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Antigens of *Euparipha pisana* (Snail)

I. Identification of Allergens by Means of in vivo and in vitro Analysis

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Abstract. The data obtained in this study suggest that eating *Euparipha pisana* (snail), a common food in Mediterranean countries, could give serious allergic reaction such as asthma. We describe here the identification and partial characterization of allergenic molecules from this new source. An aqueous extract of snail was obtained by homogenization in distilled water, centrifugation, dialysis and defatting with ethyl ether. Skin prick test (SPT) performed with the snail extract on 70 subjects allergic to the more common allergens of the Mediterranean area gave a SPT positivity in 61% of the subjects tested, with a mean value of histamine-equivalent prick (HEP) equal to 0.81 ± 0.25 ($n = 43$), while no SPT-snail-positive reactions were obtained by using the same extract on 30 not allergic subjects. To ascertain if such a sensitivity was IgE-mediated, sera from SPT-snail-positive subjects were analyzed by RAST, coupling the snail extract to polystyrene balls and to paper discs. 19% of the sera tested were RAST-positive, mean value of binding $4.8 \pm 2.8\%$ ($n = 13$), while when using sera from SPT-snail-negative subjects, the RAST mean value was $0.49 \pm 0.18\%$ ($n = 27$). Histamine release (HR) was also performed. Basophils prepared from SPT-snail-positive subjects were incubated with a snail extract. All of the SPT-snail-positive subjects gave a significant value of HR, mean value $21.8 \pm 7\%$ using $1 \mu\text{g}$ of snail extract ($n = 16$), while $1.41 \pm 1.1\%$ ($n = 10$) was the mean value obtained when SPT-snail-negative subjects were analyzed. The components of the snail extract, fractionated by sodium dodecylsulfate polyacrylamide gel electrophoresis and then transferred to nitrocellulose membrane, were studied by electroblotting using 10 sera collected from SPT- and RAST-snail-positive subjects. After a reaction with ^{125}I -antihuman IgE and autoradiography, 6 bands were capable of binding human IgE, ranging in molecular weight value from 12,000 to over 66,000 daltons. Finally, a strong cross-reactivity was detected by means of RAST inhibition, between the snail and the *Parietaria judaica*, *Dermatophagoides pharinae* and *Poa pratensis* allergens. The results obtained with four different methods provide evidence that the snail is a new source of allergenic molecules, and leads us to more extensive studies of this type of food allergy.

Introduction

It has been recognized for a long time that food can cause allergic reactions. The snail is a food commonly eaten by both children and adults in several countries. On a special occasion (Patron Saint's days) the snail in our country is part of a very popular and characteristic meal. During these days an increase in asthmatic attacks among patients usually allergic only to the more common allergens of the Mediterranean area was observed. Enquiries showed that the patients had experienced asthma attacks soon after a snail meal.

Several species of snails are living in our country, but *Euparipha pisana* is a more common one and it is the species that people usually eat.

Skin prick test (SPT) is the first test usually performed to ascertain if an extract can be an allergenic source for a specific subject. But as skin reactivity may not accurately reflect circulating food-specific IgE antibodies, because of the high proportion of positive SPT in asymptomatic subjects and vice versa [1, 2], more than one single test must be carried out to evaluate the real involvement of IgE antibody. The study reported in this paper was undertaken to define the allergenic importance of the snail, and after a

preliminary result obtained by means of SPT, three more IgE-detecting tests such as RAST, histamine release (HR) [3] and electroblotting [4] were used. The data obtained seems to suggest that the snail is a new allergenic food source containing antigens capable of binding specific human IgE antibodies.

Materials and Methods

Snail Aqueous Extract

500 g of *E. pisana* (described by O.F. Müller, 1774), classis gastropoda, family Helicidae [5], were boiled for 10 min and the whole body of the snail, separated from the spiral shell, was homogenized in distilled water. The mixture was centrifuged at 10,000 g for 10 min, and the supernatant was defatted with 1 vol of ethyl ether (v:v) at room temperature. The supernatant was then dialyzed extensively by means of Amicon apparatus, YM5 filters, cut-off 5,000 daltons. The protein concentration of the extract was measured by the procedure of Lowry et al. [6] using BSA as a standard, and the extract will be referred to throughout the paper as the snail aqueous extract.

