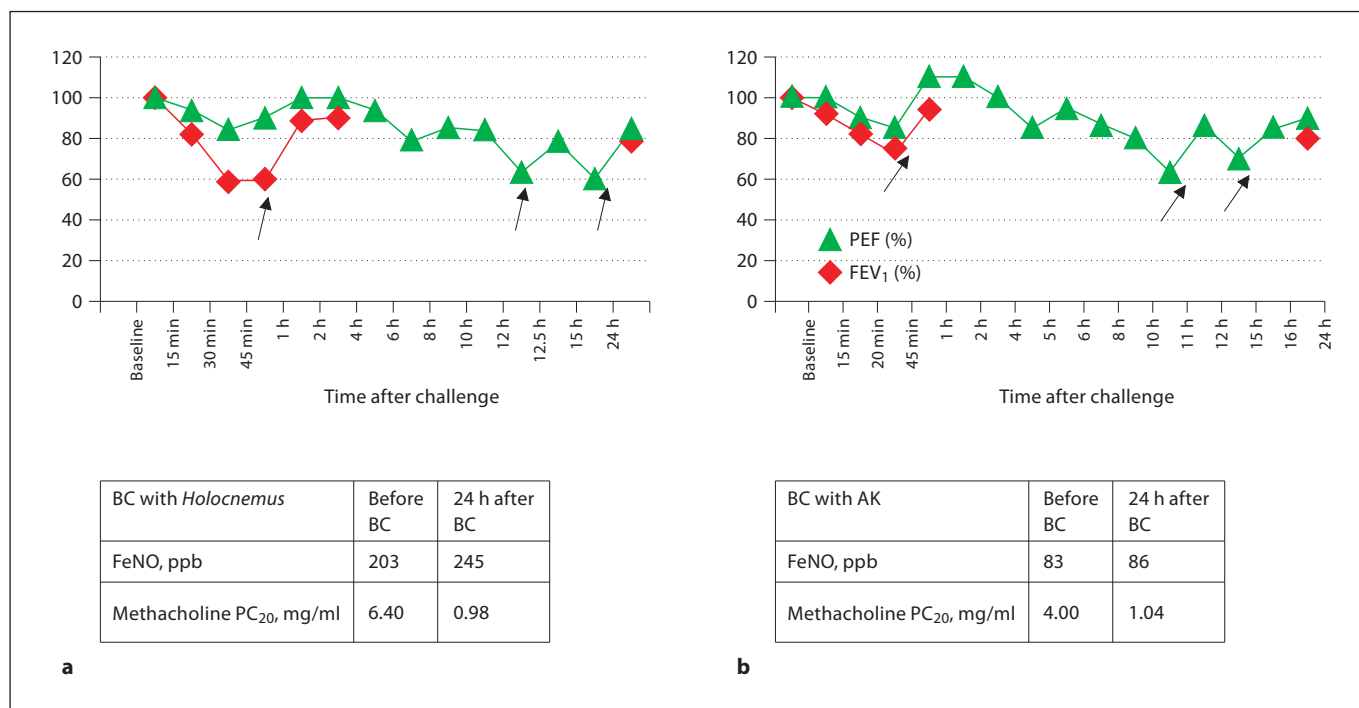


Fig. 3. **a** BC with *Holocnemus* extract (1:100 dilution). **b** BC with purified AK (1:1 dilution). Arrows indicate the administration of salbutamol (200 µg).

1/10 w/v (10- and 11-mm wheal diameter, respectively). SPTs were negative in the 5 nonexposed controls.

BC with the extract of *H. pluchei* at 1:100 dilution elicited a dual asthmatic response; there was an immediate decrease in FEV₁ of 40%, and, 12 h later, the patient experienced asthmatic symptoms at home with a documented decrease in PEF of 40% (fig. 3a). The BC with the *T. domestica* extract was negative in our patient, as were the BCs with both spider extracts performed in 2 of the control subjects.

Finally, we performed a SPT with the purified AK, which was positive (10 mm) in the patient and negative in 5 controls, followed by a specific BC with this protein at dilutions of 1:100, 1:10 and 1:1. The 1:1 dilution elicited a dual asthmatic response; there was an immediate fall in FEV₁ of 21% and, 11 h later, a decrease in PEF of 37% (fig. 3b). The results of the FeNO measurements and of the methacholine tests before and after the BC with *H. pluchei* and the AK are also shown in figure 3.

