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Evaluation of the potential allergenicity of the enzyme microbial transglutaminase using the 2001 FAO/WHO Decision Tree

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All novel proteins must be assessed for their potential allergenicity before they are introduced into the food market. One method to achieve this is the 2001 FAO/WHO Decision Tree recommended for evaluation of proteins from genetically modified organisms (GMOs). It was the aim of this study to investigate the allergenicity of microbial transglutaminase (m-TG) from *Streptoverticillium mobaraense*. Amino acid sequence similarity to known allergens, pepsin resistance, and detection of protein binding to specific serum immunoglobulin E (IgE) (RAST) have been evaluated as recommended by the decision tree. Allergenicity in the source material was thought unlikely, since no IgE-mediated allergy to any bacteria has been reported. m-TG is fully degraded after 5 min of pepsin treatment. A database search showed that the enzyme has no homology with known allergens, down to a match of six contiguous amino acids, which meets the requirements of the decision tree. However, there is a match at the five contiguous amino acid level to the major codfish allergen Gad c1. The potential cross reactivity between m-TG and Gad c1 was investigated in RAST using sera from 25 documented cod-allergic patients and an extract of raw codfish. No binding between patient IgE and m-TG was observed. It can be concluded that no safety concerns with regard to the allergenic potential of m-TG were identified.

Keywords: Allergenicity / Allergy / Decision Tree / FAO/WHO / Transglutaminase

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1 Introduction

The introduction of novel proteins into foods always carries a risk of eliciting an allergic response in patients sensitive to the protein in question or to related proteins. Furthermore, there is a risk of sensitizing predisposed individuals. There is no single-method strategy available to determine the allergenicity of a given protein, but various schemes have been proposed to test for the ability to elicit an allergic reaction in already sensitized patients. At a meeting in Rome in 2001, an expert group joined by FAO/WHO proposed a decision tree aimed at investigating biotechnologically derived proteins [1]*. The full procedure was previously tested on a genetically modified organism (GMO) protein, ice-structuring protein (ISP), in which case the protein was concluded 'not likely to be allergenic' [2].

Transglutaminase is an enzyme widely distributed in nature and can be found in diverse animal tissues, fish, and plants [3]. Natural microbial transglutaminase (m-TG) has been isolated from the organism *Streptoverticillium mobaraense* [4] and is not a GMO product. Microbial transglutaminase is homologically different from transglutaminases found in plants and animals [5]. It has a molecular mass of 38 kDa and contains no saccharide or lipid moieties [6]. m-TG catalyzes the cross-binding of protein-bound glutamine and a primary amine such as a lysine residue in protein. The enzyme acts as a texturizing agent in prepared foods adding firmness, thermal stability, water-holding capacity in addition to other qualitative changes to the food. As a food

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Abbreviations: FCS, fetal calf serum; **GMO**, genetically modified organism; **IgE**, immunoglobulin E; **Maxisorp RAST**, radioallergosorbent test using maxisorp tubes; **m-TG**, microbial transglutaminase; **NHS**, normal human serum

* A key figure of the 2001 FAO/WHO Decision Tree can be found in references 1 and 2, and at the web-address:http://www.doylefoundation.org/icsu/FAOWHO%2002001%20Decision%20Tree.htm.

enzyme, m-TG has been commercialized by the Japanese company Ajinomoto and is presently applied in seafood, meat products, noodles, pasta, dairy products, and baked goods.

We have not been able to find any cases of immunoglobulin E (IgE) sensitization to a bacterial strain, so, to our knowledge, no sera are available to use for a specific serum screen making this step of the decision tree impossible. A sequence alignment in protein databases was made to compare the amino acid sequence of m-TG to all known allergens as recommended by the 2001 FAO/WHO Decision Tree. The study demonstrated that down to a match of six contiguous amino acids there was no homology with any type of allergen, thus meeting the requirements of the decision tree. However, a match was found at the five contiguous amino acid level between m-TG and the allergen Gad c1. Due to the fact that Gad c1 is the major codfish allergen and is extremely heat-stable [7, 8], a targeted serum screen of m-TG using sera from 25 documented cod allergic patients is described in this paper. Moreover, the degradability as a result of pepsin treatment was tested.

