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Identification of New Potential Allergens From Nile Perch (*Lates niloticus*) and Cod (*Gadus morhua*)

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Abstract

Background: Globalization of the food industry has led to widespread exposure to new nondomestic fish species; therefore, identification of potential allergens is necessary in order to diagnose allergic reactions.
Objective: Contact with a patient who was allergic to Nile perch (*Lates niloticus*) prompted us to investigate the immunoglobulin (Ig) E–reactive proteins that could be allergens of this species.
Methods: 2D gel electrophoresis was used to separate the muscle proteins of *L niloticus* and *Gadus morhua*. Immunoblotting was performed with sera from 12 patients with a history of immediate-type allergic reaction to fish and from atopic and nonatopic controls. IgE-reactive proteins were detected and identified using mass spectrometry.
Results: The index patient had low levels of IgE binding to parvalbumins. However, 8 putative allergens other than parvalbumin from *L niloticus* and 5 from *G morhua* were identified. Further investigation revealed cross-sensitivity to enolase 3 from *L niloticus* in 7 of the 12 fish-allergic individuals (58%), whereas 11 of the 12 patients (92%) were sensitized to enolase 3 from *G morhua*. However, atopic control patients were also sensitized to enolase 3 from *L niloticus* and *G morhua*.
Conclusion: Identification of species-specific allergens and individual sensitization could help us to improve avoidance strategies.

Key words: 2D gel electrophoresis. *Gadus morhua*. *Lates niloticus*. Fish allergens. IgE. Western blot.

Resumen

Antecedentes: La globalización de la industria alimentaria proporciona la exposición a nuevos pescados no domésticos y se hace necesaria la identificación de los alérgenos potenciales para diagnosticar las reacciones alérgicas.
Objetivo: El objetivo de este estudio fue estudiar las proteínas fijadoras de IgE que constituyen los alérgenos de la perca del Nilo (*L. niloticus*).
Métodos: Mediante electroforesis 2D en gel se separaron las proteínas del músculo del *L. niloticus* y *G. morhua* y se enfrentaron al suero de 12 pacientes con historia de reacción inmediata a pescado, así como al suero de pacientes atópicos y controles sanos. Las proteínas reactivas a IgE fueron identificadas mediante espectrofotometría de masas.
Results: En los resultados, el paciente mostraba un índice bajo de fijación de IgE a parvalbúminas, sin embargo mostraba fijación de IgE a 8 alérgenos diferentes a la parvalbúmina de *L. niloticus* y 5 a la *G. morhua*.
Observamos una sensibilización cruzada de 7/12 (58%) de los individuos alérgicos a pescado a la enolasa-3 del *L. niloticus*, mientras que 11/12 (92%) de los pacientes estaban sensibilizados a la enolasa-3 del *G. morhua*.
Conclusión: La identificación de los alérgenos especie-específicos o de la sensibilización individual podría en el futuro mejorar las estrategias de evitación

Palabras clave: Gel electroforesis-2D. *Gadus morhua*. *Lates niloticus*. Alérgenos de pescado. IgE. Western blot.

Introduction

Fish is an increasingly common food, and consumption has been growing at a rate of 3.6% annually since 1961. When regional and national differences are taken into account, per capita consumption has risen from 19.7 kg to 27.7 kg in industrialized countries, where diets generally contain a more diversified range of animal proteins [1]. The increasing demand for fish-derived fatty acids in our diet stems from the reported beneficial effects, namely, reduced incidence of ischemic heart disease and improved prevention of allergy [2]. Food allergies are prevalent in children and adults and are one of the most common causes of life-threatening anaphylactic reactions [3]. Moreover, sensitization to food has increased [4]. Logically, the prevalence of seafood allergy is higher where seafood consumption is high [5]. However, a worldwide increase might be expected owing to technological advances in refrigeration and food preservation, which make fish products widely available regardless of their origin.

The most frequent allergen in fish, amphibians, and crustaceans is parvalbumin, a ~12-kDa heat-stable calcium-binding muscle protein, which has been identified in several fish and amphibian species and is therefore considered the main fish/amphibian panallergen [6]. As the homology of parvalbumin is high across several fish species [7,8], cross-sensitization is highly dependent on parvalbumin isoforms [6,7,9]. Based on these observations, clinical cross-reactivity has been assumed [8]. Surprisingly, however, the results of food challenge studies reveal clinical cross-reactivity in only 30% to 50% of cases [10,11]; therefore, other fish-specific allergens have been considered causative, namely, serum albumin [12] and aldehyde phosphate dehydrogenase [13], which have been detected, yet rarely characterized in detail. Some fish allergens may remain undetected, although their presence could be masked by widespread sensitization to parvalbumin [10,11]. Current recommendations for fish-allergic patients are to avoid all fish species or to try specific species following a negative skin prick test (SPT) result in a laborious oral food challenge. Consequently, new allergens must be identified in order to improve the diagnosis of fish allergy and subsequent management of fish-allergic patients [14].

