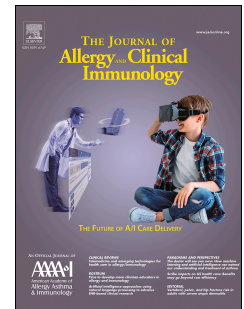


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Gal d 7 - a major allergen in primary chicken meat allergy

Christoph Klug, MSc, Wolfgang Hemmer, PhD, Patricia Román-Carrasco, MSc, Margit Focke, PhD, Santiago Quirce, MD, PhD, Teresa Boyano-Martínez, MD, Erwin Gaubitzer, PhD, Herbert Wank, PhD, Ines Swoboda, PhD



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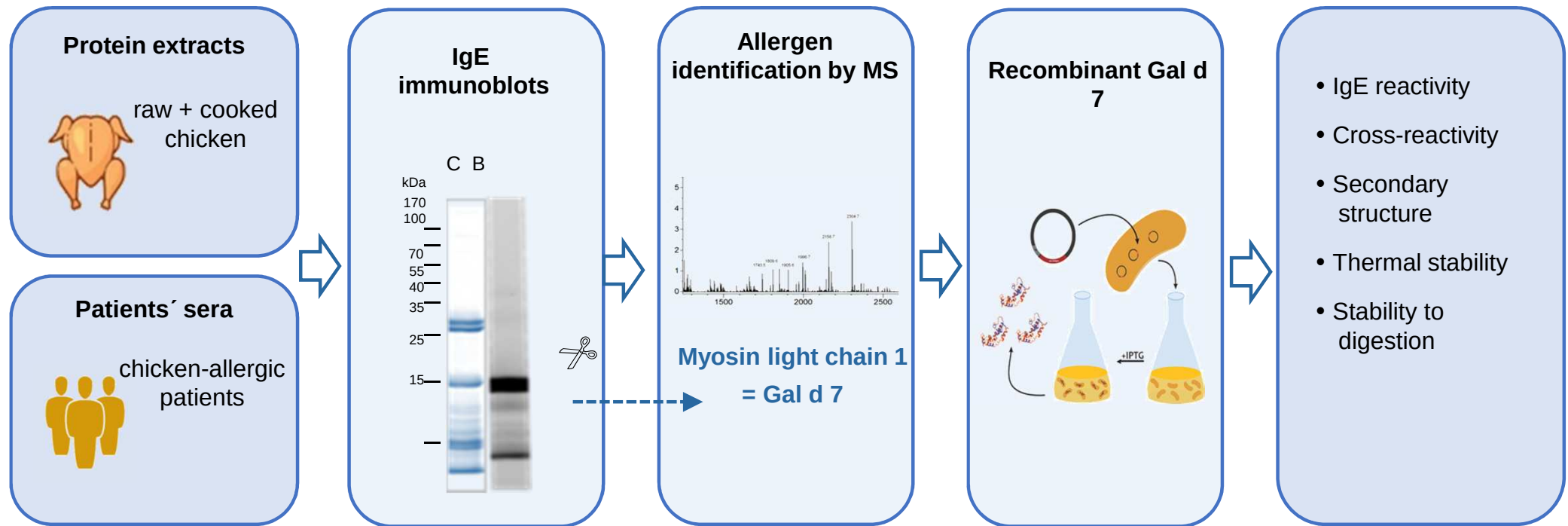
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Gal d 7 - a major allergen in primary chicken meat allergy



C: Coomassie-stained gel
 B: IgE immunoblot
 MS: Mass spectrometry

Gal d 7 - a major allergen in primary chicken meat allergy

Christoph Klug, MSc^{a,*}, Wolfgang Hemmer, PhD^b, Patricia Román-Carrasco, MSc^a, Margit Focke, PhD^c, Santiago Quirce MD, PhD^d, Teresa Boyano-Martínez, MD^d, Erwin Gaubitzer, PhD^e, Herbert Wank, PhD^a, Ines Swoboda, PhD^a

^aBiotechnology Section, FH Campus Wien, University of Applied Sciences, Campus Vienna Biocenter, Vienna, Austria; ^bFAZ - Floridsdorf Allergy Center, Vienna, Austria; ^c Division of Immunopathology, Department of Pathophysiology and Allergy Research, Center for Pathophysiology, Infectiology and Immunology, Medical University of Vienna, Vienna, Austria; ^dDepartment of Allergy, Hospital La Paz Institute for Health Research (IdiPAZ), Madrid, Spain; ^eMax F. Perutz Laboratories, Department of Structural Biology and Biomolecular Chemistry, University of Vienna, Vienna, Austria.

*Christoph Klug, MSc, is currently affiliated with MacroArray Diagnostics GmbH, Vienna, Austria.

Corresponding author:

Ines Swoboda, PhD, Biotechnology Section, FH Campus Wien, University of Applied Sciences, Campus Vienna Biocenter, Helmut-Qualtinger-Gasse 2, A-1030 Vienna, Austria. Phone: +43-1-6066877-3515, Fax: +43-1-6066877-3509, E-mail: ines.swoboda@fh-campuswien.ac.at.

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Abstract:

Background: Poultry meat can induce severe allergic reactions. So far, the molecules causing poultry meat allergy are largely unknown.

Objective: To identify and characterize poultry meat allergens.

Methods: Patients' IgE reactivity profiles to chicken muscle were analyzed in immunoblots and proteins recognized by the majority of patients were subjected to peptide mass fingerprinting. A 25 kDa IgE-reactive protein was identified as myosin light chain 1, designated Gal d 7. Recombinant Gal d 7 was produced in *Escherichia coli*. The protein's IgE reactivity was analyzed in ELISA experiments and cross-reactivity with allergens of other poultry species was assessed in inhibition immunoblots. The fold and thermal stability were evaluated by circular dichroism analysis and the enzymatic stability was investigated using *in vitro* gastrointestinal digestion assays.