2 Materials and methods

2.1 Test material

Purified microbial transglutaminase (CAS 80146-85-6, E.C. 2.3.2.13) was used in these experiments. Ten grams of m-TG preparation (about 1150 U/g) was added to 50 mL of 50 mM potassium phosphate buffer (pH 6.0), including 2 mM DTT, stirred for 3 h at 5°C, adjusted to pH 5.8 using HCl, and diluted 10 times with 20 mM sodium acetate, pH 5.8. This was then applied to a cation-exchange column (CM-Sepharose FF, 5.0 cm internal diameter × 10 cm; Amersham Pharmacia Biotech, Tokyo, Japan) equilibrated with 20 mM sodium acetate, pH 5.8. After the column was washed with 1 column volume of the same buffer, m-TG was eluted with a linear gradient of sodium chloride from 0 to 400 mM over ten column volumes at a flow-rate of 20 mL/min. Fractions, which did not have impurities, assayed by SDS-PAGE analysis, were collected and diluted 10 times with 20 mM sodium acetate, pH 5.5. Half of the fractions were applied to a cation-exchange column (SOURCE 30S, 2.6 cm internal diameter × 10 cm; Amersham Pharmacia Biotech) equilibrated with 20 mM sodium acetate, pH 5.5. After the column was washed with 1 column volume of the same buffer, m-TG was eluted with a linear gradient of sodium chloride from 0 to 500 mM over 20 column volumes at a flow-rate of 5 mL/min. Fractions, which did not have impurities, assayed by analytical reverse-phase HPLC, and low isolectric point m-TG-variants, assayed by analytical cation-exchange chromatography, were pooled. For buffer-exchange, pooled fractions was applied to a gel filtration column (Sephadex G25M) equilibrated with 20 mM sodium phosphate, pH 6.0. The final preparation was disolved in 20 mM NH_4Cl at a stock protein concentration of 5 mg/mL and frozen in aliquots.

2.2 Sequence analysis

The amino acid sequence of m-TG was previously determined by mass spectrometry and standard Edman degradation techniques [6]. Initially, a sequence comparison test was performed in 1996 using the GENETYX-MAC software on an Apple computer. The amino acid sequence data for allergenic food and nonfood proteins were drawn from the public domain databases GenBank (Release 95.0, June 1996), EMBL (Release 47.0, June 1996), PIR (Release 48.0, March 1996), and Swiss-Prot (Release 33.0, February 1996) and compared to m-TG. A second sequence comparison test was carried out in 2003 using the FASTA program. Amino acid sequence data from 1184 allergenic food and nonfood proteins were taken from the databases Swiss-Prot (2002.9.2 version), TrEMBL (2002.8.24), and PIR (2002.9.2), and compared to the sequence of m-TG.

2.3 Pepsin and trypsin resistance

The pepsin digestion was performed according to Astwood *et al*. [9]. 100 mg m-TG was added to 1.0 mL HCl, 0.03 M NaCl (pH 2.5), and preincubated for 3 min at 37°C. 2 µL 0.05% pepsin (Sigma, St. Louis, MO, USA) was added. The enzymatic reaction took place for 2 h at 37°C, and was stopped at time intervals with 40 µL 160 mM $NaCO_3$. Trypsin (Boehringer Mannheim, Mannheim, Germany) digestion was performed in 20 mM Tris-HCl (pH 7.0), and the reaction stopped by storage at −80°C. A portion of each stopped reaction mixture was later subjected to HPLC and SDS-PAGE. Reverse-phase chromatography was performed on a Vydac C_4 column (4.6 × 250 mm). 40 µL of each assay mixture was loaded onto the column followed by washing with 0.1% trifluoroacetic acid, and m-TG and its protein digestive fragments were eluted using a linear gradient of acetonitrile containing 0.1% trifluoroacetic acid (10–90% in 30 min at 1 mL/min) monitored by absorbance at 215 nm. SDS-PAGE was performed according to Laemmli [10], using a 5–20% gradient gel that was later stained with Coomassie Brilliant Blue.