We diagnosed the patient as having occupational asthma caused by allergy to AK from the cellar spider (*H. pluchei*). The patient found it impossible to change his workplace, as advised, so he was instructed to always use protective masks while working inside the warehouse or

on the pig farm. The patient's asthma symptoms are currently controlled with a combination of budesonide/formoterol (160 and 4.5 µg, respectively, every 12 h, and as reliever when needed). The patient is asymptomatic when on vacation.

Discussion

The first mention of *T. domestica* as the source of an inhalant allergen was made in 1966 [17]. Thereafter, in 1975, in a study of 87 asthmatic children [18], SPTs to spiders were positive in 40% of cases, as they were for mites (*Dermatophagoides* spp.), but the correlation with the clinical manifestations in the case of spiders was only partial.

Despite these interesting data, subsequent mentions of spiders as sources of inhalant allergens are almost inexistent.

To the best of our knowledge, we herein describe the first case of asthma caused by exposure to spiders, more precisely to the cellar spider *H. pluchei*, demonstrated by means of BC. We ruled out cross-reactivity with tropomyosin, which suggested a primary sensitization to another spider allergen. As all proteins identified in the

Tegenaria extract, which had yielded a negative BC, were hemocyanins, as well as the majority of those of *Holocnemus*, it appeared that the 17-kDa IgE-binding band was the main sensitizing allergen in this case. MS identified the protein corresponding to this band as an AK, and the BC with this purified protein was positive. The resemblance between the asthmatic responses induced by the *H. pluchei* extract and the purified AK extract can be easily observed in figures 3a and b.

At the molecular level, we identified AK as an allergen in *H. pluchei* for the first time. AKs have been described as allergens in arthropods and more recently in silkworms [19]. These enzymes (phosphotransferases), with molecular masses around 40–42 kDa, play an important role in cellular energy metabolism and maintaining constant ATP levels in invertebrate cells.

In our case, the identified protein of 17 kDa is a degraded product of AK. We suppose that the presence of dead (therefore decomposing) spiders in the stored grain and leftovers probably resulted in aerosolized (and fractioned, degraded) spider proteins, which the patient inhaled when moving the grain and peas and during pig feeding activities.

The degradation process did not seem to alter the allergenic capacity of this 17-kDa AK, as already described with other proteins, e.g. melon cucumisin [20].

Although other authors demonstrated that AKs represent a new class of cross-reactive invertebrate pan-allergens [21], this does not seem to be the case for our patient, who is not sensitized to mites, cockroach or shrimp. A possible explanation could be the existence of significant differences between the various molecular forms of the enzyme in these arthropods and the 17-kDa fraction identified here.

Moreover, the cross-reactivity between *H. pluchei* and *T. domestica* in this patient is most probably due to the hemocyanins, given the positive SPTs to both, but the negative BC with *Tegenaria* extract, which lacked the AK. Arthropod hemocyanins are copper-containing proteins that deliver oxygen in the hemolymph of many chelicerates, crustaceans, myriapods and also possibly some insect species [22]. They are multimeric proteins (each subunit weighs about 75 kDa, and they may be arranged in dimers or hexamers depending on the species), so the large size and numerous epitopes, capable of generating a substantial (delayed-type) immune response, make them ideal carrier proteins. Keyhole limpet hemocyanin is used extensively as a carrier protein in the production of antibodies for research, biotechnology and therapeutic applications [23]. Although we have not found any references to hemocyanins binding IgE in the literature, we think it was possible in the patient presented herein, also due to degradation of spider hemocyanins to smaller peptides between 20 and 70 kDa.

As has been demonstrated in numerous studies, home exposure to indoor aeroallergens is an important environmental factor in allergic sensitization and in the development and exacerbation of asthma [24]. The case reported herein involved massive workplace exposure in a farmer (online supplementary fig. 1–4, www.karger.com/doi/10.1159/000319822). Nevertheless, the abundant presence of spiders in homes is also common, which makes them an important and yet unexplored source of indoor allergens. Thus, we believe sensitization to spiders should be investigated in cases of persistent asthma due to either occupational or nonoccupational exposure.

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DRAGLINE
SILKS IN THE
WAREHOUSE.



SPIDERS (*H. PLUCHEI*) AND DRAGLINE SILKS IN THE PIG FARM.