Results: rGal d 7 represents a properly folded, predominantly α -helical protein and displays IgE binding activity comparable to its natural counterpart. IgE reactivity analysis in 28 chicken meat allergic patients revealed that Gal d 7 is a major allergen for patients primarily sensitized to chicken meat. Furthermore, Gal d 7 cross-reactive allergens were also detected in other poultry species, suggesting that rGal d 7 can be used as a diagnostic marker allergen for poultry meat allergy. The high thermal stability, refolding capacity and resistance to gastrointestinal enzymes might explain why Gal d 7 can act as a potent sensitizing agent.

Conclusion: Gal d 7 represents a novel major chicken meat allergen. Recombinant Gal d 7 could be used for diagnosis of genuine poultry meat sensitization.

Clinical Implications: Myosin light chain 1, designated Gal d 7, was identified as a major chicken meat allergen. Recombinant Gal d 7 can be used for diagnosis of patients primarily sensitized to poultry meat.

Capsule Summary: Myosin light chain 1, designated Gal d 7, represents a major chicken meat allergen for patients primarily sensitized to poultry meat and recombinant Gal d 7 can be used for diagnosis of genuine poultry meat sensitization.

Key words: poultry meat allergy, major chicken meat allergen, recombinant allergen, Gal d 7, genuine sensitization

55 **Abbreviations:**

56	BSA:	Bovine serum albumin
57	CBB:	Coomassie Brilliant Blue
58	IMAC:	Immobilized metal ion affinity chromatography
59	LC-ESI MS/MS:	Liquid chromatography ion trap mass spectrometry
60	MALDI-ToF:	Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry
61	PBS:	Phosphate buffered saline
62	PBST:	PBS containing 0.5% Tween 20
63	RT-PCR:	Reverse transcription PCR
64	SDS-PAGE:	Sodium dodecylsulfate polyacrylamide gel electrophoresis
65	SGF:	Simulated gastric fluid electrolyte stock solution
66	SIF:	Simulated intestinal fluid electrolyte stock solution
67	SSF:	Simulated salivary fluid electrolyte stock solution

INTRODUCTION

The worldwide consumption of poultry meat has increased almost three-fold in the last 50 years and poultry is one of the fastest growing agricultural sub-sectors, with the United States as the largest poultry meat producer. Extensive changes in modern farming techniques contributed significantly to the fast growth of the poultry farming industry¹. Poultry meat is recommended as an essential part of a healthy diet due to the relatively low amount of fat and the presence of high-quality proteins, of vitamins and of important micronutrients². Furthermore, in contrast to processed and red meat, poultry meat consumption is not associated with the risk of colorectal cancer³. However, meat of chicken and other poultry can induce severe IgE-mediated allergic reactions. Overall, poultry meat allergy is regarded as a rare disease. However, owing to the fact that publications on poultry meat allergy mainly refer to reports of single cases or small case series, reliable data on the prevalence of clinical poultry meat allergy are not available. Depending on the investigated patient population and the methods used for diagnosis, it is estimated that about 0.6 to 5% of food allergic subjects are affected by this disease^{4,5}.

Based on the route of sensitization and on the involved allergens two different types of chicken meat allergy can be distinguished: 1) primary chicken meat allergy, where sensitization to avian meat allergens occurs via the intestinal mucosa, and 2) secondary chicken meat allergy, where either respiratory sensitization to airborne allergens present in bird feathers and feces (“bird-egg syndrome”) or primary sensitization to egg yolk (“egg-bird syndrome”) causes allergic reactions to poultry meat due to the presence of cross-reactive allergens^{6,7}. Chicken serum albumin (Gal d 5) has been well characterized as the major, highly cross-reactive allergen involved in secondary chicken meat allergy⁸. This form of chicken meat allergy usually causes oral and gastrointestinal symptoms as well as mostly mild systemic reactions.

Much less is known about allergens involved in primary chicken meat allergy. Early studies with limited numbers of patients reported IgE binding to multiple unidentified proteins of molecular weights ranging from 5 kDa to 50 kDa^{9,10} with cross-reactivity between chicken and turkey. Later case studies suggested α - and β -parvalbumins, hemoglobin, serum albumin and myosin light chain as

allergens involved in primary chicken meat allergy¹¹⁻¹³. Recently, Kuehn et al identified in patients with concomitant allergy to both fish and chicken meat parvalbumins, enolases and aldolases as cross-reactive allergens¹⁴.

Symptoms associated with primary poultry allergy include often severe forms of the oral allergy syndrome and usually moderate systemic reactions involving the skin and the gastrointestinal tract. Interestingly and comparable to observations of fish allergic individuals, symptoms are not only triggered after ingestion of poultry meat, but also after inhalation of vapours generated during the cooking of poultry meat and after skin contact with the meat^{7,15}. Often symptoms are also induced after consumption of other avian meat, such as turkey, goose, duck or pheasant^{10,16,17}. Furthermore, also soups and processed poultry meat (e.g., sausages or ham prepared from turkey meat) as well as “hidden” poultry meat proteins in products, where pork meat is replaced by poultry meat, can trigger allergic reactions^{10,13,18}.

To get a better understanding of the allergens involved in primary sensitization to poultry meat, we analyzed IgE reactivity profiles to raw and cooked chicken muscle extracts in a large population of well-characterized meat allergic patients. Using a proteomics-based approach, we identified an IgE reactive protein of 25 kDa as the muscle protein myosin essential light chain 1 (designated Gal d 7 by the International Union of Immunological Societies allergen nomenclature subcommittee). Myosin light chains are members of the calmodulin gene family and together with myosin heavy chains they form the myosin protein complex¹⁹. IgE-reactive recombinant Gal d 7, with immunological features comparable to its natural counterpart, was produced in *Escherichia coli* and the molecule’s structures, thermal and enzymatic stability as well as its cross-reactive potential were analyzed in this study.

