The effects of gastric digestion on codfish allergenicity

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Background: In a recent murine study, we showed that impaired gastric digestion supports the induction of fish allergy by protecting the digestion-sensitive major allergen parvalbumin and thus enhancing its sensitizing properties.
Objective: The aim of the present study was to investigate whether impairment of peptic degradation might also play a role in the effector phase of codfish allergy.
Methods: The resistance of cod proteins to digestion by simulated gastric fluid was assessed *in vitro*. Gastric solutions with pH values ranging from 1.25 to 5.0 were prepared, and the influence of the pH on protein degradation was evaluated by means of SDS-PAGE and IgE immunoblotting. The allergenic potency of digested and undigested cod extract was further characterized in RAST inhibition and basophil histamine release experiments.
Results: The digestion experiments revealed that codfish proteins were degraded within 1 minute under physiologic gastric conditions. An only marginal pH shift from 2.5 to 2.75 abrogated completely the digestion of cod allergens. In RAST inhibition experiments digested cod extracts showed a reduced IgE-binding capability that was dependent on the digestion time. Moreover, peptic fragments expressed a 10,000 times reduced allergenic potency, as evaluated on the basis of histamine release from human basophils.
Conclusion: Codfish allergens have a grossly reduced ability to trigger an intestinal allergic reaction when they are physiologically degraded. Impairment of the physiologic digestion might thus lower the threshold levels of a food allergen in sensitized patients. (J Allergy Clin Immunol 2005;115:377-82.)

Key words: *Food allergy, digestion, codfish, threshold, histamine release assay, RAST inhibition*

Abbreviations used
HR: Histamine release
SGF: Simulated gastric fluid

In recent decades, the consumption of fish has gained popularity in the general western population as a result of its reported benefit in reducing artery disease[1] and decreasing mortality among myocardial infarct survivors.[2] Unfortunately, fish is also among the most frequent elicitors of IgE-mediated type I food allergy,[3] although there might be large regional differences depending on consumption patterns. It is well documented that the ingestion of fish, skin and mucosal contact, and the inhalation of aerosolized fish proteins during cooking or in an occupational setting[4,5] can cause a large variety of clinical symptoms in sensitized patients, such as urticaria, angioedema, atopic dermatitis, asthma, rhinitis, vomiting, diarrhea, and anaphylaxis.[6,7] A recent multicenter study including 678 cases of emergency department treatment for food allergy revealed that 10% of the anaphylactic reactions were caused by fish allergens.[8] Thus fish is generally accepted to be among the foods most commonly eliciting severe food anaphylaxis[9] and has been taken up in the European Union declaration guideline for allergenic foods.[10]

Only little is known about a possible pregastric absorption, but it is generally accepted that food allergy to fish might develop after sensitization through the gastrointestinal tract. ''Real'' food allergens are characterized to be abundant in the food, resistant to food processing (eg, cooking), and resistant to gastrointestinal digestion.[11] Fish antigens were previously considered to share these properties, and Gad c 1, the major codfish allergen, a parvalbumin, has always been described as a typically digestion-resistant food antigen.[12,13] However, we recently reported that parvalbumin does not show all the features thought to be typical of a classical food allergen, because it is immediately degraded under simulated gastric conditions. In a mouse model we demonstrated that parvalbumin retains its sensitization capacity only when gastric digestion is hindered. We concluded that in hypoacidic situations otherwise harmless, digestion-sensitive food proteins acquire allergenic capacity.[14]

Consequently, the aim of the present study was to investigate the effect of pH on the enzymatic digestion of codfish allergens in a situation in which mucosal mast cells and basophils already are sensitized with allergen-specific IgE.

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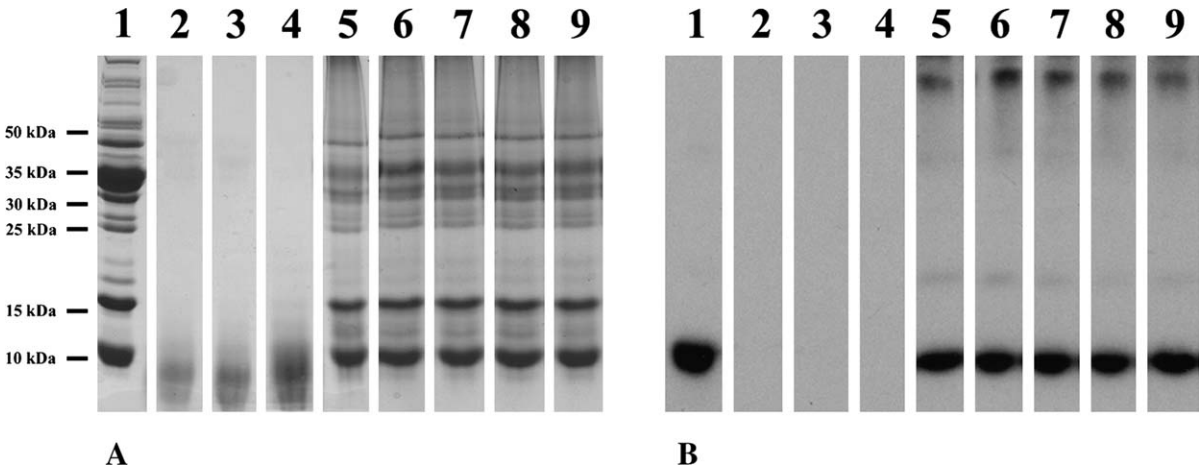

FIG 1. Increased pH in SGF experiments hampers codfish allergen digestion. Cod extract *(lanes 1)* was subjected to digestion experiments for 1 minute with pepsin at pH 1.25 *(lanes 2)*, 2.0 *(lanes 3)*, 2.5 *(lanes 4)*, 2.75 *(lanes 5)*, 3.5 *(lanes 6)*, 4.0 *(lanes 7)*, 4.5 *(lanes 8)*, and 5.0 *(lanes 9)*. **A**, Coomassie-stained SDS-PAGE. **B**, IgE immunoblot with a patients' serum pool.