We became aware of allergy to Nile perch (*Lates niloticus*) in a patient who experienced anaphylactic symptoms after occupational contact with the raw meat of this species. The patient had not previously experienced allergic reactions to *L niloticus*.

The most straightforward approach to finding new allergens involves using the sera of sensitized patients to detect allergens in protein extracts from the allergenic source. In this experimental strategy, proteins can be separated efficiently using 2D gel electrophoresis [15]. Allergens found with this approach can be used initially for species-specific or individual diagnosis of sensitization. The results of such an analysis can help us design epidemiological studies to clarify the clinical relevance of sensitization to fish allergens.

Material and Methods

Patients

Two patients (Nos. 119, 369) whose clinical data indicated allergy to seafood were recruited from the Allergy Department at the University of Leipzig, Leipzig, Germany, and 10 patients (Nos. 1, 2, 3, 118, 133, 251, 253, 364, 376, and 462) were

Table 1. Summary of Clinical Symptoms and Laboratory Data

Patient No.	Patient Specificity	Age	Sex	Clinical Symptoms	Atopy	Total Serum IgE	Specific IgE Cod, kU_A/L
119	Nile perch–allergic	24	M	AN	Yes	1058	0.72 (Gad c 1 0.15)
155	Nonatopic control	34	M	None	No	14	0
272	Atopic control	25	M	None	Yes	–	–
295		51	F	None	Yes	138.5	0.04
323		43	F	None	Yes	165	0.02
1	Cod-allergic	43	F	AN	Yes	56	10.3 (Gad c 1)
2		33	F	AN	Yes	121	2.52 (Gad c 1)
3		42	F	AN	Yes	159	0.52 (Gad c 1)
118		55	F	AN, OAS, U	Yes	1712	>100 (Gad c 1)
133		26	M	AN	Yes	5620	45.2 (Gad c 1)
251		18	F	D, A, V, abdominal pain	Yes	265	22.8 (Gad c 1)
253		27	M	AE, D, V	No	50	5.92 (Gad c 1)
364		23	F	OAS, D, A, V	Yes	700	11.6 (Gad c 1)
369		20	M	AN	Yes	393	4.05 (Gad c 1)
376		49	F	OAS, AE, D, A	Yes	223	3.82 (Gad c 1)
462		52	M	OAS, D, AE	No	49	11.5 (Gad c 1)

Abbreviations: A, asthma; AE, angioedema; AN, anaphylaxis; D, dyspnea; Ig, immunoglobulin; OAS, oral allergy syndrome; U, urticaria; V, vomiting.

Table 2. Summary of IgE-Binding Proteins Identified in *Lates niloticus* and *Gadus morhua*[a]

Spot No.	Accession No.	Protein	Taxanomy/ Organism Identified	Mass	Score	Peptides Identified	No. of IgE Reactive Sera	Allergome Code
Lates niloticus								
Ln 1	gil41056111	Phosphoglucomutase 1	*Danio rerio*	61375	890	9	2	Lat n 1
Ln 2	gil47551317	Enolase 3, (beta, muscle)	*Danio rerio*	47841	703	10	12	Lat n 2
Ln 3	gil185133138	Fast myotomal muscle actin	*Salmo salar*	42247	366	6	1	Lat n 3
Ln 4	gil21694043	Creatine kinase muscle isoform 2	*Oreochromis mossambicus*	42980	412	7	2	Lat n 4.1
Ln 5	gil156028365	Creatine kinase 1	*Paralichthys olivaceus*	42916	918	12	3	Lat n 4.2
Ln 6	gil225717412	Fructose-bisphosphate aldolase A	*Esox lucius*	39520	834	13	2	Lat n 5
Ln 7	gil222354841	Apolipoprotein AI	*Epinephelus coioides*	29184	88	3	2	Lat n 6
Ln 8	gil222088001	Adenylate kinase 1-2	*Epinephelus coioides*	29184	195	5	1	Lat n 7
Gadus morhua								
Gm 1	gil213514064	Alpha-enolase	*Salmo salar*	47348	838	12	14	Gad m 2.1
Gm 2	gil213511756	Enolase 3-1	*Salmo salar*	47615	854	11	15	Gad m 2.2
Gm 3	gil27127288	Tropomyosin	*Theragra chalcogramma*	32676	1086	18	2	Gad m 3
Gm 4	gil31322099	muscle isoform 2	*Creatine kinase Salmo salar*	42884	625	12	1	Gad m 4
Gm 5	gil7678762	Myosin light chain 1	*Theragra chalcogramma*	21355	649	13	2	Gad m 5
Gm 6	gil158705974	Nucleoside diphosphate kinase B	*Merluccius merluccius*	14368	291	3	1	Gad m 6
Gm 7	gil14531014	Parvalbumin beta	*Gadus morhua*	11618	169	14	0	Gad m 1
Gm 8	gil14531014	Parvalbumin beta	*Gadus morhua*	11618	467	43	0	Gad m 1