METHODS

Patients' sera

Sera were obtained from poultry meat allergic patients from Austria (n=16) and Spain (n=17), who were recruited from the Floridsdorf Allergy Center in Vienna and from the Department of Allergy at the La Paz University Clinic Hospital in Madrid. The demographic, clinical and serological characteristics of the patients are given in Table I. All patients reported clinical symptoms after the ingestion of poultry meat and had elevated levels of chicken meat specific IgE as measured by ImmunoCAP (Thermo Fisher, Uppsala, Sweden). Patients were classified as primary or secondary chicken meat allergic based on *in vitro* measurements of IgE reactivity to chicken meat, egg white, egg yolk and budgerigar feathers and to the major egg white (Gal d 1, Gal d 2, Gal d 3) and egg yolk (Gal d 5) allergens. All individuals with elevated IgE levels to Gal d 5 (chicken serum albumin) were classified as secondary chicken meat allergic patients (patients 1-11). In contrast, individuals lacking IgE reactivity to Gal d 5 were classified as primary chicken meat allergic patients (patients 12-33). Further details regarding the characteristics of the patients can be found in the Methods section of this article's Online Repository. All experiments were approved by the Ethics Committees of the City of Vienna (Austria) (EK-12-126-0712), and of the La Paz University Hospital (Madrid, Spain) (EK565/2007), and informed consent of all participating individuals was obtained.

Generation of anti-Gal d 7 antiserum

A polyclonal rabbit antiserum was generated in a New Zealand White rabbit by injecting 200 µg of purified rGal d 7 four times within a time frame of 70 days, using complete Freund's adjuvant for the initial injection and incomplete Freund's adjuvant for the boosts (Charles River Laboratories, Kissleg, Germany). Preimmune serum, obtained before immunization of the rabbit with rGal d 7, did not contain any rGal d 7-specific IgG antibodies and was used as a negative control serum in the ELISA competition experiments.

Preparation of protein extracts

The preparation of protein extracts from raw, cooked and grilled chicken, turkey, goose and duck muscle tissues is described in the Methods section of this article's Online Repository.

Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE), immunoblot and inhibition immunoblot experiments

For analysis of patients' IgE reactivity to protein extracts or to recombinant Gal d 7, immunoblots were carried out with sera from chicken meat allergic patients. For specific detection of Gal d 7 immunoblots were performed with the rGal d 7 specific rabbit antiserum. IgE inhibition immunoblots were performed to investigate whether rGal d 7 displays IgE reactivity comparable to its natural counterpart and to analyze whether rGal d 7 displays cross-reactivity to myosin light chains from other poultry species. The methodological details of SDS-PAGEs, immunoblots and inhibition immunoblots are given in the Methods section of this article's Online Repository.

Liquid chromatography ion trap mass spectrometry (Nano LC-ESI MS/MS) and Matrix-assisted laser desorption/ionization time-of-flight (MALDI-ToF) mass spectrometry

For identification of meat allergens LC-ESI MS/MS analyses were performed as described by Hemmer et al²⁰. The molecular weight of the recombinant protein was determined by Matrix-assisted laser desorption/ionization time-of-flight (MALDI-ToF) mass spectrometry. A detailed description of the LC-ESI MS/MS and MALDI-ToF analyses can be found in the Methods section of this article's Online Repository.

cDNA cloning and expression of recombinant chicken myosin light chain 1 (rGal d 7)

For cDNA synthesis of chicken myosin light chain, RNA was extracted from chicken breast muscle tissue and was used as a template for reverse transcription PCR (RT-PCR). The obtained cDNA coding for chicken myosin light chain, designated Gal d 7 by the International Union of Immunological Societies allergen nomenclature subcommittee, was subcloned with a sequence coding for a 6x Histidine tag (at the 3'-end) into the bacterial expression vector pET-17b (for details of cDNA cloning see the Methods section in this article's Online Repository). For expression of rGal d 7, the Gal d 7 construct was transformed into the *Escherichia coli* strain BL-21 (DE3). Upon induction of protein synthesis in liquid cultures at an OD₆₀₀ of 0.5 with isopropyl- β -D-thiogalactoside (0.5 mM), rGal d 7 was highly expressed as soluble, His6-tagged protein. Purification was carried out by native immobilized metal ion affinity chromatography (IMAC) using nickel agarose beads (Qiagen, Hilden, Germany). Purity and the molecular weight of the protein were determined by Coomassie brilliant blue staining of a 15% SDS polyacrylamide gel and by MALDI-ToF analysis. The protein concentration was determined with a Micro BCA kit (Pierce, Rockford, IL) using BSA as a standard.

Enzyme linked immuno sorbent assay (ELISA) and competition ELISA experiments

ELISAs were performed to investigate the IgE reactivity of rGal d 7. In ELISA competition experiments, the capacity of the anti-rGal d 7 antiserum to inhibit patients' IgE binding to rGal d 7 was analyzed. The methodological details of the ELISA and the ELISA competition assay can be found in the Methods section of this article's Online Repository.

Circular Dichroism (CD) analysis

CD spectra of purified rGal d 7 were recorded on an Applied Photophysics Pi Star-180 Spectrophotometer (Applied Photophysics Ltd, Surrey UK) in PBS at a protein concentration of 0.1 mg/mL using a 1-mm path-length quartz cuvette equilibrated at 25°C. Spectra were recorded with 0.2-nm resolution at a scan speed of 50 nm/min and the final spectra were corrected by subtracting the

corresponding baseline spectrum obtained under identical conditions. Results are expressed as ellipticity (mdeg) versus wavelength. Thermal denaturation and refolding experiments were performed by gradually increasing the temperature from 25°C to 95°C with a heat rate of 1°C/min and cooling back to 25°C.

Heat treatment of rGal d 7

For analysis of the heat stability of rGal d 7, the protein was heated in a thermoblock at a concentration of 0.1 mg/ml for 5 min at either 25°C, 65°C or 95°C and for 20 min at 95°C. After centrifugation (5 min, 13000 rpm) the supernatants were analyzed by SDS-PAGE and in an IgE immunoblot with a pool of sera from four chicken meat allergic individuals (patients 20, 23, 24, 26; each serum was diluted 1:40).

