

Identification of crab proteins that elicit IgE reactivity in snow crab-processing workers

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Background: The expanding snow crab-processing industry has resulted in increased numbers of workers at risk of occupational allergy.

Objective: Our study is to identify relevant allergenic proteins in cooked snow crab meat (CM) and crab water (CW) used for cooking for improved remediation, diagnosis, and treatment.

Methods: Extracts were prepared from CM extracts, CW extracts, and an air-filter collection near the crab cooker. Of the 207 workers, 24 with the highest IgE antibody reactivity to CM and CW extracts, as determined by using RASTs, were tested for reactivity to nitrocellulose membranes containing CM and CW proteins separated with SDS-PAGE. A 3-serum pool was similarly incubated against nitrocellulose-bound proteins from air samples collected near the crab cooker.

Results: Of the 207 sera tested, 27 and 39 sera exhibited positive IgE antibody reactivity ($\geq 2\%$) to CM and CW extracts, respectively. Twenty-two of 24 sera with the highest RAST activity ($\geq 3.5\%$ binding) demonstrated IgE binding to multiple

proteins (13.6-50 kd). A majority of the sera reacted to 4 proteins: 79% and 71% to a 34.0-kd protein, 79% and 42% to a 25-kd protein, 67% and 71% to an 18.5-kd protein, and 75% to a 14.4-kd protein in both CM and CW extracts, respectively. The pool of IgE-positive sera blotted against the air-filter extract reacted to 14.4-, 18.5-, 34.0-, 43.2-, and 50-kd proteins present in both crab extracts.

Conclusion: Four major IgE-reactive proteins were identified in CM extracts, CW extracts, and air-filter eluate. Analysis of any potential association of protein reactivity with disease suggested crab proteins at 34.0 and 14.4 kd might be more relevant. (J Allergy Clin Immunol ■■■■;■■■:■■■-■■■.)

Key words: Crab, occupational allergy, RAST, IgE reactivity, immunoblot

The increased demand for healthy, protein-rich food in the modern diet has generated a substantial increase in the consumption of seafood. Americans alone consumed 1.612 trillion kilograms of crustaceans in 2005.¹ With this increased demand has come a dramatic growth in the harvesting and processing of seafood, including snow crab. A number of older, often poorly ventilated fish-processing plants in the Canadian provinces of Newfoundland and Labrador were converted to snow crab (*Chionoecetes opilio*)—processing plants to meet this demand during the past decade. By 2004, more than 400 shellfish-processing plants along the eastern Canadian coast used an estimated 22,000 laborers, making snow crab the second most commonly processed shellfish (approximately 100,000 metric tons in 2004) in Eastern Canada.²

The cleaning, steaming, washing, sawing, cracking, and crushing of snow crabs within these plants can routinely expose many workers to crab proteins in the form of dust, steam, vapor, and crab meat (CM).³ These conditions place the plants' usually seasonally employed laborers at risk for occupational IgE-mediated allergic disease.⁴⁻⁶ Respiratory illnesses in these workers negatively affect their quality of life and, for those forced to give up their jobs, imposes economic burdens on them and their families because of limited employment alternatives and infrequent access to health specialty services. Often rural and remote plants can result in affected workers remaining at their jobs, making it difficult for them to obtain a specific diagnosis and access to workers' compensation.³

Previous studies have shown the primary source of antigen exposure in seafood processing to be the inhalation of aerosolized proteins ranging in molecular weight from 10 to 70 kd.⁷ In the crab industry these aerosols often contain primarily crab exoskeleton,

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Abbreviations used

CM: Crab meat

CW: Crab water

OA: Occupational asthma

muscle protein, gills, and internal organs with lesser amounts of background material, such as cellulose, synthetic fibers, and inorganic particles.⁸ At least 30% of these particles have diameters of 5 μ m or less and thus lie within the respirable range.³ Collaboration with the snow crab-processing industry and regulatory agencies of Newfoundland and Labrador has helped to identify the sources and relative intensity of antigen exposure to modify plant design, processing procedures, and safety equipment, and practices to improve work safety are an important goal. This study is the first description assessment of the importance of snow CM and CW allergens in snow crab allergy and identification of aerosolized allergens from snow crab cooking water.