Abbreviation: Ig, immunoglobulin.
[a]The identified proteins are listed with the spot number (taken from Figure 3), cross-species proteins, NCBI accession number, MS/MS-Mowse Score, and number of peptides used in the Mowse search.

recruited from the Laboratory of Clinical Biochemistry and the Centre for Occupational and Environmental Allergy at Haukeland University Hospital, Bergen, Norway (clinical data are summarized in Tables 1 and 2). Serum from a healthy nonatopic individual (155) who tolerated fish and had no history of allergy or serological sensitization served as a control. We also analyzed serum from atopic patients (allergic rhinitis: 182, 272, 295, 323) who tolerated fish. Total and specific immunoglobulin (Ig) E values were determined using the CAP FEIA System (Phadia) (Table 2). The cutoff value for a positive result was 0.35 kU_A/L. No double-blind, placebo-controlled food challenge was performed because of the inherent risk of anaphylaxis.

Skin Prick Testing

SPTs were performed according to the guidelines of the European Academy of Allergology and Clinical Immunology on skin tests with raw fish. Reactions were determined after 15 minutes by measuring the 2 perpendicular diameters of the wheal. A mean wheal diameter larger than 3 mm of the negative control (0.9% saline solution) was considered positive. Histamine chloride 10 mg/mL was used as a positive control.

Direct Enzyme-Linked Immunosorbent Assay

Enzyme-linked immunosorbent assay (ELISA) was performed using 96-well microtiter plates (Dynatech Laboratories Inc) coated with 1 μg of allergen in 100 μL of 0.1 M sodium carbonate buffer (pH 9.6) per well and incubated overnight at 4ºC. After washing 5 times with Tris buffered saline (TBS) (pH 7.4) containing 0.05% Tween-20, 100 μL of undiluted human serum or 1000-fold diluted rabbit serum was added and incubated overnight at 4ºC. After washing, antihuman IgE or antirabbit IgG conjugated to alkaline phosphatase was added and incubated for 2 hours at room temperature. Once the plates were washed, the color reaction was developed using Sigma FAST pNPP alkaline phosphatase substrate (Sigma Chemical Co.), and absorbance was read at 405 nm after 30 minutes.

Preparation of Fish Protein Extracts

Small amounts of muscle tissue (60-70 mg) from *L niloticus* and *G morhua* were cut from deep-frozen fish and lysed in 100 μL of buffer (20 mM HEPES [pH 7.2], 10% glycerol, 1% triton X-100, 1 mM EDTA, 0.5% protease inhibitor, and 0.5% endonuclease). For complete disruption of cells, samples were additionally sonicated for 30 seconds 3 times on ice (duty cycle 40%, output control 3). The suspensions were centrifuged at 4°C for 10 minutes at 16 000*g* and the supernatants collected in new tubes. Protein concentrations were then estimated using the Lowry protein

assay. If samples were not processed directly, they were kept frozen at –20°C until further usage.

Rabbit Antisera

Rabbit polyclonal antibodies against purified parvalbumins (Gad c 1 and Sal s 1) were prepared as described elsewhere [16].