Simulated gastric and intestinal digestion of chicken meat

In order to evaluate the stability of Gal d 7 to gastrointestinal digestion, cooked chicken meat was subjected to stimulated *in vitro* digestion following the harmonized INFOGEST method published by Minekus et al.²¹ with minor modifications. A description of the methodological details can be found in the Methods section of this article's Online Repository.

RESULTS

Patients' IgE reactivity profiles to blotted raw and cooked chicken meat extract

Aqueous protein extracts of raw and cooked chicken breast were separated by 12% SDS-PAGE. A comparison of panels A (raw meat) and B (cooked meat) in the Coomassie stained gels (Fig 1, lanes CBB) showed that cooking reduces the number of soluble proteins in the higher molecular weight ranges of the extract (above 40 kDa). When probing nitrocellulose blotted extracts with sera from the chicken meat allergic individuals, two different IgE reactivity profiles could be distinguished (group 1 and group 2 in Fig 1) corresponding to the classification of patients into primary and secondary meat allergic individuals (Table I). In the raw extract, group 1 patients predominantly recognized only one protein of ~70 kDa protein (Fig 1, A), which was no longer recognized in the cooked extract (Fig 1, B). A more complex binding pattern was observed in patients of group 2, which recognized multiple IgE reactive bands between 13-30 kDa as well as proteins in the higher molecular range in the raw extract (Fig 1, A). In the cooked chicken extract, most of these individuals exhibited strong IgE binding to a ~23 kDa protein, which appeared as a double band in some cases, and, to a lesser extent, to a ~13 kDa protein (Fig 1, B). Control immunoblots performed with buffer only (PBS) or with the serum of a non-allergic individual (NA) revealed some unspecific binding of the secondary antibody to proteins of ~65 kDa and ~37 kDa in the raw extract, but not to proteins in the cooked extract.

Identification of myosin light chain as an IgE reactive protein by mass spectrometry analysis

For identification of the IgE reactive proteins, the 70 kDa protein from the raw extract as well as multiple low molecular weight protein bands, most notably the 23 kDa and 13 kDa proteins in the cooked extract, were excised from the Coomassie stained gel and subjected to trypsin digestion, HPLC separation and LC-MS/MS analysis. As expected, the 70 kDa protein, predominantly recognized by group 1 patients, was identified as chicken serum albumin. Our experiments confirmed the thermolabile nature of this allergen, since no IgE reactive bands corresponding to serum albumin were recognized by patients' IgE in the cooked meat extract (Fig 1, B). Peptides of both the 23 kDa

and 13 kDa proteins were identified as originating from the essential light chains of myosin, MLC 1f (UniProt accession number P02604) and MLC 3f (UniProt accession number P02605).

These light chains are part of the myosin protein complex and represent two variants of the essential chain of myosin, which originate from one gene as a result of alternative transcription and splicing^{22,23}. As can be seen in the amino acid sequence comparison in Fig E1 in this article's Online Repository, the long form (MLC 1f), has a proline-alanine rich N-terminus of 42 amino acids, which is not present in the short form (MLC 3f). However, in the 150 amino acids long overlapping region the sequences of MLC 1f and MLC 3f are almost identical. Since some patients (e.g., patients 17, 28, 29) exhibited IgE reactivity only to the 23 kDa protein, our efforts focused on the molecular cloning of MLC 1f.

Gal d 7 represents a major allergen for patients primarily sensitized to chicken meat

For generation of recombinant myosin light chain 1, a cDNA coding for MLC 1f was cloned with a C-terminal hexahistidine tag into the expression vector pET-17b. *Escherichia coli* expressed recombinant MLC 1f was purified by affinity chromatography to >95% homogeneity, as assessed by SDS-PAGE (see Figure E2 in the Online Repository) and MALDI-ToF analysis (data not shown). The molecular weight determined by MALDI-ToF analysis corresponded to the molecular weight calculated based on the amino acid sequence (21,722 Da), confirming that an authentic recombinant protein had been produced. MLC 1f was designated Gal d 7 and was submitted to the International Union of Immunological Societies allergen nomenclature subcommittee (<http://www.allergen.org/>).

A first analysis of the IgE binding capacity of rGal d 7 in immunoblots with sera from chicken meat allergic patients (patients 24, 28, 30) (Fig 2, A) demonstrated that rGal d 7 represents an IgE reactive protein. In addition to the 23 kDa protein of rGal d 7, another protein of ~50 kDa was recognized by patients' IgEs. Since the 50 kDa protein was also detected by a polyclonal rabbit antiserum generated against rGal d 7 (data not shown), it was concluded it represents the dimeric form of Gal d 7.

To analyze, whether rGal d 7 displayed IgE binding capacity comparable to its natural counterpart, inhibition immunoblots were performed (Fig 2, B). Upon pre-incubation of a serum pool (sera 24-28) with rGal d 7, IgE binding to the 23 kDa protein, representing Gal d 7 (= MLC 1f), but also to the 13 kDa protein, representing MLC 3f was inhibited. This suggests that rGal d 7 contains all the IgE binding epitopes present in both essential myosin light chains. In contrast, pre-incubation of the serum pool with Gal d 5 (CSA) did not affect IgE binding, indicating the specificity of the inhibition.

An ELISA performed with 28 sera (Fig 2, C) showed that 77% of the patients with genuine chicken meat allergy (group 2) were positive to rGal d 7, suggesting that Gal d 7 represents a major chicken meat allergen. Interestingly, rGal d 7 was also recognized by four patients with secondary meat allergy (patients 1, 9, 10 and 11), which might suggest double-sensitization to Gal d 5 and Gal d 7. A comparison of the IgE levels to rGal d 7 measured by ELISA (Fig 2, C) with the IgE titers to chicken meat determined by ImmunoCAP (Table I) showed a lack of correlation between the results: e.g., patients 17, 19 and 24 displayed high IgE titers to rGal d 7, but rather low IgE levels to chicken meat. The reason for this lack of correlation is most likely the fact that the chicken meat ImmunoCAP contains only raw chicken meat, whereas our initial immunoblots results showed that the IgE reactivity of the ~23 kDa protein band of MLC 1f (Gal d 7) was much stronger in cooked chicken meat (Fig 1, B).