2.4 Serum samples

Sera from 25 patients with clinically documented allergy to codfish were obtained from the Allergy Center of Odense University Hospital, Denmark, and the Allergy Clinic of the National University Hospital of Copenhagen, Denmark,

and used for the targeted serum screening. The patients were diagnosed according to the guidelines published by the European Academy of Allergy and Clinical Immunology [11, 12]. All had a positive skin prick test to cod, and were positive in double-blind placebo-controlled food-challenges [13]. However, patients with a history of anaphylaxis or a very severe reaction to ingestion of minute amounts of codfish were not challenged for ethical reasons.

2.5 Codfish extracts and antibodies

Raw codfish meat was extracted in distilled water (1 g/mL) using an automated extractor (Stomacher 80, high speed for 2 min) and centrifuged for 10 min at $2000 \times g$. The extract was kept at -20 °C until use. The protein content was determined to be 15.8 mg/mL by the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA, USA) using bovine serum albumin (BSA) as a standard. A monoclonal antibody against m-TG was produced by Ajinomoto Co. in male balb/c mice and affinity-purified [14]. Monoclonal mouse anti human T cell, T3 (Dako, Glostrup, Denmark) (conc. 210 mg/L) was used as a negative control.

2.6 RAST and RAST inhibition

The targeted serum experiments were all carried out using individual setups of the radioimmunoassay (RIA) and the related method radioallergosorbent test (RAST), which has been previously described by Poulsen *et al*. [15, 16]. Briefly, Maxisorp tubes (Nunc, Roskilde, Denmark) were coated with 250 µL 2 µg/mL antigen (m-TG or codfish) diluted in phosphate-buffered saline (PBS), and incubated overnight (o/n) at 37°C for cod and 4°C for m-TG. Lower incubation temperatures along with the addition of 20 mM NH_4Cl were used in all steps containing m-TG to inhibit the enzymatic activity of the protein. The tubes were washed three times between all incubation steps with washing solution (PBS with 0.1% v/v Tween). The coated tubes were blocked with 500 µL cell culture medium (RPMI 1640) including 10% v/v fetal calf serum (FCS). 250 µL sample serum diluted in washing solution to an appropriate concentration was added and the tubes incubated for 3 h at room temperature on a vibrator. 250 µL anti-human IgE-I^{125} diluted 12 times in washing solution was added to the washed tubes and incubation took place overnight at room temperature (4°C for m-TG) on a vibrator. After washing, the γ-radiation was measured for 3 min on a 1471 Wizard counter (Wallac, Allerød, Denmark). All experiments were performed in duplicate. Inhibition experiments were performed in similar fashion to the direct RAST described above except that the serum samples were preincubated with antigen (1+1) o/n at 4°C before the inhibited sample was added to the maxisorp tubes [17]. A RIA was set up to measure total human IgE [18] with all experimental details as described above, except tubes were coated with 100 µL 2.8 µg/mL rabbit anti-human IgE (Dako) and the samples were dilutions of normal human serum (NHS) with IgE concentrations from 6000 to 23 pg/mL in RPMI w. 10% FCS. The tubes were finally incubated with 100 µL anti-human IgE-I^{125} diluted 1 : 6 in RPMI containing 10% FCS. A second RIA was established to confirm the binding of m-TG to the maxisorp surface. Tubes were coated with 100 µL of a number of concentrations of m-TG and the enzyme detected with 100 µL of a monoclonal antibody (mAb) against m-TG in various dilutions in RPMI w. 10% FCS. 100 µL of rabbit anti-mouse (Dako) labeled with I^{125} diluted to a total count of 100 000 cpm in RPMI w. 10% FCS was added to each tube and left to incubate as previously described.