METHODS

Snow crab extracts

Cooked snow crab (908 g) purchased commercially (P. Janes & Sons, Hant's Harbour, Newfoundland, Canada) was blended with 500 mL of PBS (pH 7.2, 0.01 mol/L) in a commercial blender (3 $\times$ for 1 minute each). The slurry was stirred for 1.5 hours at 4°C and then centrifuged at 10,000g. The supernatant was concentrated by means of ultrafiltration (YM1 filter; molecular weight > 1000 d; Amicon Corp, Lexington, Mass) to a volume of 100 mL. Centrifugation of this concentrate at 80,000g and 4°C resulted in the recovery of 56 mL of supernatant (CM extract) at 33.6 mg/mL protein, as assayed by using the Bradford method.⁹

A 2-L water sample was taken from a boiler of a Newfoundland crab-processing facility after cooking 50,000 lbs of snow crab and freezing for shipment. The water was thawed and concentrated (YM1 filter), and the resultant concentrate was centrifuged (80,000g), all at 4°C. Sixty-five milliliters of supernatant-concentrated CW extract at 12.5 mg/mL total protein was recovered.⁹

Area air samples were collected with the Air Sentinel (Quan-Tec-Air, Inc, Rochester, Minn) at a flow rate of 3 L/s by using a polytetrafluoroethylene filter (0.3 μ m at 99% efficiency) over the samples and placed near the cooker exhaust of a Newfoundland plant, allowing filter steam from the active cooker to be collected for 2 hours. The filter was removed and extracted overnight in 250 μ L of 10% SDS solution (liquid filter extract).

Subject sera

In 2002-2003, a total of 215 workers (representing approximately 45% of the total workforce) from 4 snow crab-processing plants in Newfoundland and Labrador invited to participate in health assessments for occupational asthma (OA) and allergy consented to the use of their sera for linked research into OA and allergy to snow crab. The sera of 196 of these workers were analyzed in the present study. The comprehensive study performed in Newfoundland and Labrador was described elsewhere.⁴ A diagnosis of highly probable OA was based on work-related respiratory symptoms, positive skin prick test results, or specific IgE measurements to crab allergens with or without positive peak expiratory flow monitoring; these criteria were met in 39 workers. The study protocols had been approved by the Ethics committee of Hôpital du Sacré-Coeur de Montréal and of Memorial University, St John's Newfoundland and Labrador.

In another survey done in Quebec (Îles de la Madeleine) in 1982 and 1984, we assessed 303 of 313 workers in 2 crab-processing plants. In 54 of these workers, the diagnosis of OA was confirmed by using specific inhalation challenges, a combination of positive peak flow monitoring and significant changes in bronchial responsiveness to histamine, or both.^{5,6} The sera of 11 of

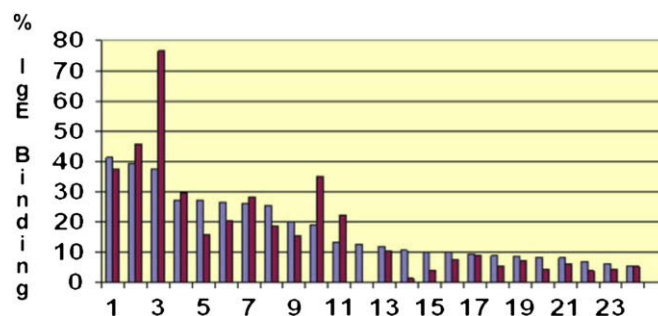


FIG 1. RAST values (percentage IgE binding) of snow crab workers to CM (red) and CW (blue) extracts.

these workers were used in the present study (206 from Newfoundland and 11 from Quebec).

Sera drawn for 207 of the snow crab processors included in those surveys were used.