Gal d 7 is present in all skeletal chicken muscle tissues and displays cross-reactivity to allergens from different poultry species

Immunoblots with the polyclonal anti-rGal d 7 rabbit antiserum (Fig 3, A) demonstrated that Gal d 7 was also present in chicken legs (lane 2) and wings (lane 3). In addition, myosin light chain proteins MLC 1f (at around 23 kDa) and MLC 3f (at around 13 kDa) were as well detected by the anti-rGal d 7 antiserum in skeletal muscle tissue from turkey (lane 4), goose (lane 5) and duck (lane 6). IgE inhibition immunoblots (Fig 3, B) further showed that pre-incubation of the sera with rGal d 7 abolished or at least reduced IgE binding to the myosin light chain proteins present in other poultry species. This proved the high cross-reactivity between the different myosin light chain proteins and

suggested that rGal d 7 contains most of the IgE binding epitopes occurring in myosin light chains of the investigated poultry species. Comparison of the amino acid sequences of the different poultry myosin light chains revealed sequence identities of more than 95% (data not shown), which is consistent with the observed cross-reactivity.

Immunization of rabbits with rGal d 7 induces IgG antibodies that block patients' IgE binding to rGal d 7

In order to investigate whether the anti-rGal d 7 antiserum can inhibit the binding of human IgEs to rGal d 7, competition ELISA assays were performed (Table II). Pre-incubation of plate bound rGal d 7 with anti-rGal d 7 IgG antibodies prior to incubation with patients' sera caused a significant reduction of IgE binding to rGal d 7. These IgE inhibition rates ranged between 30% and 78% (mean 53%) and correlated with the levels of Gal d 7-specific IgE antibodies present in the patients' sera, since patients with higher anti-rGal d 7 IgE levels (e.g., patients 11, 30 and 31) also displayed a more pronounced blocking effect (78%, 74% and 78%) (Table II).

Heat treatment does not affect the refolding capacity and IgE reactivity of rGal d 7

To investigate the effect of heat treatment on rGal d 7, we first analyzed the molecule's structural stability by circular dichroism (CD) spectrometry (Fig 4, A). The far-UV spectrum of rGal d 7 is characterized by two minima at around 208 and 223 nm and a maximum at 195 nm (strong blue line), which is typical for a folded alpha helical protein. Investigation of the thermal stability by increasing the temperature to 95°C resulted in a gradual denaturation of the protein, where spectra first showed less pronounced minima at 208 and 223 nm and then displayed shifts of the minima to shorter wavelengths (indicated by the transition from blue to red lines). The spectrum recorded at 95°C (strong red line) displayed a minimum at 200 nm, which is characteristic for unfolded proteins. However, upon cooling to 25°C, the protein completely refolded (dashed blue line), indicating a high thermal stability and intrinsic refolding capacity of the protein.

For analysis of the influence of heat treatment on the IgE binding capacity of rGal d 7, the recombinant molecule was heated to 55°C, 65°C and 95°C for 5 to 20 min. An SDS-PAGE and an IgE immunoblot performed with a serum pool (patients 17, 20, 21, 22) indicated that heating neither affected the protein's integrity nor its IgE reactivity (Fig 4, B). Patients' IgE antibodies also still recognized the rGal d 7 dimer (at around 50 kDa), indicating that the molecule's capacity to form dimers was also retained.

We further evaluated the stability of Gal d 7 under conditions typical for food preparation and either boiled or grilled chicken breast, since heat is transferred differently during these two cooking methods. Extracts of raw, boiled and grilled chicken meat prepared under native (n) and denaturing (d) conditions as well as the cooking water (C) were then analyzed for the presence of Gal d 7 in immunoblots (Fig 4, C). The anti-rGal d 7 antibody recognized myosin light chain in the native extracts of boiled and grilled chicken breast, but not in the extract of raw meat (lanes n). This corroborated our previous experiments and proved the high stability of Gal d 7 under conditions reflecting the preparation of a meal. Interestingly, preparation of the extracts under denaturing conditions (lanes d) resulted in solubilization of Gal d 7 and of the 13 kDa protein representing MLC 3f also in the raw meat sample. Gal d 7 was largely absent in cooking water (lane C), indicating no loss of the molecule during the cooking process.

Effect of *in vitro* digestion with simulated salivary (SSF), gastric (SGF) and intestinal (SIF) fluid on the stability and IgE reactivity of Gal d 7

To investigate the gastrointestinal stability of Gal d 7, *in vitro* digestion experiments were performed with simulated salivary (SSF) and gastrointestinal fluids (SGF and SIF) following the standardized static *in vitro* digestion method published by Minekus et al²¹. To mimic the process of digestion as close as possible, cooked chicken breast was minced, suspended in SSF (0) and then exposed for different times to SGF (0 min = G₀, 5 min = G₅, 15 min = G₁₅, 30 min = G₃₀, 60 min = G₆₀) and to SIF (5 min = D₅, 30 min = D₃₀, 60 min = D₆₀). An immunoblot performed with the anti-rGal d 7 antiserum (Fig 5, A) showed that Gal d 7 was neither digested in SSF (0) nor in SGF (G₀ to G₆₀) and remained

339 stable even after 60 min of gastric digestion (G_{60}). An IgE immunoblot carried out with a pool of sera
340 from chicken meat allergic individuals (patients 19, 29, 30, 32) further demonstrated that the protein
341 also retained its IgE binding capacity and was in this way most likely able to sensitize allergic
342 individuals (Fig 5, B). However, after addition of pancreatic enzymes Gal d 7 was apparently
343 degraded already after 5 min of digestion (Fig 5, A, B), since neither the anti-rGal d 7 nor patients'
344 IgE antibodies recognized the molecule in samples that had been exposed to SIF (D_5 , D_{30} , D_{60}).