3 Results

3.1 Comparison with known allergens

Two independent sequence alignments in protein databases were made in 1996 and 2003 to compare the amino acid sequence of m-TG to all known allergens as recommended by the 2001 FAO/WHO Decision Tree (Table 1). Both database searches showed that the enzyme has no homology with any known allergens down to a match of six contiguous amino acids, which meets the requirements of the decision tree. However, the database search in 1996 showed a match at the five contiguous amino acid level (sequence: SFDED) between m-TG and the major cod-allergen Gad c1 (parvalbumin). A targeted serum screen of m-TG using sera from 25 documented cod-allergic patients was performed in order to rule out the possibility of cross-reactivity. Surprisingly, when the sequence alignment was repeated in 2003, the match between m-TG and Gad c1 was not found. However, there was a match between m-TG and three new allergens at the five contiguous amino acid level. One is a pollen allergen from Japanese cedar tree (accession No. P18632), another is from the paper wasp, an American species (accession No. Q9U6V9), and the third is a pollen allergen from Japanese cypress (accession No. Q96385). None of the three are food allergens.

3.2 Targeted serum screening

3.2.1 m-TG does not interfere with IgE immunoreactivity

Due to the fact that transglutaminase has the ability to cross-bind proteins, a preliminary experiment was performed to test if m-TG would interfere with the immunoreactivity of human IgE during incubation in the RIA system.

Table 1. Sequence comparison tests in 1996 and 2003

Time of search	Number of residues	Food allergens	Accession No.	Nonfood allergens	Accession No.
1996	6	None	–	None	–
	5	Codfish (β-parvalbumin)	A94236	None	–
2003	6	None	–	None	–
	5	None	–	Major Cry j1 I precursor	P18632
				Hyalurono-glucosaminidase pre	Q9U6V9
				Major pollen allergen Cha o 1 pre	Q96385

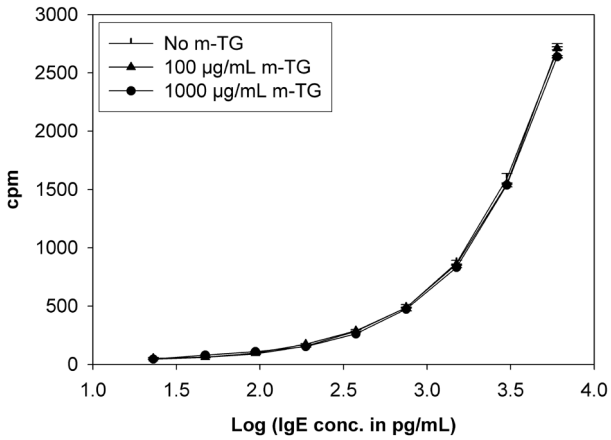

Figure 1. RIA for total IgE. Standard dilutions of a NHS pool with IgE concentrations 2-fold from 6000 to 23 pg/mL mixed with two different concentrations of m-TG or no m-TG.

Three identical dilution series of NHS with a known content of IgE were pre-adsorbed over night at 4°C with 1000, 100 or 0 µg/mL soluble m-TG, respectively. The three curves of total IgE were identical indicating that the binding capacity of IgE was not altered in our system even in the presence of high amounts of m-TG (Fig. 1).

3.2.2 m-TG binds to the maxisorp surface

The binding of m-TG to the solid-phase maxisorp was confirmed by use of a mAb. The antibody specific for m-TG was compared to a mAb not reacting with the enzyme (irrelevant ab) and buffer as negative controls. Three coating concentrations of m-TG were compared to two dilutions of the mAbs. This experiment demonstrated that m-TG binds efficiently to a maxisorp surface, and the background signal was low when the mAb was sufficiently diluted (Fig. 2a). Furthermore, it was tested whether soluble m-TG or codfish extract was able to inhibit the binding between coated m-TG and the mAb. Results show that the binding was strongly inhibited by soluble m-TG at levels higher than 0.1 µg/mL, whereas no cross-reactivity between the mAb and up to 100 µg/mL of codfish protein was observed (Fig. 2b).