Isoelectric Focusing, 2D Gel Electrophoresis, and Western Blotting

For 2D sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), 200 μg of protein per gel was precipitated with acetone at –20°C for 15 minutes. The precipitates were then centrifuged at 16 000*g* for 15 minutes and washed once with acetone [17]. The resulting protein pellets were air dried, then resuspended in 135 μL of DeStreak rehydration solution with 0.5% immobilized pH gradient (IPG), pH 3-10 nonlinear (NL) buffer (vol/vol) (GE Healthcare). The solution was centrifuged for 15 minutes at 16 000*g* to remove precipitates, and loaded on 7-cm Immobiline DryStrips, pH 3-10 NL (GE Healthcare). After rehydration, proteins were focused overnight using an IPGphor electrophoresis unit (GE Healthcare) for 14 000 W/h. Gels were stained with Coomassie Brilliant Blue G-250 (Roth) for identification of protein spots. Alternatively, the proteins were transferred onto a nitrocellulose membrane (0.2 μm, Schleicher & Schuell) by electroblotting using a Western blot chamber (XCell II Blot Module, Invitrogen) and 10 mM of CAPS buffer, pH 11. A current of 1.5 mA/cm² was applied for 90 minutes at room temperature. Successful blotting was checked using Ponceau S staining of the membrane (0.2% Ponceau S in 3% TCA). The membranes were then rinsed with water, calibrated for 30 minutes in Tris-buffered saline Tween (TBST: 20 mM Tris, 500 mM NaCl, pH 7.5, 1% Tween-20) and blocked with TBST + 5% milk powder overnight. As a negative control for immunological detection, the membranes were incubated only with secondary antibody coupled with alkaline phosphatase (goat antihuman IgE antibody [Sigma-Aldrich]). Membranes were left shaking in antibody solution (1:2000 in TBST + 5% milk powder) for 1 hour at room temperature before being washed 3 times for 10 minutes. The bound antibody was then detected by staining with ECL solution (Promega). For detection of allergenic proteins, membranes were initially incubated with patient sera. In brief, the membrane was incubated in a dilution of 1:20 serum in TBST + 5% milk powder at 4°C overnight. After washing 3 times for 5 minutes in TBST, secondary antibody incubation and detection was performed as described above for the negative control.

Identification of Proteins by Mass Spectrometry

Protein spots of interest were cut from polyacrylamide gels and digested overnight using trypsin (Sigma-Aldrich) as described elsewhere [18]. The resulting peptides were eluted out of the gel, concentrated by vacuum centrifugation, and analyzed using a hybrid mass spectrometer (LTQ Orbitrap XL ETD, ThermoElectron) equipped with a nanoelectrospray ion source (ThermoElectron) and coupled to a nano–high-performance liquid chromatography system (NanoLC-Ultra 2D, Eksigent Technologies).

The mass spectrometry (MS) raw data files were converted to Mascot generic format using the ProteinQuant Suite. A database search was then conducted using the MS/MS ion search (MASCOT, http://www.matrixscience.com) against all metazoan entries of NCBInr (GenBank) with trypsin digestion and up to 2 missed cleavages (carbamidomethyl and oxidation products) as fixed modifications and with peptide tolerance ± 1.2 Da, MS/MS tolerance ± 0.6 Da, and a peptide charge of +1, +2, and +3 as variable modifications.

Results

A 24-year-old cook (No. 119, index patient) reported anaphylactic reactions upon contact with raw Nile perch and upon ingestion of cooked salmon (*Salmo salar*), although other fish were tolerated. SPT using raw fish was positive for *L niloticus* and *S salar*. Intriguingly, the result of SPT with cod (*G morhua*) was negative. Specific IgE results revealed low concentrations for *G morhua* (0.72 kU_A/L) (Table 1) and *S salar* (0.81 kU_A/L). The patient had a history of atopy with allergic rhinitis to grass pollen, rye, and mugwort. One-dimensional immunoblotting of *G morhua* and *L niloticus* did not reveal an IgE-reactive band indicative of parvalbumin, although it did generally reveal faint bands in extracts of *L niloticus*. Therefore, direct IgE ELISA was performed and compared with sera from a pool of 3 other fish-allergic patients (Nos. 253, 376, 462) (Figure 1).

The pooled sera of fish-allergic patients contained specific IgE against *L niloticus* and *G morhua* at various levels. Interestingly, IgE levels in the index patient were low or undetectable for the parvalbumins Gad c 1 and Sal s 1 compared to extracts of *G morhua*, *S salar*, and *L niloticus* (Figure 1A and B). Antigenic cross-reactivity between *L niloticus*, *G morhua*, and *S salar* was demonstrated using polyclonal IgG from rabbit anticod and rabbit antisalmon sera. IgG from rabbit anticod recognized antigens in *L niloticus* extracts with the same intensity as rabbit antisalmon (Figure 1C).

Detection of Reactive IgE From L niloticus and G morhua

Since SPT revealed no reactivity to *G morhua* and determination of IgE revealed only low reactivity to *G morhua* and parvalbumins (Sal s 1 and Gad c 1) in the index patient, we decided to perform a global search for allergens other than parvalbumin in *L niloticus* and to compare them with allergens from *G morhua*. To elucidate the general presence of the allergens other than parvalbumin, 11 other patients with clinical data supporting allergy to fish were also analyzed (summarized in Table 1). Three atopic patients (Nos. 272, 295, and 323), and 1 nonatopic control (No. 155) were included.