DISCUSSION

In this study, we describe the identification and characterization of myosin light chain 1, designated Gal d 7, as the first major poultry meat allergen. Using IgE immunoblots in combination with peptide mass fingerprinting we identified a 23 kDa and a 13 kDa muscle protein, which were the dominant proteins recognized by chicken meat allergic individuals in cooked meat, as different splice variants of myosin light chain 1. Since some patients displayed IgE reactivity only to the 23 kDa, but not to the 13 kDa protein, we assumed that the long splice variant contained all the IgE binding epitopes. Therefore, we focused on the long splice variant and isolated a cDNA coding for the 23 kDa protein from chicken muscle mRNA and expressed it in *E. coli* as a recombinant protein. Analysis of the IgE reactivity of the recombinant protein in immunoblots and ELISAs showed that recombinant Gal d 7 (rGal d 7) indeed represents an IgE reactive protein. The fact that rGal d 7 was able to block patients' IgE binding to its natural counterpart in immunoblot inhibition experiments, showed that the recombinant allergen contained all the IgE binding epitopes present in natural Gal d 7. Since IgE binding to both proteins, the 23 kDa and the 13 kDa protein, were inhibited, it can be assumed that rGal d 7 contains all the epitopes from both myosin light chains.

A distinction between primary and secondary chicken meat allergy based on medical history, clinical characteristics, bird exposure history or skin prick testing is not always unambiguously possible. Currently no reliable serological markers exist to distinguish between these two types of food allergy in clinical practice. Therefore, genuine chicken meat allergy, although certainly a rare condition, might often be overlooked. In this study, we decided to classify our patients as primary or secondary chicken meat allergic based on the absence or presence of specific IgE levels to the chicken serum albumin (Gal d 5), together with specific IgE reactivity to the major egg white allergens, to egg yolk and to bird feathers (Table I). IgE immunoblots demonstrated that most Gal d 5 positive patients (secondary meat allergic patients) only reacted with a heat-labile protein of ~70 kDa in chicken meat extracts (Fig 1), identified by peptide mass fingerprinting as chicken serum albumin, but rarely ever with Gal d 7 or other thermostable proteins. In contrast, Gal d 5 negative patients (primary chicken meat allergic patients) recognized several IgE reactive low molecular weight proteins in raw meat,

and after cooking, they predominantly displayed IgE reactivity to a 23 kDa and a 13 kDa protein (Fig 1), identified by us as myosin light chains (Gal d 7). ELISA and immunoblot experiments showed that 77% of the patients from this group displayed IgE reactivity to rGal d 7, which identifies Gal d 7 as a major chicken allergen for genuinely sensitized patients.

Immunoblots with a rabbit antiserum raised against rGal d 7 allowed analysis of the expression of Gal d 7 in different skeletal muscle tissues and showed that the protein was not only present in chicken breast, but also in legs and wings, the other frequently consumed chicken tissues, suggesting that Gal d 7-sensitized patients need to avoid any part of the chicken. The anti-rGal d 7 antiserum also enabled detection of related proteins in turkey, goose and duck. IgE immunoblots showed that these Gal d 7-related proteins were recognized by patients' IgE antibodies and that rGal d 7 was able to inhibit IgE binding to these allergens. These findings indicate the strong cross-reactivity between Gal d 7-related proteins of different poultry species and show that rGal d 7 contains the majority of IgE binding epitopes present on these proteins. These data further provide a molecular basis for the observation that poultry meat allergic patients often react to several poultry species¹⁶.

Investigation on the impact of heat on rGal d 7 by SDS-PAGE, IgE immunoblotting and circular dichroism spectroscopy showed that even a heat treatment of 95°C for 20 minutes did not affect the allergen's integrity and IgE binding capacity (Fig 4, B). Interestingly, the remarkable thermal stability of natural chicken myosin light chain 1 and myosin light chain 3 has already been observed before²⁴ and has resulted in the selection of these two molecules as biomarkers for the detection of chicken contaminants in meat mixes²⁴. Furthermore, also the behaviour upon heat treatment of chicken breast myosin light chain proteins in the myosin protein complex has already been investigated and it has been shown that the light chains dissociate at around 40°C from the complex and remain soluble, whereas the heavy chains denature and form insoluble aggregates^{25,26}. It is conceivable that the dissociation of the essential light chains from the heavy chain during the cooking process and their resistance to aggregation enhance the potential of essential light chains to sensitize susceptible individuals, since protein solubility is regarded a prerequisite for genuine food allergens. The dissociation of the light chains from the myosin complex upon heating might further cause the

unmasking of previously inaccessible IgE binding epitopes and might lead in this way to the increased IgE reactivity observed in cooked as compared to raw chicken meat (Fig 1).

It is known that allergens sensitizing via the gastrointestinal tract require high resistance to gastrointestinal digestion²⁷. It further has been suggested that the resistance of the allergens not only depends on their intrinsic characteristics, such as their sequence, structure and posttranslational modifications, but also on the composition of the food matrix²⁷. We therefore investigated the stability of Gal d 7 to *in vitro* simulated gastric and intestinal digestions in the chicken meat matrix. Immunoblots performed with the anti-rGal d 7 antiserum showed that Gal d 7 was highly resistant to gastric digestion (Fig 5, A). IgE immunoblots with patients' sera further demonstrated that the IgE binding capacity of the protein was also not affected by the exposure to gastric conditions (Fig 5, B). However, under intestinal conditions Gal d 7 was rapidly degraded. It might well be that low molecular weight fragments generated during intestinal digestion still contain linear IgE binding epitopes that have sensitizing capacity. Immunoblots do not allow the detection of such small fragments, but inhibition ELISA experiments showed that IgE binding to ELISA plate bound rGal d 7 was significantly reduced by pre-incubation of patients' sera with meat samples after intestinal enzymatic digestion, indicating the presence of IgE reactive peptides in these samples (data not shown).