RASTs

All 207 crab workers' sera were tested for IgE antibodies to CM and CW extracts by using a RAST, according to the method of Ceska and Lundkvist.¹⁰ Crab proteins were coupled to cyanogen bromide-activated filter paper discs at 25 μ g per disc, incubated with 100 μ L of sera per disc, washed, and further incubated with iodine 125-labeled goat anti-human IgE (DiaMed, Windham, Me) at 15,000 counts per minute per disc. The assay was performed in duplicate with appropriate controls. The results are expressed as percentages of IgE bound to the individual discs.

Immunoblotting

The proteins of the CM, CW, and air-filter extracts were separated by using discontinuous SDS-PAGE and transferred to cyanogen bromide-activated nitrocellulose membrane (Scheicher & Schuell, Dassel, Germany). Of the 207 sera tested, the 24 sera with the most significant IgE-binding values were blotted against the meat and water membranes overnight. A pool of the 3 sera with highest IgE antibody reactivity to crab were blotted against the filter extract to accommodate the limited amount of extract available. Each membrane was cut into 5-mm-wide strips, washed, and incubated with 15,000 cpm per strip of iodine 125-labeled goat anti-human IgE for 24 hours. After washing and drying, the membrane strips were exposed to autoradiographic film (Amersham International, Little Chalfont, Bucks, United Kingdom) for 6, 10, and 18 days in the absence of light at -70°C to identify and grade all reactive bands. The varying periods of incubation were used so that individual bands to which reactivity occurred (and not obscured by more reactive bands with overexposure) could be detected, as well as to have a way of assessing the intensity of reactivity. Bands that were barely detectable or showed little reactivity even at the greatest exposure were given a grade of 1. Bands to which significant reactivity occurred but did not appear to be of the strongest intensity were given a grade of 2. Only those bands to which maximal reactivity occurred, generally in the earliest incubation period, were given a grade of 3. These blots were viewed and graded independently by 3 reviewers, and the grading is a composite of their review. Generally, the results of all the reviewers were in agreement; for the few bands in which this did not occur, the result with the greatest number of reviewers in agreement was chosen. Molecular weight markers and extracts were visualized separately by using Colloidal Gold Total Protein Stain (Bio-Rad Laboratories, Hercules, Calif).

Statistical analysis

Exploratory univariate linear regression analyses were performed to identify any potential associations between quantified subject protein reactivities and RAST scores using data from the 24 samples with significant IgE antibody reactivity to crab allergens on which immunoblotting was done. The proportions of individuals with high, medium, low, or no sera binding were

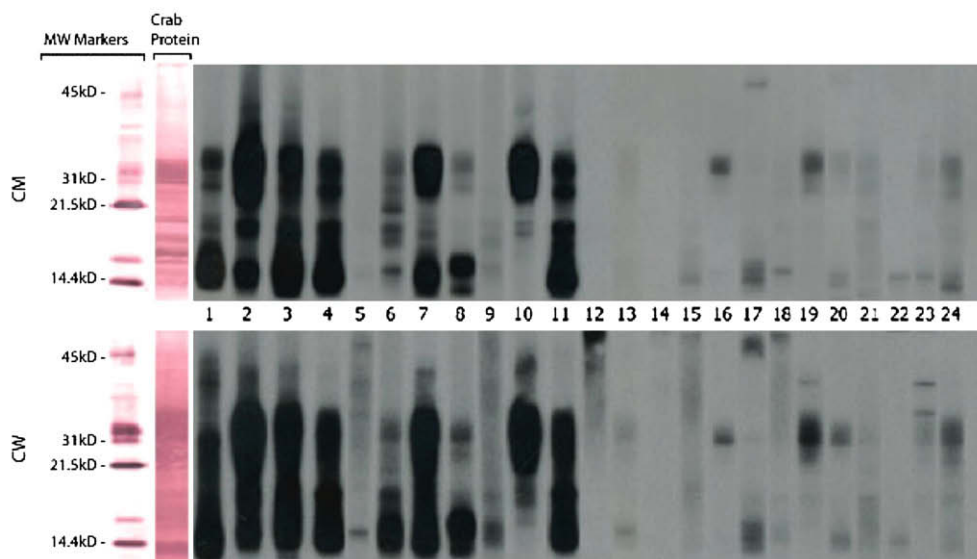


FIG 2. Autoradiograph at day 9 of all sera incubated against CM (upper panel) and CW (lower panel) extracts.

computed for each protein studied. Statistical analyses were performed with SPSS 15.0 software (SPSS, Inc, Chicago, Ill). A *P* value of less than .05 was considered statistically significant.