In order to perform a global assessment of allergens from *L niloticus* and *G morhua*, protein extracts from muscle tissue were separated by 2D gel electrophoresis. Gels were either stained with Coomassie Blue (Figure 2A and E) or blotted and probed with serum from patients (Figure 2B-D and F-H). A

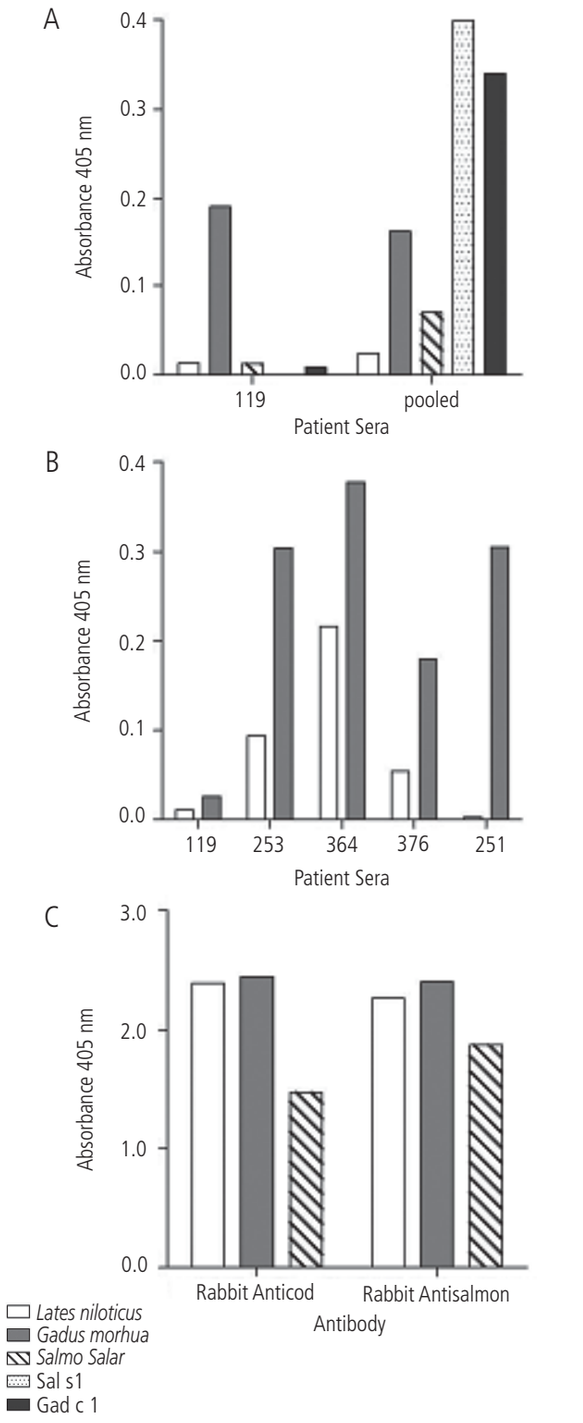

Figure 1. Enzyme-linked immunosorbent assay (ELISA) of different fish extracts that are reactive to sera from fish-allergic patients. A and B, Immunoglobulin E reactivity towards extracts from *Lates niloticus*, *Gadus morhua*, and *Salmo salar*, as well as towards Gad c 1 and Sal s 1 was analyzed using direct ELISA. C, The antigenic cross-reactivity of *L niloticus*, *G morhua*, and *S salar* was demonstrated using polyclonal IgG from rabbit anticod and rabbit antisalmon sera.

similar pattern and overall staining intensity makes it possible to draw a semiquantitative comparison of immunoreactivity. The protein patterns on the 2D gels show a predominant pearl necklace–like structure at around 40 kDa with a pI of 7. The protein composition of the muscle tissue exhibited a limited number of protein spots (171 for *L niloticus* and 117 for *G morhua*) and was dominated by metabolic enzymes. This finding is consistent with other reports on the skeletal muscle tissue of fish [19]. Figure 2B-D and F-H illustrate the results of the IgE immune-reactive spots with the serum from the index patient (No. 119), a patient with general fish allergy (No. 118), and the nonatopic control (No. 155). The negative control (detection without prior incubation with serum) was proven to be negative. The qualitative and quantitative differences in immune-reactive signals between patients and extracts point to differences in the allergenic proteins between the various species.

Identification of Allergens From L niloticus and G morhua

For the identification of allergens, the IgE-reactive protein spots from the Western blots were matched with the corresponding spots in the master gels (Figure 3A and B). The spots were identified using MS, and the standard parameters for unambiguous identification were applied (data summarized in Tables 2 and 3). Since none of the species examined have been sequenced, allergens were detected using cross-species identification [20] based on homologous sequence tags. This approach usually leads to limited overall probability scores, although not in this case, since identification was unambiguous for all spots. In all cases, the differences were greater than half of the score of the identified protein; therefore, the fundamental doubt over whether a spot might contain more than 1 protein can be excluded.