Skin Test

A skin test was performed by means of SPT on 70 subjects allergic to the more common allergenic sources, and on nonallergic subjects as a control. An aqueous crude extract of snail at a protein concentration of 1.6 mg/ml was used in the SPT. The histamine-equivalent prick (HEP) was calculated as previously reported [7]. Histamine-HCl 1 mg/ml (1 HEP) was used as a positive reference and a saline solution, 0.9% NaCl, as a negative control.

Human Sera

Serum samples were obtained from 70 subjects allergic to the more common Mediterranean allergens (i.e. *Parietaria judaica*, grass, *Dermatophagoides pteronyssinus* and *pharinae*, olive, etc.). Sera from nonallergic subjects were also collected and used for in vitro tests either as single serum or as a pool. The sera collected were stored at -20°C until used.

RAST, RAST Inhibition and Total IgE

RAST was performed as described by Wide et al. [8] with minor modifications. As solid phase we used polystyrene balls 6.4 mm diameter (Allergofere®, Lofarma, Italy) coated with the snail aqueous extract. The IgE binding values were determined by addition of 50 µl rabbit antihuman IgE ¹²⁵I (Sferikit®, Lofarma, Italy), and the bound radioactivity was measured in a γ-counter (Packard 800). Sera from SPT-snail-negative subjects were used as a control. The results obtained with polystyrene balls as solid phase were confirmed by using paper discs CNBr-activated and coated with the snail aqueous extract. RAST inhibition was performed as described earlier [9]. The concentration of total IgE for each serum was determined with Sferikit® (Lofarma, Italy) as suggested by the instructions supplied by the manufacturer.

Histamine Release Assay

Histamine was measured by a single-isotope enzymatic microassay as described by Taylor et al. [3]. Basophils from subjects

SPT-positive to a snail aqueous extract were tested for their capability to release histamine on challenge with the snail aqueous extract. The enzyme histamine methyltransferase was prepared from kidney of male rats as described elsewhere [10]. S-adenosyl-L-methionine 1 mCi, purchased from Radiochemical Centre, Amersham, UK, was used. Total blood histamine was detected by boiling for 10 min 100 µl of blood diluted with 100 µl of 0.05 M phosphate buffer pH 7.9. Spontaneous release was calculated by incubation of the blood with buffer alone. The amount of histamine released on challenge with snail extract was expressed as percentage of total histamine after subtraction of spontaneous release.

Electroblotting

Snail aqueous extract (40 µg) was fractionated by means of 8% sodium dodecylsulfate polyacrylamide gel electrophoresis (PAGE-SDS) as previously described [4]. The separated antigens/allergens were transferred from the separation gel to a nitrocellulose membrane (Transblot 0.45 µm) by electrophoretic transfer as described elsewhere [11]. The unoccupied protein binding sites on the nitrocellulose membrane were blocked by equilibration for 60 min at 37°C in Phadebas RAST buffer containing 0.5% Tween 20. The nitrocellulose membrane with blotted proteins were cut into strips 0.5 cm wide, and incubated overnight with 500 µl of patient serum diluted to 3 ml in RAST buffer containing 0.5% Tween 20. After a washing step, the strips were incubated with ¹²⁵I-antihuman IgE (Phadebas RAST) 200,000 cpm/strip in 3 ml of RAST buffer overnight at room temperature. The strips were then air dried at 4°C and subjected to autoradiography on Kodak XL-1 X-ray film for 1, 3, or 10 days.