Recently allergen-specific immunotherapy has also become an area of research in food allergy and different routes of application of food allergens (oral, sublingual, epicutaneous and injection immunotherapy) are under investigation²⁸. Even though the immune mechanisms underlying successful immunotherapy are not yet fully understood, clinical improvement is accompanied by the production of allergen-specific, protective IgG antibodies. We therefore investigated the ability of rGal d 7 to induce the generation of IgG antibodies in rabbits, which would be able to inhibit allergic patients IgE binding to Gal d 7. We indeed found that rGal d 7-specific IgGs reduced allergic patients IgE binding to Gal d 7, suggesting that there might be the possibility to use our recombinant molecule for allergen-specific immunotherapy of Gal d 7 sensitized patients in the future.

425 In summary, using a proteomics based approach we identified myosin light chain 1, designated Gal d
426 7, as a major chicken meat allergen, especially for patients primarily sensitized to chicken meat.
427 Recombinant Gal d 7 was produced as a folded, IgE reactive protein and it was shown to contain the
428 majority of IgE binding epitopes present in natural poultry species, which suggests that the molecule
429 can be used for diagnosis of patients primarily sensitized to poultry meat. The observed remarkable
430 thermal stability, refolding capacity and resistance to salivary and gastrointestinal enzymes might
431 explain why Gal d 7 can act as a potent sensitizing agent for poultry meat allergic individuals.

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FIGURE LEGENDS

FIG 1. IgE reactivity patterns of chicken meat allergic patients to chicken meat extract. Raw (A) and cooked (B) chicken meat extract was separated by SDS-PAGE and either stained with Coomassie brilliant blue (CBB) or blotted onto nitrocellulose and exposed to sera from 29 meat allergic patients, to the serum of a non-allergic individual (NA) or to buffer only (PBST). Bound IgE antibodies were detected with anti-human IgE antibodies. Based on their IgE reactivity profile patients were categorized in two groups (Group 1 and Group 2). Molecular weights (kDa) are indicated in the left margin.

FIG 2. IgE binding capacity of recombinant Gal d 7. **A**, Nitrocellulose-blotted rGal d 7 was exposed to sera from three chicken meat allergic patients (patients 21, 24 and 26), or to the serum of a non-allergic individual (NA) or to buffer only (PBST). Bound IgE antibodies were detected with anti-human IgE antibodies and molecular weights are indicated in the left margin. **B**, Inhibition of IgE binding to nitrocellulose-blotted cooked chicken meat extract after pre-incubation of a pool of patients' sera (patients 24-28) with rGal d 7 or with chicken serum albumin (CSA), or buffer (untreated). Molecular weights are indicated in the left margin. **C**, Prevalence of IgE binding to rGal d 7 as determined by ELISA. ELISA plate-bound rGal d 7 was exposed to 28 sera of chicken meat allergic patients (Patients) belonging to Group 1 or Group 2, or, for control purposes (Controls), to sera from patients allergic either to birch pollen, dog dander or fish (C1 to C3) or to buffer only (C4, C5). Mean OD values corresponding to IgE binding were measured by ELISA and are indicated on the y-axis. The dashed line represents the mean value of the negative controls (control sera and buffer) plus the twofold standard deviation.

FIG 3. Presence of Gal d 7 in different chicken skeletal muscles and of Gal d 7 cross-reactive allergens in other poultry species. **A**, Nitrocellulose-blotted extracts from chicken breast (1), leg (2) and wing (3) as well as from turkey breast (4), goose leg (5) and duck leg (6) were exposed to the rabbit anti-rGal d 7 antiserum. Molecular weights are indicated in the left margin. **B**, Extracts from cooked turkey (T), goose (G), and duck (D) skeletal muscle were separated by SDS-PAGE and blotted onto nitrocellulose. Nitrocellulose membranes were exposed to patients' sera (patients 11 and

17) that had been pre-incubated with rGal d 7 (+rGal d 7) or with buffer only (untreated). Molecular weights are indicated in the left margin.

FIG 4. Effect of heat treatment on rGal d 7. **A**, The effect of heat treatment on the structure of rGal d 7 was determined by far-UV circular dichroism analysis. The spectra were recorded in a temperature scan from 25°C (strong blue line) to 95°C (strong red line) and at 25°C after cooling (dashed blue line) and are indicated by a transition from blue to red lines with increasing temperature. They are displayed as ellipticity (mdeg, y-axis) at given wavelengths (nm, x-axis). **B**, The effect of heating on the stability and IgE reactivity of rGal d 7 was assessed in a Coomassie stained SDS-PAGE (CBB staining) and by IgE immunoblotting (α -IgE immunoblot). Heat treatment was performed at 25°C (25), 65°C (65) and 95°C (95) for 5 min or 20 min. Nitrocellulose-blotted rGal d 7 samples were exposed to a pool of patients' sera (sera 17, 20, 21, 22) and bound IgE antibodies were detected with anti-human IgE antibodies. Molecular weights (kDa) are indicated in the left margin. **C**, The effect of cooking, grilling and the addition of the denaturing agent urea on native Gal d 7 was analyzed in a Coomassie stained SDS-PAGE (CBB staining) and in an immunoblot performed with the rabbit anti-rGal d 7 antiserum (α -rGal d 7 immunoblot). Raw, cooked and grilled chicken meat was extracted under native (n) or denaturing conditions (d). Cooking water (C) was also analyzed. Molecular weights are indicated in the left margin.

FIG 5. Effect of simulated gastric and intestinal digestion on the stability of Gal d 7. Samples from cooked chicken breast extracted in simulated salivary fluid (O) and exposed for 0, 5, 15, 30 and 60 min to simulated gastric fluid (G_0 , G_5 , G_{15} , G_{30} , G_{60}) and for 5, 30 and 60 min to simulated intestinal fluid (D_5 , D_{30} , D_{60}) were blotted onto nitrocellulose and either incubated with the rabbit anti-rGal d 7 antiserum (α -rGal d 7 immunoblot) (**A**) or with a pool of patients' sera (α -IgE immunoblot; sera 19, 29, 30, 32) (**B**). Molecular weights are indicated in the left margin.