RESULTS

Of the 207 sera tested, 27 and 39 exhibited positive IgE antibody reactivity ($\geq 2\%$ binding, as determined by means of RASTs) to the CM and CW extracts, respectively. Twenty-four sera with the highest binding activity, as shown in Fig 1 (ranging between 3.5% and 45.6% binding), were chosen for immunoblotting. RAST reactivities to both CM and CW extracts were observed in all sera, and the negative control sera (not shown) from unexposed individuals showed negative results. Twenty-three of these sera were from workers with a highly probable diagnosis of OA, and the 24th serum (subject # 12) was from a worker with a negative diagnosis of OA. Thirteen of the 24 positive sera were from Newfoundland workers, and the remaining 26 of 39 subjects with highly probable OA had negative or low IgE antibody activity (between 2% and 3.5%) but positive skin prick test responses to crab allergens (wheal diameter ≥ 2 mm in 14 and ≥ 3 in 7) or a positive peak expiratory flow monitoring result ($n = 1$). Eleven of the 24 positive sera studied here were from Quebec workers; the other 43 of 54 workers with OA had low IgE antibody or no RAST investigations.

Of the 24 positive sera chosen by means of immunoblotting, 11 exhibited IgE binding by means of RASTs to 1 or both extracts of 20% or greater, whereas 3 had binding of greater than 40%. By using workers' sera, the immunoblots demonstrate IgE binding to multiple proteins ranging in molecular weight from 13.6 to 50.1 kd in both CM and CW extracts (Fig 2). Examples of sera with a variety of reactivities to CM or CW extracts are shown. The similarities and differences of individual sera to CM and CW extracts are shown in Fig 3. Several of the patterns of reactivity in these figures might vary from that seen in Fig 2 because of different incubation periods.

The 2 allergolograms in Fig 4 summarize the sera's IgE reactivity with individual proteins in CM or CW extracts (Table I). Reactivity to a range of proteins from 13.6 to 50.1 kd was

demonstrated: 34.0-kd protein, 80% for CM and 71% for CW extracts; 18.5-kd protein, 67% for CM and 71% for CW extracts; and 14.4-kd protein, 75% for CM and CW extracts. Curiously, the 80% reactivity to a 25.1-kd protein in CM extracts diminished substantially to 42% for CW extracts. Overall, a higher amount of IgE binding was observed against specific proteins in the CW compared with the CM extracts.

The 3-donor pool of IgE-positive sera blotted against the air-filter extract reacted to several proteins (14.4, 18.5, 34, 43.2, and 50.1 kd), all of which were detected in both CM and CW extracts (Fig 5).

Using quantified protein reactivity against the CM extract, we found several proteins (43.2, 34, 25.1, 21.1, 18.7, and 14.4 kd) to exhibit definite positive associations with RAST scores (β coefficients from regression analysis ranging from 6.60 to 15) with statistical significance (*P* values ranging from .03 to .001, Table II). Unfortunately, we cannot definitively relate protein reactivity to disease because we have not screened all subjects. Indeed, relatively few subjects have data on protein binding ($n = 24$), and among them, 23 have a diagnosis of highly probable OA. However, for CM extract of 34 kd and CM extract of 14.4 kd, there was a tendency for increased protein reactivity in subjects with a diagnosis of highly probable OA: high binding was seen in 10 subjects to CM extract of 34 kd and 9 to CM extract of 14.4 kd, medium binding was seen in 6 subjects to CM extract of 34 kd and 3 subjects to CM extract of 14.4 kd, low binding was seen in 3 subjects to CM extract of 34 kd and 6 subjects to CM extract of 14.4 kd, and no binding was observed in 4 subjects to CM extract of 34 kd and 5 subjects to CM extract of 14.4 kd, whereas in the 1 subject with improbable or negative OA, there was no binding. A similar but less striking pattern was seen for CM extract of 25.1 kd. However, for the other bands (CM extract of 13.6 kd, CM extract of 17.5 kd, CM extract of 20.2 kd, CM extract of 21.1 kd, CM extract of 43.2 kd, and CM extract of 50.1 kd), the pattern was different: there was no binding in at least 50% of subjects. For CM extract of 18.5 kd, subjects were evenly distributed across the levels of binding. There is thus a suggestion that CM extract of 34 kd and CM extract of 14.4 kd might be more relevant to the disease.