Results

Screening of Patients' Snail Hypersensitivity by SPT and RAST

The snail used in this study was the *E. pisana*. 70 subjects allergic to the more common allergens of the Mediterranean area were tested by SPT with a snail aqueous extract. If we consider the reaction produced with the histamine-HCl solution (1 mg/ml) as 1 HEP, then the snail extract has 1 HEP of allergenic potency when it elicits a prick test reaction with the same D + d/2 value. The results of this analysis are reported on table I. 61% of the subjects tested were SPT-positive to the snail extract, with a HEP value higher than 0.50, presence of erythema and wheal with a diameter higher than 5 mm. The serum of each patient tested by SPT was collected and the total and specific IgE (RAST) for the snail's antigens were detected (table I). Of 70 sera tested, only 13 (19%) showed IgE specific for the snail antigens bound to the balls, while the total IgE values were poorly correlated both to HEP and RAST values (table I). The symptomatology with an asthma attack after a snail meal, among

Table I. Histamine-equivalent prick values, total serum IgE levels, and snail-specific IgE values

Patient	HEP	PRIST IU/ml	RAST % binding	Patient	HEP	PRIST IU/ml	RAST % binding
1	0.88	220	0.1	36	0.68	1,350	0.4
2	n	72	0.2	37	n	60	0.6
3	n	170	0.1	38	0.85	480	0.6
4	0.73	220	2.5	39	n	44	0.5
5	0.76	120	0.1	40	n	23	0.6
6	0.56	165	0.6	41	1.42	230	13.1
7	1.05	64	0.6	42	0.68	530	0.5
8	0.83	145	0.1	43	n	850	0.4
9	0.83	13	0.1	44	1.25	240	0.5
10	n	72	0.5	45	0.76	650	0.5
11	n	220	0.3	46	n	34	0.5
12	1.60	255	0.3	47	n	27	0.5
13	n	390	0.4	48	0.84	105	0.6
14	n	350	0.6	49	0.58	190	0.4
15	1.00	1,350	2.6	50	0.57	30	0.6
16	0.58	195	4.2	51	0.61	3,200	3.9
17	0.83	24	0.6	52	0.75	37	0.6
18	0.56	40	3.0	53	0.55	12	0.6
19	0.73	290	3.1	54	n	140	0.6
20	n	170	0.1	55	n	105	0.6
21	0.75	22	0.2	56	0.52	220	0.7
22	n	95	0.6	57	1.00	77	2.6
23	0.57	210	0.7	58	n	33	0.6
24	n	24	0.6	59	n	120	0.6
25	0.73	105	0.7	60	0.78	105	0.5
26	1.05	145	0.6	61	0.58	53	0.6
27	0.64	3,000	5.4	62	0.66	1,650	6.9
28	1.00	750	4.2	63	0.68	1,000	5.0
29	1.00	370	6.7	64	n	310	0.8
30	n	800	0.6	65	n	47	0.6
31	0.82	230	0.7	66	n	50	0.5
32	1.40	85	0.8	67	n	96	0.6
33	n	145	0.6	68	n	150	0.1
34	0.72	88	0.6	69	1.27	230	0.6
35	0.56	38	0.5	70	n	69	0.6

n = Negative.

the SPT-snail-positive subjects was present only in 15% of the patients analyzed. No gastrointestinal symptoms were described by the patients. As a control, 30 nonallergic subjects were tested by SPT using the snail extract, and none showed positivity. An analysis of the distribution of the more common Mediterranean allergens among the SPT-snail-positive subjects showed a distribution similar to that observed among an allergic population [12] in the Mediterranean area (i.e. *P. judaica* > grass > *Dermaphagoides* > olive, etc.).

HR Analysis

Blood basophils were prepared from SPT-snail-positive and RAST-snail positive patients challenged with 1 μ g of the snail aqueous extract. Goat antihuman IgE (1 μ g) was used as an internal control. The results reported in table II show that all the patients positive with SPT and RAST to the snail extract had also basophils capable of releasing histamine on challenge with the same snail extract. Hemocyanin (1–10 μ g) from Keyhole limpet (*Megathura crenulata*) was also used in the assay to ascertain if it was respon-

Table II. HR values obtained on challenge with snail extract, hemocyanin and antihuman IgE basophils prepared from subjects allergic and nonallergic to snail

Donors	Net HR, %		
	anti-IgE	snail extract	hemocyanin
HEP(+)¹; RAST(+)²			
4	20	10	1.5
18	17	20	2.0
19	23	12	0.8
28	27	31	0.9
41	40	25	2.3
51	28	12	3.0
62	ND	20	1.8
63	26	30	1.0
HEP(+); RAST(-)			
7	20	12	ND
12	15	20	ND
26	26	22	0.8
31	25	30	ND
32	18	32	ND
44	31	25	ND
45	16	20	ND
69	20	28	ND
HEP(-); RAST(-)			
2	10	2.3	ND
10	19	4.1	ND
24	15	0.8	ND
33	12	1.2	ND
39	15	1.1	ND
40	16	0.5	ND
54	8	0.2	ND
65	12	0.8	ND
66	14	1.6	ND
70	13	1.5	1.2

ND = Not determined.