Since IgE-reactive parvalbumin has been detected in 116 vertebrate species, it is noteworthy that it was not found in the 2D Western blots from the present study. In order to assure that we did not overlook even minimal antiparvalbumin IgE immune reactivity, we analyzed all spots, including those with only slight immunoreactive signals after serum incubation, even though they were thought to be isoforms because of their localization in the gel. We also confirmed that parvalbumin was present in the gels and identified 2 parvalbumin-positive spots in *G morhua* (Figure 3B, Spots 7 and 8).

For *L niloticus*, 8 distinct IgE binding proteins were identified. The most frequently detected IgE-reactive protein was enolase, to which 8 out of 12 patients (66.6%) were sensitized. Creatine kinase 1 was found in 3 of the 12 patients (25%). Less frequently detected IgE-reactive proteins were fructose-bisphosphate aldolase A (1 of 12, 8%), fast myotomal muscle actin (1 of 12, 8%), creatine kinase muscle isoform 2 (1 of 12, 8%), apolipoprotein A1 (1 of 12, 8%), and adenylate kinase (1 of 12, 8%) (allergome codes, Table 2). The atopic control patients (Nos. 272, 295, and 323) also exhibited reactive IgE to enolase 3. One of these patients also revealed IgE binding to fructose-bisphosphate aldolase and creatine kinase 1 (No. 272).

The 4 IgE-reactive proteins detected for *G morhua* were enolase, tropomyosin, mysosin light chain, and nucleoside diphosphate kinase B. As with *L niloticus*, the most frequently

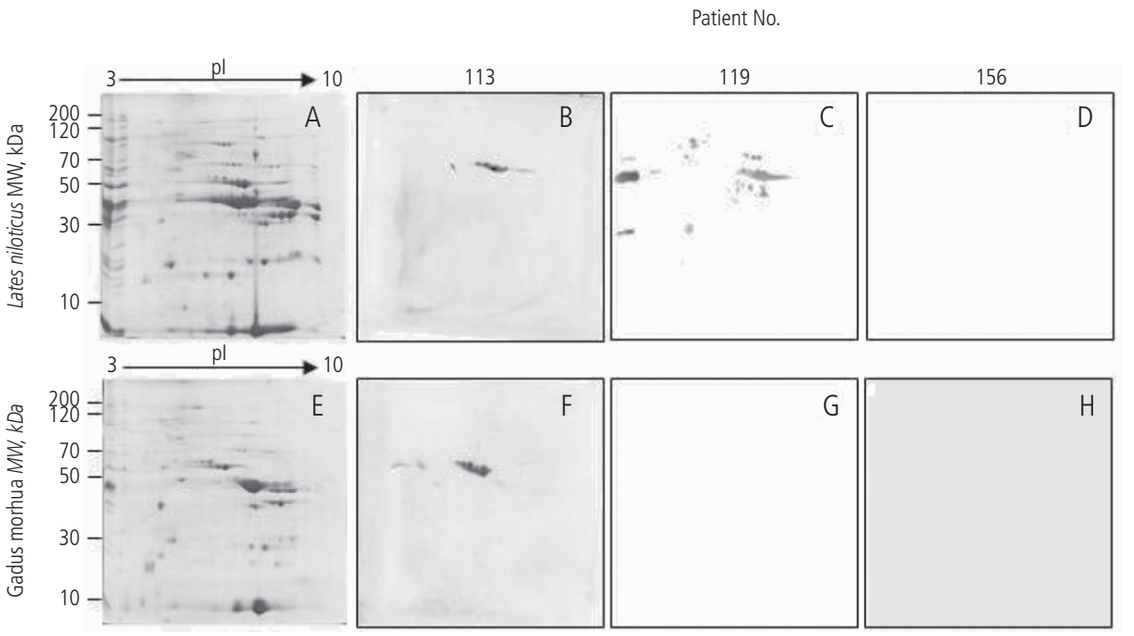

Figure 2. 2D blots with extracts from *Lates niloticus* and *Gadus morhua*. Representative IgE immune reactive spots by sera from 2 patients and the nonatopic control are displayed. MW indicates molecular weight.

Table 3. Summary of the Signals Obtained From Incubation of the Western Blots With Patient Sera

Species	Spot No./Allergen	Immunoglobulin E Signals in Western Blot Detection															No. of IgE-Reactive Sera
		119	155	272	295	323	1	2	3	118	133	251	253	364	369	376	462	
Lates niloticus																	
	1. Phosphoglucomutase 1	++						++										2
	2. Enolase 3	++		+	++	++		++	++	++			++	++	++	++		11
	3. Fast myotomal muscle actin	+																1
	4. Creatine kinase muscle isoform 2	+																1
	5. Creatine kinase 1	++		+				++	++									4
	6. Fructose-bisphosphate aldolase A			+				+										2
	7. Apolipoprotein A1	+																1
	8. adenylate kinase 1-2							++										1
Gadus morhua																	
	1. Alpha-enolase			++	++		++	++	++	+	++	++	++	++	++	++	++	14
	2. Enolase 3-1			++	++	++	++	++	++	++	++	++	++	++	++	++	++	13
	3. Tropomyosin										++							1
	4. Creatine kinase muscle isoform 2																	0
	5. Myosin light chain 1										++							1
	6. Nucleoside diphosphate kinase B										++							1

+, weak signal in Western blot; ++, strong signal in Western blot.