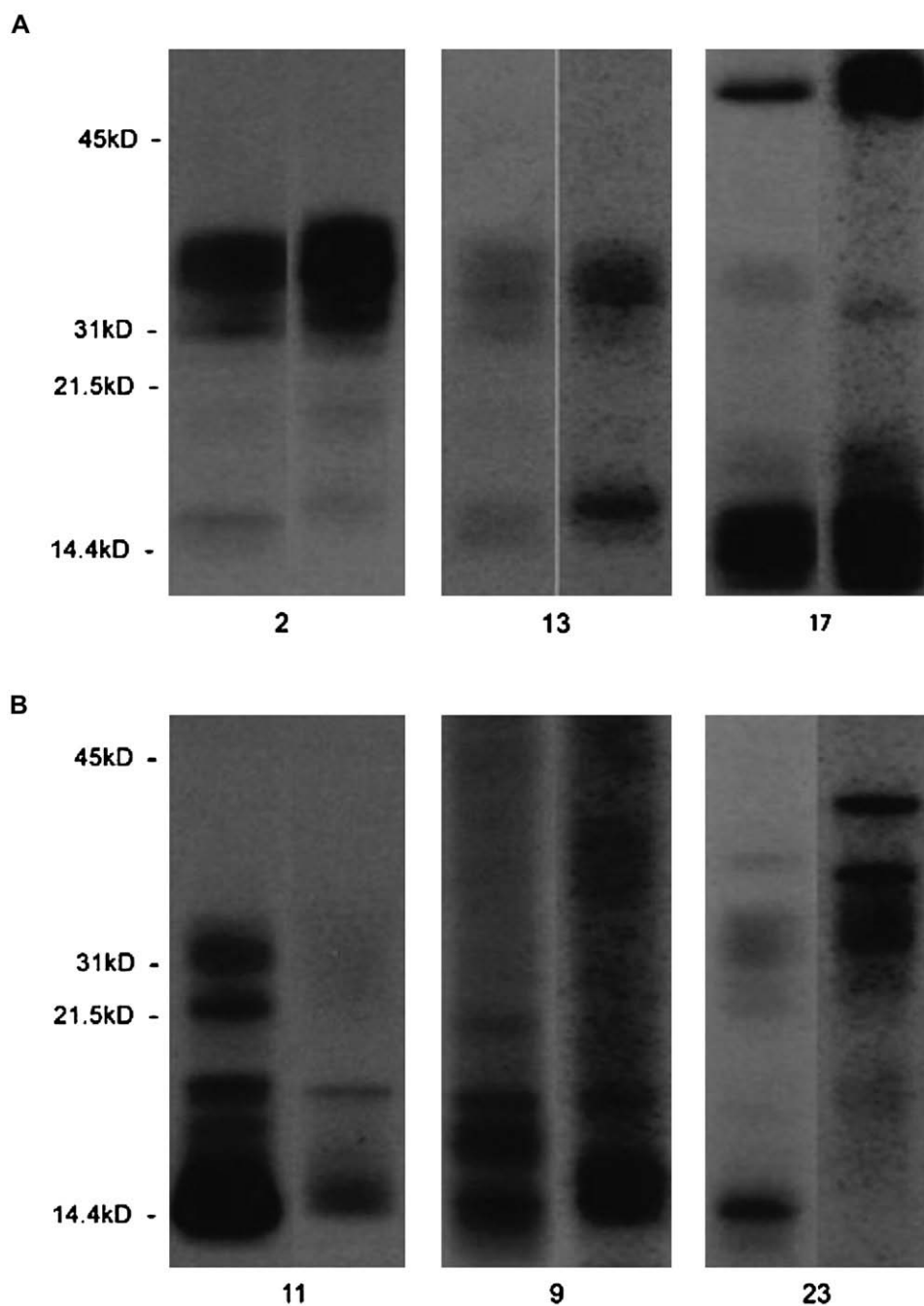


FIG 3. Examples of IgE antibody reactivity of individual sera that reacted to similar (A) or different (B) proteins in CM (left column) and CW (right column) extracts.