¹ Donor positive to SPT with snail extract.

² Donor with snail-specific IgE.

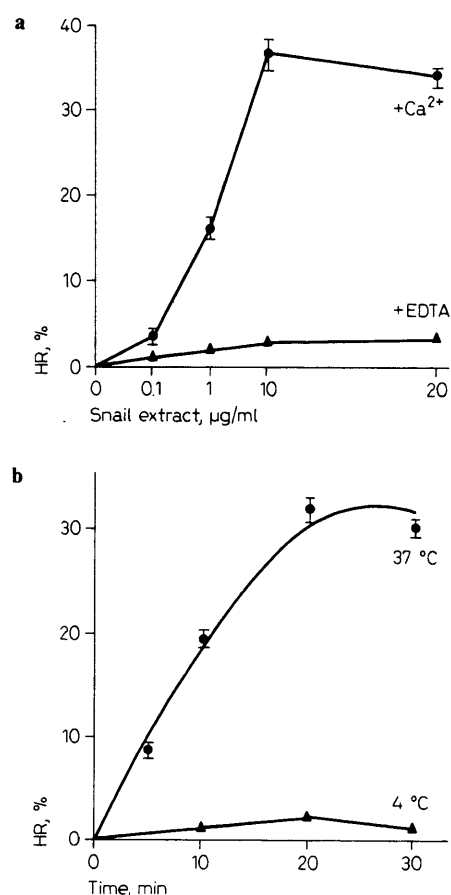


Fig. 1. Calcium (a)- and temperature (b)-dependent HR induced by snail extract from basophils of SPT- and RAST-snail-positive subjects. HR was performed in Tris-ACM buffer, pH 7.4, containing: 0.025 M Tris, 0.12 M NaCl, 0.005 M KCl, 6×10^{-4} M CaCl_2 , 0.001 M MgCl_2 and 0.3 mg/ml bovine serum albumin. The final concentration of the disodium EDTA into the assay mixture was 2 mM. The temperature-dependent curve (b) was performed by using 10 μg of the snail extract. HR was expressed as a percentage of the total cellular content of the amine; all values were corrected for the spontaneous release occurring in the absence of any stimulus. The values reported are the mean of triplicate determinations from four different sensitive donors.

sible for the allergic reaction. As reported in table II, the hemocyanin used was not capable of inducing any HR from the basophils of the subjects analyzed. When SPT-snail-positive, but RAST-snail-negative patients were tested by using HR, still a significant positive HR value was obtained, suggesting a correlation between the SPT and HR results (table II). As a control, 10 SPT- and RAST-snail-negative subjects were analyzed by HR, and no positive values of HR were obtained (table II). These results suggest that all

the subjects ($n = 16$) positive to the snail extract by means of SPT were also positive by HR analysis, with a mean value of $21.8 \pm 7\%$, while the subjects negative to the snail extract by both SPT and RAST were also negative by HR, with a mean value of $1.41 \pm 1.1\%$ ($n = 10$). The positive HR value obtained on challenge with the snail aqueous extract was further analyzed to avoid the possibility of a cytotoxic effect or the involvement of an aspecific nonimmunologic release. The HR was performed in a medium made Ca-

Fig. 2. SDS-PAGE (8%) pattern of protein markers (first column) and snail extract (40 μ g) (second column) after protein-staining with CBB. The other strips represent detection of specific IgE-binding components of snail extract on nitrocellulose membrane strips, using individual patients' serum and isotope detection by 3 days autoradiography. Numbers at the bottom of the nitrocellulose strips indicate the different individual sera used, P the pool of 10 sera from nonallergic subjects.