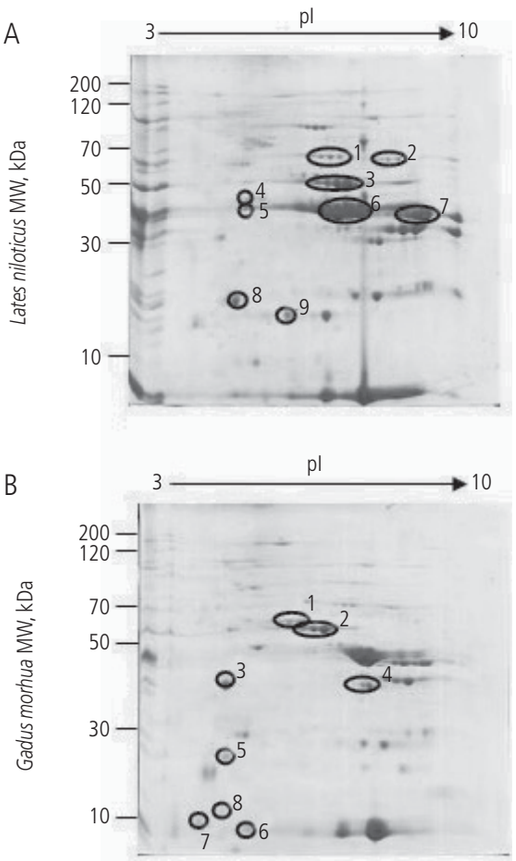

Figure 3. Preparative gels of protein extracts from *Lates niloticus* and *Gadus morhua*. Two hundred micrograms of protein was applied to 2D gel electrophoresis, and the total protein amount was stained with Coomassie Brilliant Blue. Additionally, 10 µg of protein was applied to 1D gel electrophoresis on the left hand side of each gel. The total protein amount was stained with Coomassie Brilliant Blue.
All proteins identified as allergens by Western blot detection are encircled. Additionally, parvalbumin (spots 7 and 8) was identified as a control in *G morhua* using tandem mass spectroscopy. We most often found protein identities in groups. MW indicates molecular weight.

found IgE-reactive protein for *G morhua* was enolase. Enolase 1 and/or enolase 3 anti-IgE was detected for all 11 cod-allergic patients (100%). Furthermore, 1 of the 12 (8%) fish-allergic patients had anti-IgE against tropomyosin or myosin light chain 1. Nucleoside diphosphate kinase B was detected in only 1 patient (8%).

Discussion

We report a patient with immediate-type allergy to *L niloticus* and demonstrate that the allergy was caused by cross-reactive fish allergens other than parvalbumin. The low-level or absence of binding to Sal s 1 and Gad c 1 indicated that allergens in *L niloticus* other than parvalbumin were recognized. Phosphoglucomutase, muscle actin, and apolipoprotein A1 were reactive in this patient, although they did not react to the sera of cod-allergic patients, thus indicating species-specific sensitization towards potential allergens of *L niloticus*. Enolase 3 was the main cross-reactive IgE-binding protein in *L niloticus* in 7 of the 12 patients (58%) and in *G morhua* in all 11 cod-allergic patients (100%). However, atopic control patients were also sensitive to enolase 3 from *L niloticus* and *G morhua*.