DISCUSSION

It is well documented that snow crab-processing workers are at risk of IgE antibody reactivity to proteins present in the snow crab.³⁻⁷ Although this reactivity is known to lead to occupational crab allergy, including asthma, there is little information about the crab proteins that elicit these reactions. Our study demonstrated that 3 snow crab proteins (the 14.4-, 18.5-, and 34-kd proteins) with IgE reactivity to approximately three quarters of the sera tested are present in both CM and CW extracts. Even sera with low levels of IgE, as determined by means of RASTs,

demonstrated high reactivity to some of these proteins, particularly the 14.4- and 34-kd proteins.

A fourth major protein (25 kd) was detected mainly in CM extract (almost twice as many workers reacted to this protein in CM extract compared with CW extract). Reactivity to 2 other proteins, 17.5 and 20.2 kd, was completely unique to the CM extract. These proteins might not be readily solubilized by the cooking process, and the cooking could alter the proteins' migration (causing them to appear as allergens of different molecular weight) or their reactivity with IgE antibodies.

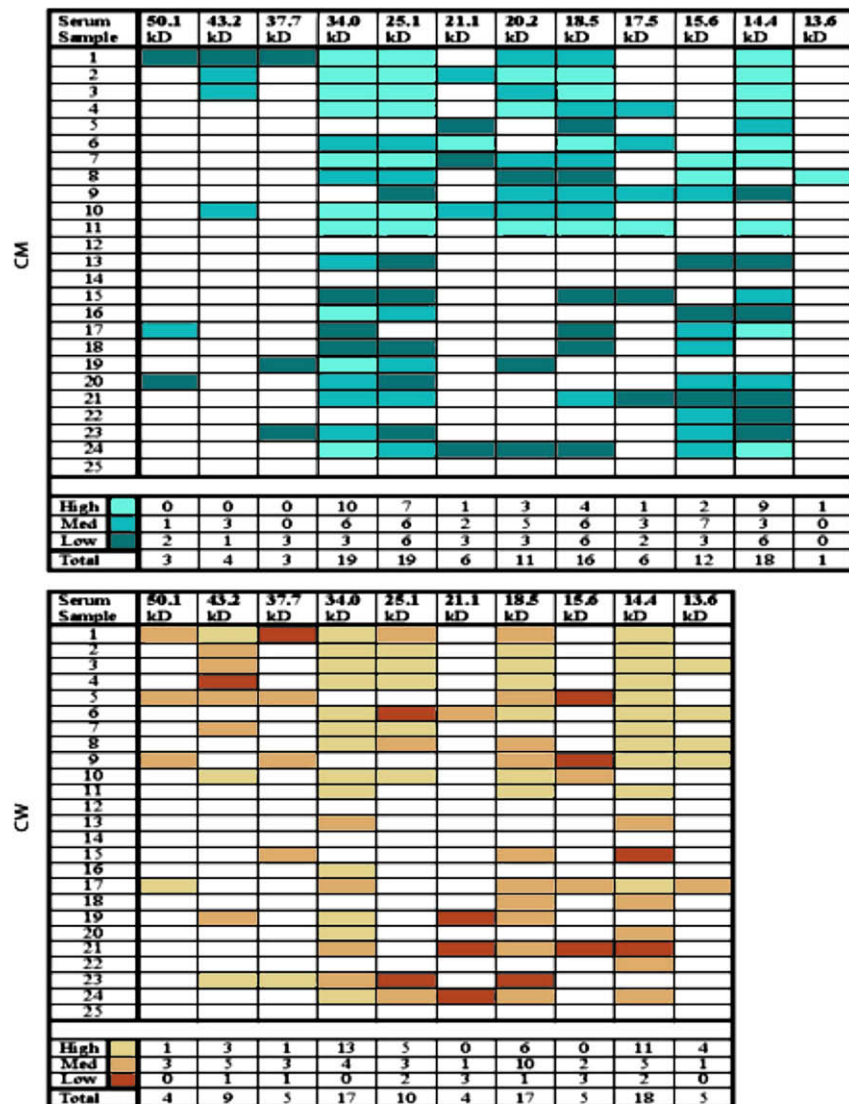


FIG 4. Allergologram of sera against CM (A) and CW (B) extracts. The shown shadings correspond to high, medium, and low individual sera binding (labeled by row) to individual proteins (labeled by column). Serum sample 25 is from a nonexposed control subject with negative RAST results. The results are tabulated in Table I.

Identification of these proteins and those in other bands by means of amino acid sequence analysis should resolve this issue.