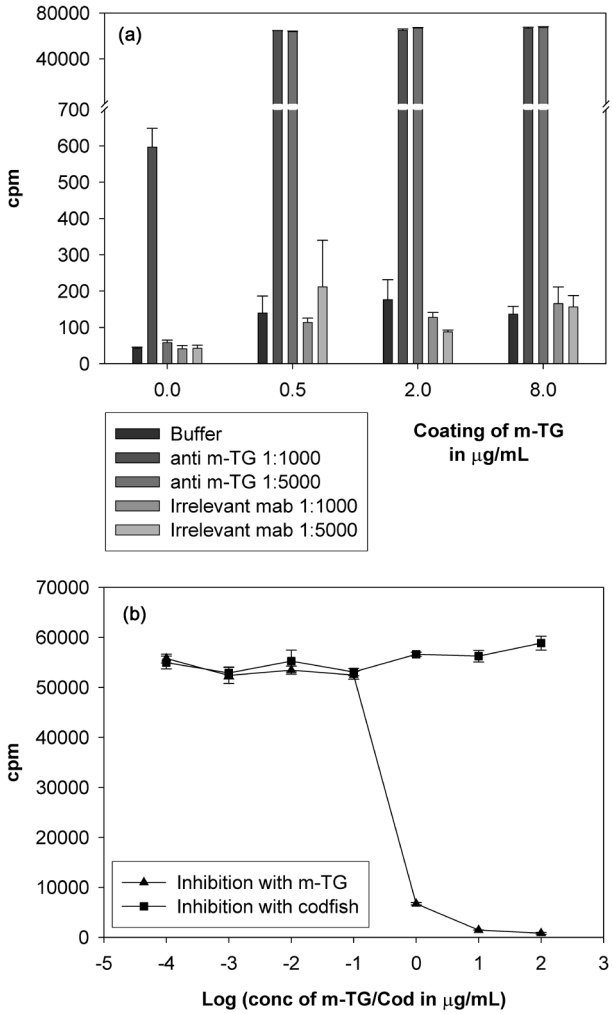

Figure 2. (a) Confirmation of m-TG binding to maxisorp by a monoclonal ab against m-TG in a RIA setup. Three coating concentrations of m-TG were tested against two dilutions of the mAb. The negative controls were buffer and an irrelevant mAb, mouse anti human T-cell receptor. (b) Binding between m-TG and the mAb inhibited with m-TG as a positive control and codfish extract as a negative control.

3.2.3 Testing of targeted sera against m-TG

The 25 codfish allergic sera were initially tested in the maxisorp RAST against a codfish extract to confirm their con-

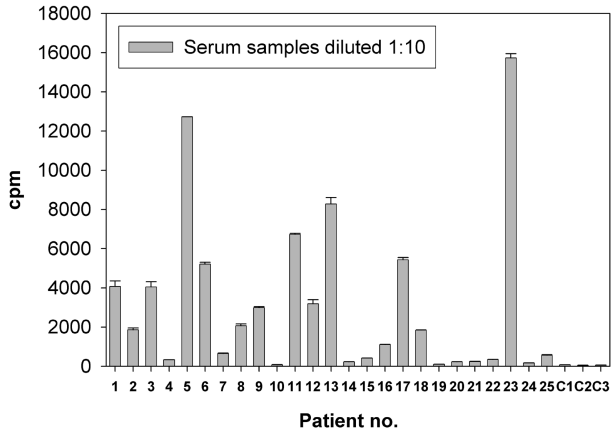

Figure 3. Direct codfish RAST on 25 codfish allergic patients. Three negative controls were included, C1 was a pool of normal human serum and C2 and C3 were sera from individual patients allergic to allergens other than codfish.

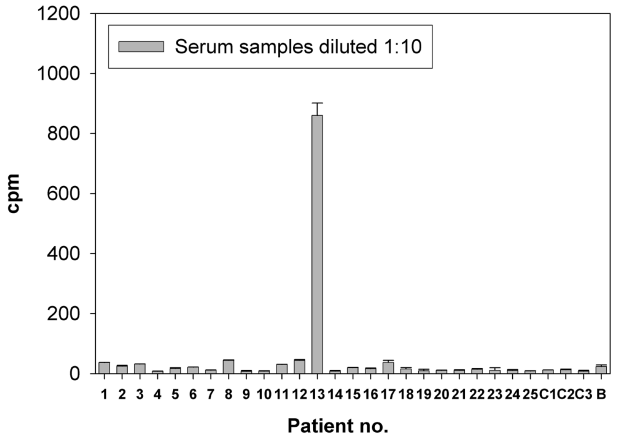

Figure 4. Direct m-TG RAST on 25 codfish allergic patients and three negative controls (same as in Fig, 3).