Sensitization to individual fish species caused by allergens other than parvalbumin has already been reported [21,22]. To date, none of the proteins found in the present study have been reported to be allergens for *L niloticus* and *G morhua*. The association between sensitization to parvalbumins in *G morhua* and fish allergy with clinical manifestations seems to be clear-cut [23,24]. Nevertheless, controversy exists with regard to cross-sensitization and the recommendation that allergic patients avoid all fish species. Oral challenge performed in adults showed that the parvalbumin Gad c 1 was a reliable marker for fish allergy, although patients could tolerate other species with no adverse reactions [8,10,11,25]. The finding that fish-allergic patients tolerate some fish species has been explained by the presence of different concentrations of parvalbumins in different species, eg, high concentrations in cod but low concentrations in tuna [26,27]. Despite the similar features of parvalbumins from different species, these proteins have been recognized as allergens only in fish and in frog. Parvalbumins from higher vertebrates are not bound by human IgE [28]. It is compelling that in a diverse taxonomic class such as osteichthyes (bony fish), which is extremely diverse and consists of over 29 000 species, a single conserved protein accounts for major allergenicity. In 11 of the 12 fish-allergic patients (92%), IgE against cod parvalbumin was detected by ELISA. Although no specific IgE binding for parvalbumin was detected by 2D immunoblotting, parvalbumin was present in the gels. The discrepancy between the results of ELISA and 2D gel electrophoresis with fresh preparations of fish can be explained in part by differences in sample preparation for 2D immunoblotting and ELISA. Furthermore, IgE binding to parvalbumin is affected by calcium concentration and treatment of protein extracts with periodate; however, the sample buffer in SDS-PAGE diminishes the proteins' secondary structure, and metal binding has been successfully used to detect parvalbumin [6]. It is interesting to note here that deglycosylation of parvalbumin, which is actually a very stable allergen, led to reduced binding of IgE [6].

The antigenic cross-reactivity of *L niloticus*, *G morhua*, and *S salar* was demonstrated using polyclonal IgG from rabbit anticod and rabbit antisalmon sera. IgG from rabbit anticod recognized and bound to *L nilticus* extract–specific antigens more than rabbit antisalmon. Only 1 of the IgE-binding proteins of *L niloticus*, namely, enolase 3, was also detected in most of the cod-allergic patients and sensitized 100% of the patients who were allergic to *G morhua*. This finding may either contribute to the known cross-reactivity between fish species [8,10,11] or may be nonspecific owing to a polyclonal IgE response in atopic patients, since enolase was also bound in atopic control patients (No. 272, 295, 323) with no clinically apparent fish allergy. Previous studies demonstrated

IgE binding to fish protein in atopic patients who tolerated fish [8,23]. However, enolase has been described as an important allergen from various molds and some plants [29,30]. Cross-reactivity of enolase might not be surprising, since it is highly conserved in eukaryotic and prokaryotic species, with the result that an identity of 83% was found even between the protein sequences of *Tetraodon nigroviridis* and humans [31]. Increased enolase gene expression in response to stressors, including exposure to heavy metals, environmental variation, and infection, has been experimentally tested and reported in fish following lipopolysaccharide challenge [32]. This expression is particularly interesting, since it may ultimately also affect the allergenicity of the ingested fish [4].

The second most frequent potential allergen, creatine kinase, belongs to the ATP:guanido phosphotransferase family (AF049). Creatine kinase plays a key role in regulating the metabolism of adenosine triphosphate, which is the primary source of energy in animals and is also abundant in fish muscle [33]. No allergenic potential has been reported to date for creatine kinase; however, it belongs to the same guanidine family of kinases as the known crustacean allergen arginine kinase, which is considered a highly cross-reactive nonvertebrate panallergen [34]. Interestingly, the primary, secondary, and tertiary structures of creatine kinase and arginine kinase are very similar [35].

The third most abundant allergen, fructose bisphosphate aldolase, belongs to the AllFam family (AF144). Fructose-bisphosphate aldolases are glycolytic enzymes that catalyze reversible aldol condensation of glyceraldehyde 3-phosphate and dihydroxyacetone phosphate to yield fructose-bisphosphate. IgE against fructose-bisphosphate aldolase was found in patient 272 (non–fish-allergic patient) and patient 2. Fructose bisphosphate aldolase was bound by IgE in a Taiwanese midge (For t 2) and *Candida albicans* (Cand a FPA) [36,37].

The apolipoprotein A1 determined as an IgE-reactive protein from *L niloticus* belongs to the lipoprotein family (AF092). Although no sensitization towards apolipoprotein A1 has been described in fish to date, lipoproteins are reported to be allergenic in dust mites (Der p 14) and chicken (Gal d apoI). Here, only the IgE of the index patient (No. 119) bound to apolipoprotein A1 in *L niloticus*, not that of the other fish-allergic patient. No sensitization to phosphoglucomutase, muscle actin, pyruvate kinase, and adenylate kinase has been reported in other species.

In conclusion, our data suggest that phosphoglucomutase, muscle actin, and apolipoprotein A1 are putative *L niloticus*–specific allergens. Strong cross-reactivity was detected for enolase from *L niloticus* and *G morhua*. Enolase may be either a minor allergen or a clinically irrelevant sensitization, since IgE binding also occurred in atopic control patients. Identification of species-specific allergens or individual sensitization may be of value when deciding which species of fish can be tolerated by fish-allergic patients. Furthermore, identification of clinically irrelevant IgE binding may improve the specificity of in vitro diagnosis of fish allergies.

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