A comparison in the allergologram of IgE reactivity intensity between identical proteins in the CM and CW extracts indicates a higher level of IgE binding to the CW extract. Although this pattern is slightly apparent in Fig 2, which was captured at 9 days of incubation, increased incubation periods revealed this trend to a higher extent. Additionally, the number of interactions with a lower level of reactivity occurred more frequently for the CM extract (38 bands) than the CW extract (13 bands). Excluding the 2 proteins unique to the cooked meat, only 3 proteins (the 15.6-, 25.1-, and 21.1-kD proteins) had less reactivity to the CW extract than to the CM extract. This suggests that some proteins present in the CW extract, although from the same origin as those in the CM extract, appear to be more reactive, and thus the CW extract should be used as a source of crab extracts. One possible

explanation is that these proteins are more water soluble than other meat proteins, and thus there could be a higher concentration of these particular proteins present in the CW extract.

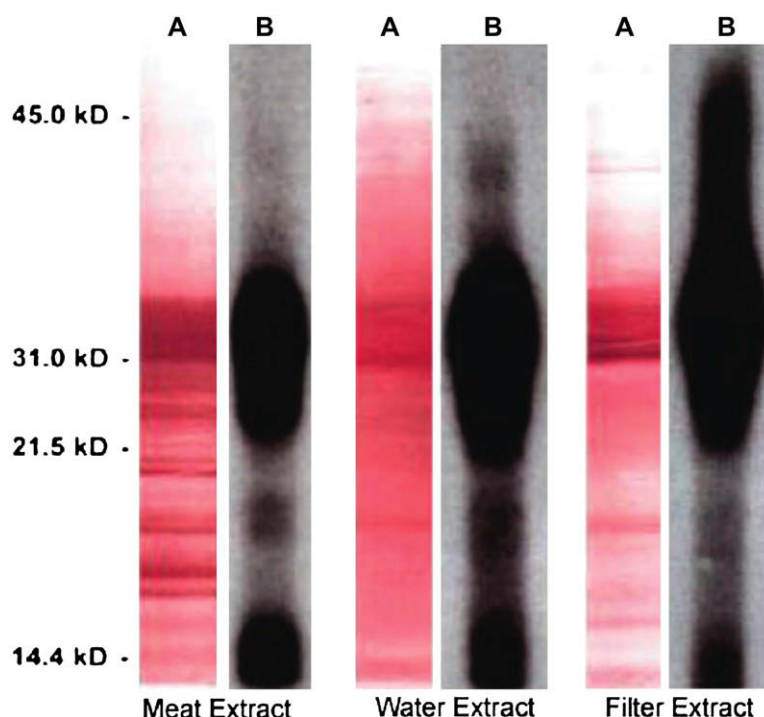
The nature of these IgE-reactive crab proteins has yet to be determined. The 34-kD protein is probably snow crab tropomyosin because of its molecular weight, and crab tropomyosin has been shown to be a major component of aerosolized allergens by means of mass spectrometric analysis.^{11,12} Recent studies have shown that the muscle protein tropomyosin is an important allergen in a number of invertebrates, including lobsters,^{13,14} squid,¹⁵ snail,¹⁶ oyster,¹⁷ house dust mite,^{18,19} and cockroach.^{20,21}