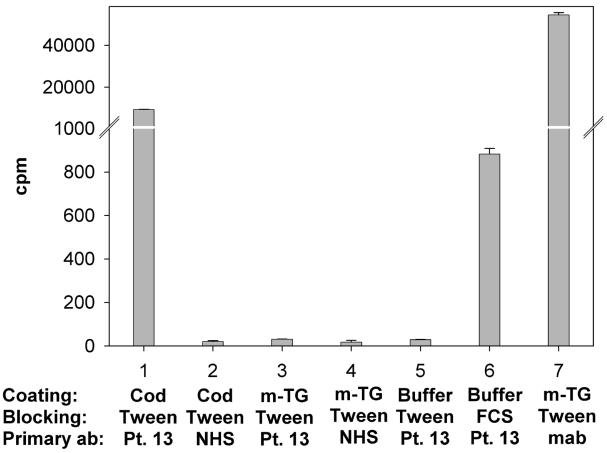

Figure 5. Different setups of the RIA/RAST system to establish if serum IgE from patient 13 binds directly to m-TG or to the FCS in the blocking solution.

tent of cod-specific IgE in our system. NHS and two sera negative for codfish in the Pharmacia cap test were included as negative controls, and all sera were tested in four dilutions (1 : 10, 1 : 30, 1 : 100, and 1 : 300). 24 of 25 sera tested positive against codfish in our RAST system (Fig. 3), and they all demonstrated a dose-response relationship upon dilution (not shown).

An experiment was made to test for cross-reactivity between m-TG and codfish by attempting to inhibit the binding between patient IgE and codfish with three dilutions of m-TG (100, 10, and 1 μg/mL). Serum from patients 1 and 17 was not included due to lack of sufficient quantities of serum, and serum from patient 10 was also left out since it was negative in the direct cod RAST. No inhibition was seen in any of the 22 tested sera demonstrating no cross-reactivity (results not shown). The binding between patient IgE and cod was easely inhibited by preincubating the serum samples with codfish extract as demonstrated for four of the highest affinity patient sera (patient 5, 11, 13, and 23) (results not shown).

The 25 cod sera and the three control sera were subsequently tested in a direct RAST against coated m-TG. Unexpectedly, one of the 25 sera reacted positively to m-TG although the signal was ten times lower than for codfish (Fig. 4). Further investigations revealed a previous positive Pharmacia cap test to cow meat along with codfish and other food allergens. Since all tubes in the experiment had been incubated with a blocking solution containing 10% v/v FCS, a RAST experiment was made to determine if the patients' IgE was reacting with m-TG or to FCS (Fig. 5). The serum reacted negatively to the m-TG-coated tubes when the blocking solution was replaced with washing solution (PBS with 0.1% Tween 20), but bound to tubes coated with FCS alone. It was concluded, based on these results, that the serum from patient 13 does not bind to m-TG, but to FCS.

3.3 Digestibility of m-TG

The stability of m-TG against well-known digestive enzymes from the stomach and intestine, namely pepsin and trypsin, was examined. Pepsin completely cleaved m-TG into small fragments within 1 min at pH 2.5 (Fig. 6). Trypsin failed to cleave all of the m-TG as a band was observed at the undigested position even after 48 h. Analysis by HPLC confirmed these results (not shown). Furthermore, m-TG was analyzed for pH-stability alone, and was found to loose all enzymatic activity within 1 min at pH 2.5 (not shown).

