

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

In recent decades, the consumption of fish has gained popularity in the general western population as a result of

Abbreviations used

HR: Histamine release

SGF: Simulated gastric fluid

its reported benefit in reducing artery disease¹ 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 anaphylaxis⁹ 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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Supported by grant 10326 of the Austrian National bank "Jubiläumsfond," by a fellowship award of the EAACI, and in part by grant SFB-F018#8 of the Austrian National Science Foundation (FWF).

Received for publication August 20, 2004; revised October 15, 2004; accepted for publication October 18, 2004.

Available online January 4, 2005.

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0091-6749/\$30.00

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doi:10.1016/j.jaci.2004.10.029

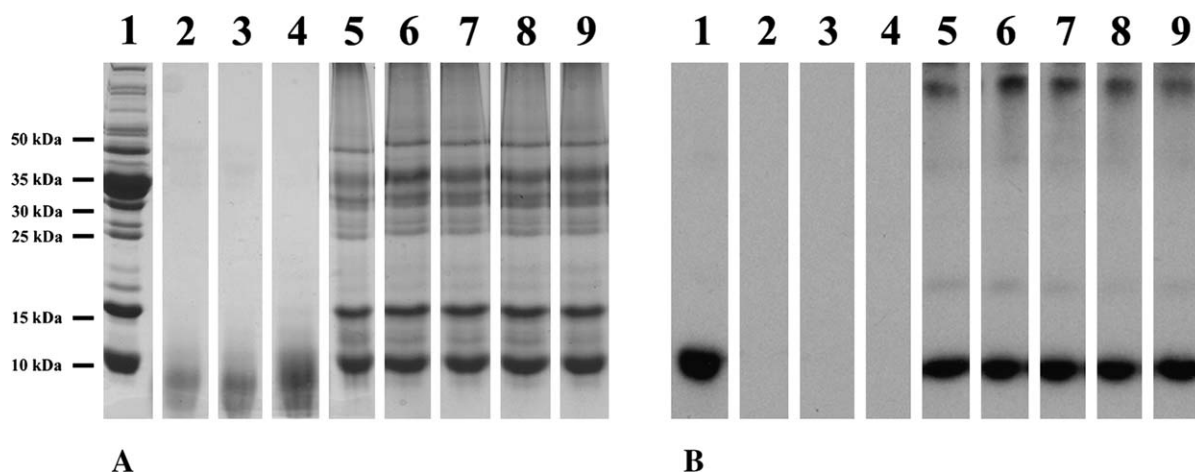


FIG 1. Increased pH in SGF experiments hampers codfish allergen digestion. Cod extract (lanes 1) was subjected to digestion experiments for 1 minute with pepsin at pH 1.25 (lanes 2), 2.0 (lanes 3), 2.5 (lanes 4), 2.75 (lanes 5), 3.5 (lanes 6), 4.0 (lanes 7), 4.5 (lanes 8), and 5.0 (lanes 9). **A**, Coomassie-stained 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 Na₂S₂O₃. After 2 centrifugation steps (325g, 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 Lammeli¹⁸ 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

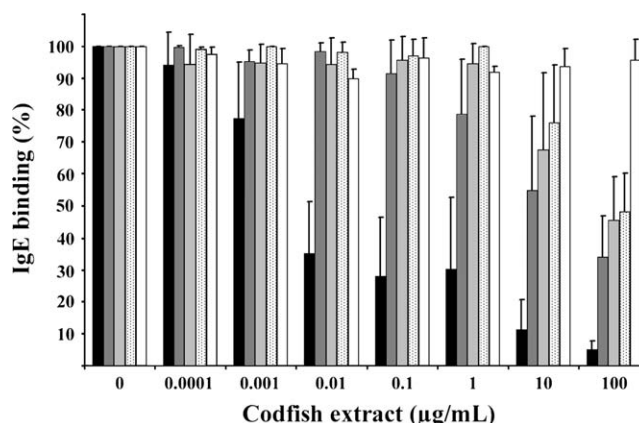


FIG 2. The dose-dependent inhibitory capacity of codfish extract is reduced by digestion with SGF in a time-dependent manner. Codfish samples were left undigested (■) or were incubated with pepsin at pH 1.25 for 1 minute (■), 15 minutes (□), and 1 hour (□). The concentrations of proteins applied in RAST inhibition experiments are shown on the x-axis. Uninhibited IgE binding was set at 100%, and the sample values were compared with that value. No inhibition occurred with peanut extract, an irrelevant control antigen (□). The mean values of the evaluation with 3 serum samples are presented, and error bars indicate SEMs.

washed away, and the released, glass fiber-bound histamine level was determined by using the fluorometric o-Phthal-di-aldehyde method. The results are expressed as the percentage of net HR relative to the total cellular content.