METHODS

Cod extract preparation

Codfish extract was prepared as described previously.[15] Fresh cod meat was grounded in liquid nitrogen and extracted overnight at 4°C in 10 mM phosphate buffer, 2 mM EDTA, 0.01% pepstatin A, and 3 mM NaN_3. After 2 centrifugation steps (325*g*, 15 minutes, 4°C), supernatants were dialyzed (dialysis membranes cutoff 6000-8000; Spectra/Por, Huston, Tex) in distilled water overnight at 4°C. The dialyzed proteins were frozen at −70°C and freeze-dried. Extract quality was confirmed by means of SDS-PAGE, and the protein concentration was determined according to the method of Bradford.[16]

Patients' sera

Serum samples of 3 patients with cod allergy from the Allergy Department of the National University Hospital Copenhagen, Denmark, were included in our study. Cod allergy was diagnosed on the basis of detection of cod-specific IgE antibodies (patient 1, 1.87 kUA/L; patient 2, 2.61 kUA/L; patient 3, 255 kUA/L) in the CAP-FEIA system (Pharmacia & Upjohn, Uppsala, Sweden), positive skin test reactivity, and positive oral provocation test results.

In vitro digestion experiments

For simulated gastric fluid (SGF) assays, protein preparations (1 mg/mL) were incubated at 37°C with 0.87 g/L pepsin (Sigma, St Louis, Mo) during continuous agitation.[17] The pH of SGF was adjusted to 1.25, 1.5, 1.75, 2.0, 2.25, 2.5, 2.75, 3.0, 3.1, 3.2, 3.3, 3.4, 3.5, 3.6, 3.7, 3.8, 3.9, 4.0, 4.25, 4.5, 4.75, and 5.0 with 0.1 N NaOH. At every pH, the enzymatic reaction was determined after 1 minute, 15 minutes, and 1 hour by neutralizing the pH and cooling the samples on ice. For control purposes, the degradation effect of acid without enzymes was tested.

SDS-PAGE and IgE immunoblots

Digested cod protein extracts were evaluated by means of SDS-PAGE according to the methods of Lammeli[18] by Commassie brilliant blue staining, and the molecular weight was calculated by using prestained protein standards. The separated proteins were blotted onto nitrocellulose.[19] Blots were saturated with blocking buffer containing 0.5% dried milk powder and incubated overnight at 4°C with sera from patients or healthy control subjects. Bound IgE antibodies were detected with iodine 125–labeled anti-human IgE antibodies (IBL, Hamburg, Germany) and visualized by means of autoradiography.

RAST inhibition experiments

The RAST inhibition experiments were performed according to the method previously described.[20] Adsorbing tubes (Nunc, Roskilde, Denmark) were coated by the addition of 300 μL of a 2 μg/mL codfish extract in PBS overnight at 37°C. For the inhibition experiment, patients' sera were incubated with digested and undigested codfish extracts, as well as the control antigen peanut extract, at different concentrations overnight at 4°C. After washing with PBS-Tween and blocking with RPMI–10% FCS for 2 hours at room temperature, the inhibited serum was added for 3 hours at 37°C. Tubes were washed and incubated with 300 μL of iodine 125–labeled anti-human IgE (Pharmacia RAST, Uppsala, Sweden) 1:12 in PBS-Tween (approximately 10,000 cpm) overnight. After 3 washing cycles, bound radiolabeled antibodies were measured (Wallac Wizard 1470 Automatic Gamma Counter; PrekinElmer, Wellesley, Mass).

Histamine release assay

Histamine release (HR) from basophil leukocytes was performed as previously described.[21] In brief, buffy coats of different healthy donors were screened for their responses to an anti-IgE antibody by using glass fiber–coated microtiter plates. Donor basophils with an anti-IgE–induced HR of greater than 30% were selected for further experiments. PBMCs were isolated with Lymphoprep (Nycomed Pharma Holding AS, Roskilde, Denmark). After intensive washing with Pipes buffer, cell-bound IgE was released from cells with a stripping buffer containing 20 g/L potassium chloride and 0.37 g/L sodium hydrogen phosphate at pH 3.55. Washed cells were then incubated with sera from patients with codfish allergy for 1 hour at 37°C to passively sensitize basophils. After 3 washing cycles, cells were pipetted into allergen-coated glass fiber microtiter plates (Reference Laboratory, Copenhagen, Denmark). For this purpose, 12 dilutions, with a dilution factor of 3.5 starting with a 0.1 mg/mL codfish allergen stock solution, were prepared and incubated with PBMCs under releasing conditions at 37°C for 60 minutes. Cells were