Investigation of crab tropomyosin by Leung et al²² identified a 34-kD protein, designated as Cha f 1, in the claw meat of the crab *Charybdis feriatius*, which elicited IgE reactivity in the sera of subjects with food allergy. Amino acid sequencing identified the 34-kD molecule to be crab tropomyosin. Thus we suggest that Chi o 1 is the homologous protein in snow crab and

TABLE I. Summary of CM and CW proteins that bound IgE antibodies of crab workers

MW (kd)	Meat: 24 sera samples					Water: 24 sera samples				
	High	Medium	Low	Total	% Samples positive	High	Medium	Low	Total	% Samples positive
50.1	0	1	2	3	13	1	3	0	4	17
43.2	0	3	1	4	17	3	5	1	9	38
37.7	0	0	3	3	13	1	3	1	5	21
34.0	10	6	3	19	79	13	4	0	17	71
25.1	7	6	6	19	79	5	3	2	10	42
21.1	1	2	3	6	25	0	1	3	4	17
20.2	3	5	3	11	46					
18.5	4	6	6	16	67	6	10	1	17	71
17.5	1	3	2	6	25					
15.6	2	7	3	12	50	0	2	3	5	21
14.4	9	3	6	18	75	11	5	2	18	75
13.6	1	0	0	1	4	4	1	0	5	21
Totals	38	42	38	118		44	37	13	94	

MW, Molecular weight.

**FIG 5.** Protein bands (A) and reactivity of pooled IgE-reactive snow crab workers' sera (B) against crab meat, water and filter extracts.

the major snow crab allergen. Other proteins eliciting responses might be different allergens of lesser importance or perhaps fragments or aggregates of this protein. Confirmation of Chitinase as the major snow crab allergen tropomyosin requires demonstration by means of amino acid sequencing of homology to Chitinase.

The pool of IgE-positive snow crab-processing worker sera reacted to several proteins (14.4, 18.5, 34, 43.2, and 50.1 kd) present in the processing plant air-filter extract. The presence of snow crab allergens on this filter confirms that these proteins become aerosolized during crab processing. Aerosolization of crab proteins during processing has been previously studied,²³⁻²⁵ but this is the first characterization of these proteins. The

origin of these proteins is probably from CW because 2 bands unique to CM (17.5 and 20.2 kd) were not present in the filter extract.

Although these results are preliminary, they suggest an association to CM proteins at 34.0 and 14.4 kd and disease. These are 2 proteins to which most of the sera from workers with OA reacted in both CM and CW extracts and 2 major proteins present in air sampling during crab processing. This hypothesis needs to be confirmed by further studies.

Identification of the snow crab proteins that elicit IgE reactivity in the snow crab workers, as determined in this study, will aid in the diagnosis of allergic reactions in crab workers. Identification of the allergenic crab proteins will also aid in the characterization

TABLE II. Association between levels of sera reactivity to individual proteins in cooked CM extract and RAST results, as assessed by means of univariate linear regression analysis

Proteins	Univariate analysis	
	β	P value
CM 50.1 kd	−0.68	.91
CM 43.2 kd	15.32	<.001
CM 37.7 kd	0.87	.92
CM 34 kd	6.60	.003
CM 25.1 kd	9.36	<.001
CM 21.1 kd	7.31	.03
CM 20.2 kd	9.79	<.001
CM 18.5 kd	9.68	<.001
CM 17.5 kd	2.01	.55
CM 15.6 kd	−3.73	.16
CM 14.4 kd	5.49	.013

of various crab proteins present in the plant environment and their clinical relevance. Ultimately, plant design and processes might be altered to reduce exposure to allergenic proteins, with outcomes measured by using objective quantitative evidence obtained from bioaerosol collections. Thus crab-processing plants should be able to test for and locate the offending proteins in the work environment, monitor these environments for early exposure detection, develop methods to minimize these proteins in the work environment, and reduce workplace exposure and sensitization.

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Clinical implications: There is a suggested association of reactivity to crab proteins at 34.0 and 14.4 kd with disease. This will aid in the diagnosis and treatment of crab workers.

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