RESULTS

Digestion experiments

The resistance of codfish extract to peptic digestion was determined in an *in vitro* SGF assay and analyzed by means of SDS-PAGE (Fig 1, A) and IgE immunoblotting (Fig 1, B). An IgE immunoblot with a serum pool of the patients included in this study showed that their IgE antibodies bound to a 10-kd protein band in undigested codfish extract (Fig 1, B, lane 1), most likely the major fish allergen parvalbumin Gad c 1. Under digestion at physiologic conditions ($\leq$ pH 2.5), all proteins were degraded to small fragments within 1 minute (Fig 1, A, lanes 2-4) and lost their IgE-binding capability (Fig 1, B, lanes 2-4). Acid without enzymes did not show any degradation effects on codfish proteins (data not shown). Interestingly, a small pH shift from 2.5 to 2.75 resulted in a complete change of digestion properties of the gastric fluid on codfish allergens (Fig 1, A, lanes 5-9). In these samples IgE bound to a 10-kd protein and to some minor bands at higher molecular weights (Fig 1, B, lanes 5-9). These protein patterns remained stable, even after 1 hour of incubation. No IgE reactivity was observed when testing with the serum of a nonallergic control subject (data not shown).

RAST inhibition experiments

A quantitative estimation of the IgE-binding capacity of digested extracts was performed in a competitive RAST inhibition assay by using sera from the 3 patients. The results depicted in Fig 2 show that undigested cod extract inhibited IgE binding to the coupled antigen in a concen-

TABLE I. RAST inhibition experiments with digested and undigested codfish extract

Extract	IC50 (µg/mL)	Activity reduction*
Undigested codfish	0.0056	0
1-min digestion	16.08	2900
15-min digestion	31.79	5700
1-h digestion	60.92	10,900

IC50, 50% Inhibitory concentration.

*The activity reduction was calculated by dividing the 50% inhibitory concentration of digested extracts by the 50% inhibitory concentration of undigested codfish extract. The mean values of 3 experiments are presented.

tration-dependent manner. IgE binding was inhibited by digested cod extract only when applying 1000-fold to 10,000-fold higher concentrations. No inhibition was observed when performing the experiment with an irrelevant peanut allergen. The 50% inhibitory concentration of each extract and the activity reduction by digestion was calculated. Only 1 minute of gastric digestion resulted in a 2800-fold reduction of inhibitory capacity when compared with untreated cod extract. This influence of the gastric protease on the allergenicity of cod proteins was time dependent, resulting in a 5000-fold reduction after 15 minutes of digestion and in a 10,000-fold reduction after 1 hour of enzymatic degradation (Table I).

HR experiments

The ability to induce HR from human basophils was determined to further investigate the properties of digested and undigested extracts as elicitors of allergic reactions. We performed HR experiments with stripped basophils from nonallergic donors passively sensitized with sera from the patients with codfish allergy. HR of up to 26% (patient 1; Fig 3, A), 35% (patient 2; Fig 3, B), and 50%