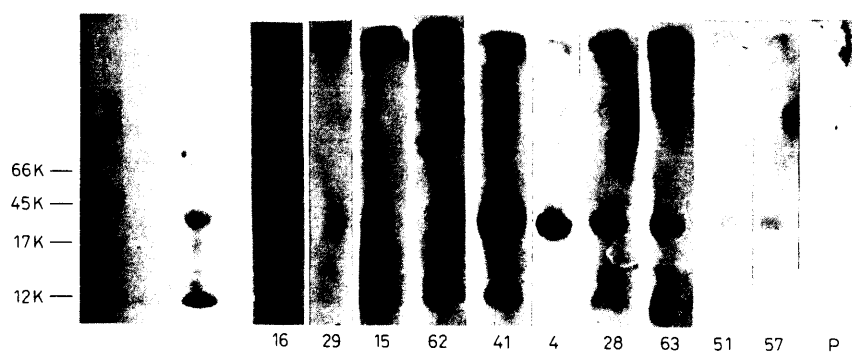


Table III. Patients' sera ($n = 10$) IgE binding to the identified allergen bands separated on PAGE-SDS and transferred to nitrocellulose membrane

Patients' sera	Snail allergen band and estimated molecular weight (daltons)					
	1	2	3	4	5	6
	> 66,000	> 66,000	66,000	24,000	15,000	12,000
16	×			×		×
29	×			×		
15	×				×	×
62	×			×	×	×
41	×	×	×	×		×
4	×			×		
28	×			×		×
63	×	×	×	×	×	×
51				×		
57				×		

free by the addition of ethylenediamine tetraacetate (EDTA) or at 4°C instead of 37°C. The results reported in figure 1a,b show that in presence of EDTA or at 4°C no HR was detected.

Allergenic Composition of the Snail Extract

To determine the allergenic composition, 40 μ g of the snail aqueous extract were fractionated by means of 8% PAGE-SDS, and then transferred by electroblotting to nitrocellulose membrane. The results re-

ported suggested that of at least 9 protein bands showed in the second column (fig. 2) stained with CBB, 6 were capable of binding IgE molecules. However, a great variability regarding the IgE-snail-specific composition was observed among the different sera analyzed. In fact, if the sera 63 and 41 were capable of binding 6 and 5 protein bands, respectively, the serum 29 was capable of binding two snail extract components, and sera 51 and 57 only 1 protein band. The molecular weight range of the allergens was from > 66,000 to 12,000 daltons and only one allergen corresponding to the band 4 was capable of binding IgE of almost all the sera ($n = 10$) tested (fig. 2). As a control a pool of 10 sera obtained from SPT-, RAST- and HR-snail-negative subjects was used in the same analysis, and the results reported in figure 2 as P support the specificity of the IgE binding observed.

Cross-Reactivity with Other Mediterranean Allergens

To detect cross-reactivity of the snail allergens with the more common allergens of the Mediterranean area, RAST inhibition experiments were carried out by using polystyrene balls coated with the snail aqueous extract. The sera 28, 62 and 63 of patients allergic to the snail extract were used as source of snail-specific IgE, while the allergen sources tested were *P. judaica*, *Poa pratensis*, *D. pharinae* and egg white. The results reported in table IV suggested a high cross-reactivity of the snail allergens with the allergens derived from *P. judaica*, *P. pratensis* and *Dermatophagoides*. No cross-reactivity was detected with the allergen derived from the egg white.

Table IV. Cross-reactivity between snail and some Mediterranean allergens

Sera	RAST		RAST inhibition	
	allergen	%	allergen	%
28	snail	4.2	snail	47
	<i>D. pharinae</i>	0.4	<i>D. pharinae</i>	14
62	snail	7	snail	70
	<i>D. pharinae</i>	17	<i>D. pharinae</i>	44
	<i>P. pratensis</i>	46	<i>P. pratensis</i>	69
	<i>P. judaica</i>	20	<i>P. judaica</i>	70
	egg white	0.9	egg white	0
63	snail	5	snail	91
	<i>D. pharinae</i>	26	<i>D. pharinae</i>	66
	<i>P. pratensis</i>	45	<i>P. pratensis</i>	76
	<i>P. judaica</i>	31	<i>P. judaica</i>	74
	egg white	0.5	egg white	0

RAST inhibition was performed by polystyrene balls coated with the snail aqueous extract. 30 μ l of allergic serum were incubated for 3 h with 30 μ l of each allergen reported above. Phosphate-buffered saline was used as a control. The mixture was then added to the coated balls following the normal RAST procedure. The percentage of inhibition was calculated as previously described [9].