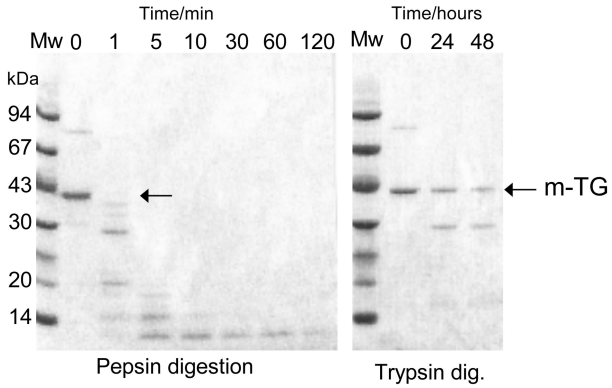

Figure 6. Digestability studies of m-TG using pepsin or trypsin enzymes. The enzyme reactions were stopped at set times and a portion of the reaction mixtures were assayed by SDS-PAGE.

4 Discussion

Novel proteins that are introduced into foods can cause problems with regard to allergenicity in two different ways. They can elicit an allergic response in people that are already sentitized to the same protein or a structurally-related protein, or they can induce an IgE response in susceptible individuals. No predictive methods currently exist that can completely assure that a protein lacks these abilities, but various strategies have been proposed. The FAO/WHO 2001 Decision Tree, proposed by expert consultation in Rome, uses a weight of evidence approach to assess the likelihood that a novel protein will prove allergenic.

Bacterial proteins have not yet been known to induce reactions or to sensitize. However, we decided to put m-TG through a risk evaluation using the decision tree from FAO/WHO. The first step was a database search to compare the sequence of m-TG to all known allergens which was performed in 1996 and repeated in 2003. There was no match down to six contiguous amino acids in either search, which meets the requirements of the decision tree, and strongly indicates that m-TG is not likely to elicit reactions in already sensitized individuals as a result of IgE cross-reactivity. However, the database search in 1996 found a match at the five contiguous amino acids level between m-TG and the major codfish allergen Gad c1 (sequence: SFDED). This led to a precautionary serum screen of m-TG using sera from 25 documented cod-allergic patients. No cross-reactivity between m-TG and an extract of codfish was found. Surprisingly and inexplicably, when the sequence alignment was repeated later in 2003, the match between m-TG and Gad c1 (A94236) was not reported by the FASTA algorithm in spite of the fact that Gad c1 (but not Gad m1 (AAK63087), in which the corresponding position contains the sequence SFDHK) is reported to contain the above sequence. However, a match was identified between m-TG and three new allergens at the five contiguous amino acid level. The difference in search results may be due to errors or changes to the allergen databases or to the search algorithms used by the FASTA program. Unfortunately, allergen databases may contain a certain number of errors, and a better validated allergen database is much needed [19]. This point is discussed in an accompanying paper in this volume (Poulsen: Allergy assessment of genetically modified foods). This study has already shown that a match of 5 amino acids between two proteins does not give rise to any IgE cross-reactivity. With this in mind, we were less concerned about the three new non food allergens found in 2003.

Calculations demonstrate that an individual could consume up to 8 – 13 mg m-TG (90% percentiles) per day depending on the age of the consumer. The lowest dosage of protein known to have caused an allergenic reaction in a sensitized individual in one single challenge session is 0.1 mg [20]. Since the consumption data is based on intake of several m-TG containing food products over a period of 24 h, it is likely that the maximum ingested amount will be close to the lowest reported dose that has induced food allergic reactions in the most sensitive patients. Unfortunately, there are still only limited data in the literature on the lowest adverse effect levels, so the above figures should be considered very cautiously.

Though no methods exist that can predict the ability of a protein to sensitize, digestability studies and animal models can provide useful information to form a judgment on the aspect. Animal models were not used in this study because available models are not yet sufficiently evaluated and validated. m-TG was completely broken down into small fragments within 1 min standard pepsin treatment under stomach-like conditions, suggesting that it is unlikely to sensitize susceptible individuals.

Trypsin treatment was also employed in this study, although not required by the decision tree, and the results showed that trypsin was not able to breakdown m-TG. Perhaps the acidic conditions during pepsin treatment denature the protein, thus making it more susceptible to proteolytic attack by pepsin. Another possible explanation is that no trypsin cleavage sites are available on the surface of m-TG.

In conclusion, microbial transglutaminase meets the requirements of the 2001 FAO/WHO Decision Tree and, with regard to the allergenic potential, no safety concerns have been identified.

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