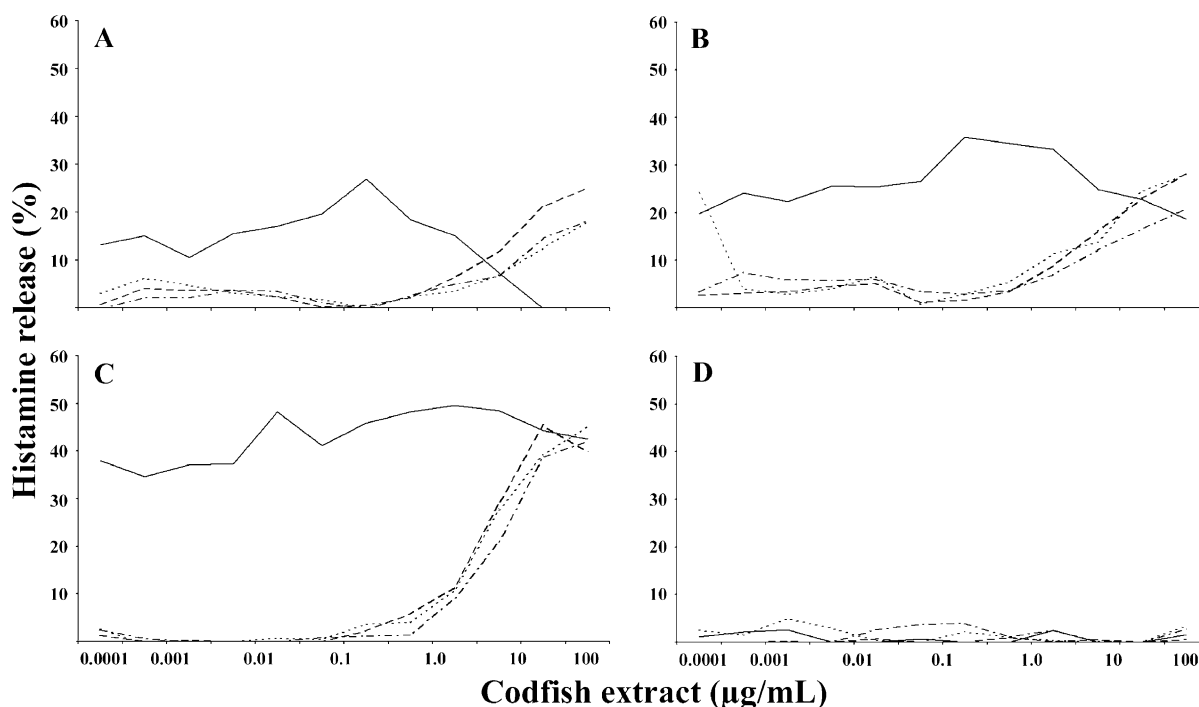


FIG 3. Dose-dependent HR triggered by digested or undigested codfish extract. The assay was performed with stripped human basophils from a healthy donor passively sensitized with the serum of patient 1 (**A**), patient 2 (**B**), patient 3 (**C**), and a nonallergic control patient (**D**). Lines (undigested —, 1-minute digested ----, 15-minutes digested - · - · -, and 1-hour digested - · - · -, cod extract) indicate the relative percentage of released histamine calculated from the total histamine content of lysed cells, which was set at 100%.

(patient 3; Fig 3, C) relative to the total histamine content was induced by undigested cod extract. Also, low amounts of undigested protein extracts (0.1 ng/mL) induced significant HR. By using the digested cod extract samples, HR was observed only in high concentrations. No HR was observed when the serum of a nonallergic control patient was used for sensitization of donor cells (Fig 3, D).

DISCUSSION

Recently, we described a correlation between intake of antiulcer drugs and the induction of IgE-mediated food allergy. Reduced acidity impairs peptic digestion, protects digestion-labile proteins, and enhances their sensitization capacity.¹⁴

The aim of the present study was to determine the digestion stability of codfish proteins, which are among the most frequent elicitors of severe IgE-mediated food-adverse reactions, and to investigate the role of gastric digestion in the effector phase of type I food allergy in sensitized individuals.

A large variety of codfish proteins has been reported to harbor allergenic properties, but Gad c 1 seems to be the major allergen.²²⁻²⁵ These fish proteins have always been considered to show the features of so-called “real” food allergens,^{12,13} which exhibit resistance to heating and pro-

teolysis.^{26,27} Paradoxically, in our *in vitro* assay simulating gastric fluid, proteins of a codfish extract were degraded within 1 minute. Other dietary compounds, like fruits and vegetables that are known to be digestion-labile food antigens,²⁸ are mainly elicitors of local symptoms as an oral allergy syndrome.^{29,30} In contrast, fish ingestion triggers life-threatening systemic reactions in sensitized patients,⁷ even if it is obviously quickly degraded.

By varying pH during the simulated gastric digestion, it could be demonstrated that a marginal pH shift strongly reduced the digestive properties of gastric fluids on codfish allergens. An increase of the pH from 2.5 to 2.75 abrogated the digestion of cod proteins completely. These data are in line with our previous observations in a murine model of food allergy. Antiulcer drugs causing hypoacidity supported IgE induction against food proteins.¹⁴

It is well established that the gastric acidity plays an important role in digestion because the different pepsins exhibit optimal proteolytic activity at low pH.³¹ Furthermore, acidity per se has an important influence on digestion by acidic hydrolysis.³² Thus the physiologic digestive function of the stomach depends to a great extent on the gastric acid secretion. In consequence, conditions with low net acid output, like, for example hypoacidity as a physiologic feature in newborns, atrophic gastritis, or partial gastrectomy, might critically affect the digestive

capacity of the stomach. Moreover, dyspeptic disorders are treated with highly effective and long-lasting antilulcer drugs, which can almost totally inhibit acid secretion^{33,34} and increase the gastric pH to an average of 5.³⁵ Under these circumstances, the digestion of the fish proteins and other food items might be abolished, and intact allergenic proteins might enter the gut with a subsequent systemic absorption and elicitation of generalized allergic symptoms.^{36,37}

Our working hypothesis is that hypoacidity might have an effect also in the effector phase of food allergy in already sensitized individuals. The result of our competitive RAST inhibition assay indicated indeed that a 2000-fold increased concentration of digested cod extract was needed to achieve the same IgE inhibitory capacity as seen with undigested cod proteins. These findings were even more pronounced after enzymatic degradation of longer than 1 minute. The average stomach transit rate constant calculated by a gastrointestinal transit kinetic model was shown to be 2 hours.³⁸ Thus during an average transit process, the IgE-binding capacity of codfish is 10,000-fold reduced. Not only serologically, but also in HR assay, high amounts of the digested cod proteins were needed to initiate mediator release, whereas the native allergen revealed releasing capacities in very low allergen doses.

Our findings are interesting with respect to the efforts that are being undertaken to establish threshold levels for allergenic foods. In a recent hypothesis article, the threshold doses resulting in a reaction of one in a million in highly sensitized patients were calculated statistically for a number of food allergens on the basis of previously published data.³⁹ Furthermore, very recently, a consensus protocol has been published to estimate the lowest observed adverse effect level and no-observed adverse effect level.⁴⁰ These estimates might have to be reevaluated for patients who do not exhibit physiologic gastric functions.

From our data, we conclude that allergenic properties of fish proteins can be increased tremendously by impaired gastric digestion. In highly sensitized individuals, when IgE is already formed and fixed to effector cells through the high-affinity IgE receptor, insufficient digestion might remarkably enhance the risk of experiencing more severe allergic reactions.

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