Discussion

The results obtained in this study indicate that the snail can be the cause of allergic reactions in some people eating this food. Asthma was the symptom correlated with the ingestion of snail and SPT performed with a snail aqueous extract showed a significant positivity in these subjects. When 70 subjects already allergic to other allergens were skin-prick tested with the snail extract, the percentage of positivity was 61%, while only 15% had experienced an asthma attack on ingestion of snail. Gastrointestinal symptoms were not reported by the patients analyzed. The remaining percentage of SPT-snail-positive but asymptomatic subjects had at least once in their life eaten snail without any particular reaction. When nonallergic people were tested by SPT with the snail extract, no reaction was observed, suggesting that the positivity observed among the allergic patients was not due to some aspecific irritant molecule contained in the snail extract. Due to the percentage of asymptomatic subjects with a SPT positivity to the snail extract, additional analytical methods were used to control the

reliability of the SPT results. A RAST analysis was made on the sera of the 70 subjects described above and only 19% of the sera showed a significant RAST positivity to the snail extract. The reason for the apparent difference between the results of RAST and SPT is unclear at present but it may be related to problems regarding the binding step of the allergens to the polystyrene balls, or to a different sensitivity of the two methods used, the in vivo SPT being about 1,000 times more sensitive than the in vitro RAST [13]. In fact when a different very sensitive test such as the HR was used, all the subjects positive for SPT were also positive for HR. Since it has been reported in the literature that hemocyanin from *Helix pomatia* was capable of inducing synthesis of IgE [14], we tested such a molecule in the HR assay to ascertain if it was the molecule responsible for the allergic reaction. The results obtained suggest that this hemocyanin was not capable of inducing release of histamine from the basophils of the subjects analyzed and that the allergenic activity detected in the snail extract was probably not due to the hemocyanin. Hemocyanin from *M. crenulata* was also used in SPT on the same SPT-snail-positive subjects, and no reaction was obtained [data not shown], supporting the conclusion that hemocyanin was not involved in the allergic reaction. However, we have not tested the hemocyanin prepared from the same snail so the role of the hemocyanin in these allergic reactions cannot be completely ruled out. The remarkable thermal stability of snail allergens suggested by the significant allergenic activity after the boiling step is well comparable with a similar property shared by the pollen allergens [15]. Although research directed specifically at isolating food allergens has been limited as compared to the efforts devoted to the pollen allergens [16, 17], a thermal stability has also been described for peanut allergens [18, 19]. To establish the allergenic composition of the snail extract, it was separated by PAGE-SDS and, after a transfer to nitrocellulose membrane, it was tested with some sera of SPT- and RAST-snail-positive subjects. 6 different allergens were detected. 1 serum contained IgE for all the 6 allergens (serum 63) and this result was well correlated with the RAST value. A great variability was also observed among the different sera regarding the capability of binding different components of the snail extract, and this is very common also with other allergenic extracts already described [20]. One component of the snail extract, the allergen 4, reacted with almost all the sera tested and

it could represent one major allergen, but further exploration with larger numbers of sera is necessary. The molecular weight values (12,000–66,00 daltons) of the snail allergens were also in the range reported for other pollen allergens [20]. A high cross-reactivity among the snail allergens and the allergens from *P. judaica*, *D. pharinae* and *P. pratensis* was detected by means of RAST inhibition, and work is in progress to better characterize this point.

In conclusion, the study reported here seems to support that the snail is another source of food allergy; this has been confirmed by using four different methods of analysis; at least six allergens are present in the snail extract used and one could be classified as a major allergen.

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