TABLE I. Demographic and clinical data of chicken meat allergic patients*

Patient number	Nation	Age (yr)/gender	Symptoms with chicken meat	SPT with chicken meat	Total IgE (kU/L)	sIgE by ImmunoCAP (kU/L)**				sIgE by ISAC112 (ISU-E)**				Other food allergies
						Chicken meat	Egg white	Egg yolk	Budgerigar feather	Gal d 1	Gal d 2	Gal d 3	Gal d 5	
1	S	14/M	OAS	+	852	34.6	>100	96.8	12.4	27.0	8.5	2.8	10.0	Egg white and yolk
2	S	25/F	OAS	+	834	1.42	71.5	41.9	3.44	5.7	3.0	1.1	1.7	Egg white and yolk
3	S	39/M	OAS	+	1651	3.54	>100	74.6	5.10	11.0	24.0	5.7	2.8	Egg white and yolk, apple, peach
4	A	14/F	OAS	+	866	3.59	2.66	4.44	13.5	0	0	0	1.8	Egg white and yolk
5	A	43/F	N, CU	+	1420	2.94	33.8	7.58	89.1	0	0	1.6	7.1	Egg yolk
6	S	29/M	U	+	445	7.32	0.86	15.3	7.42	0	0	0	1.0	Egg yolk
7	S	10/M	U	+	1798	59.3	>100	>100	26.1	18.0	14.0	12.0	24.0	Egg white and yolk
8	S	7/M	U	+	2118	82.2	>100	>100	23.8	2.7	5.9	2.1	7.7	Egg white and yolk
9	S	8/M	RC,U,V	+	1283	36.6	>100	>100	9.55	17.0	4.9	1.2	2.6	Egg white and yolk
10	S	5/F	OAS	+	N/A	1.43	10.7	6.90	4.10	1.1	0	0.5	0.5	Egg white and yolk, lentil, nuts
11	A	51/F	N,V	+	2000	3.51	2.03	5.77	9.92	0	0	0	3.7	Egg yolk
12	S	6/F	U	+	653	3.30	0.87	0.61	0.10	0	0	0	0	Egg white and yolk, rabbit meat, fish, milk
13	S	9/F	OAS	+	438	1.00	0.38	0.12	0.03	0	0	0	0	Rabbit meat, fish, peach
14	S	33/M	EE	+	958	1.44	1.38	1.16	0.22	0	0	0	0	Fish, peanut, soy
15	S	5/F	OAS	+	765	1.28	30.7	8.02	0.44	9.0	0	0	0	Egg white and yolk, fish, milk, nuts
16	S	5/F	OAS	+	2093	5.85	>100	28.6	5.33	15.0	1.2	8.1	0	Egg white, fish, walnut, kiwi
17	S	12/M	OAS	+	2695	3.33	9.96	6.66	3.00	2.9	0.5	3.8	0	Egg white and yolk, shellfish, banana
18	A	28/M	OAS	±	38	0.36	0.00	0.01	0.00	0	0	0	0	Fish
19	A	31/M	RC	+	50	2.29	0.12	0.06	0.01	0	0	0	0	Fish
20	S	8/M	OAS	+	2750	9.13	12.63	11.22	2.40	3.6	0	2.3	0	Egg white and yolk, shellfish, nuts
21	S	2/M	U	+	416	8.50	29.7	4.68	0.51	2.0	0	0	0	Egg white, fish, lentil
22	A	18/M	D, U, V	+	454	3.23	0.14	0.02	0.00	0	0	0	0	-
23	A	3/M	U,V	+	118	1.85	0.56	0.56	0.04	0	0	0	0	-
24	A	4/F	AE	N/A	960	5.01	0.67	0.91	0.09	0	0	0	0	Egg white
25	A	23/M	U	+	190	4.96	0.65	0.25	0.03	0	0	0	0	-
26	A	21/M	D, OAS	+	553	2.41	0.05	0.12	0.02	0	0	0	0	Fish
27	A	5/F	ANG, D, N, OAS, U	+	132	3.40	0.90	0.72	0.14	0	0	0	0	-
28	A	25/M	ANG, OAS, U	+	61	2.87	0.03	0.62	0.02	0	0	0	0	-
29	S	12/M	AP, OAS	N/A	1154	5.60	0.21	0.33	0.09	0	0	0	0	Fish
30	A	19/M	ANG, OAS, U	+	400	35.9	3.11	0.00	3.96	0.5	0	0	0	Egg yolk, lamb meat, fish, lentil, pea
31	A	25/M	AP, N	+	1015	1.52	0.04	0.06	0.03	0	0	0	0	-
32	A	71/F	U	N/A	694	7.66	0.02	0.02	0.01	0	0	0	0	Fish
33	A	29/F	OAS, U	N/A	220	2.05	1.27	0.65	0.03	0	0	0	0	Fish

* Based on their IgE reactivity profiles patients above the horizontal line (patients 1 – 11) were diagnosed with egg-bird/bird-egg syndrome, whereas patients below the line (patients 12 – 33) were diagnosed with primary chicken meat allergy.

** sIgE against chicken meat, egg white, egg yolk and budgerigar feather were determined by ImmunoCAP (kU/L); sIgE against Gal d 1, Gal d 2, Gal d 3 and Gal d 5 were determined by ISAC112 (ISU-E); in case of the ISAC112 results “0” always stands for “< 0.3”.

S, Spain; A, Austria; M, male; F, female.

AE, exacerbation of atopic eczema; ANG, angioedema; AP, abdominal pain; CU, contact urticaria; D, dyspnea; EE, eosinophilic esophagitis (diagnosed by biopsy); N, nausea; OAS, oral allergy syndrome; RC, rhinoconjunctivitis; U, urticaria; V, vomiting.

N/A, not available.

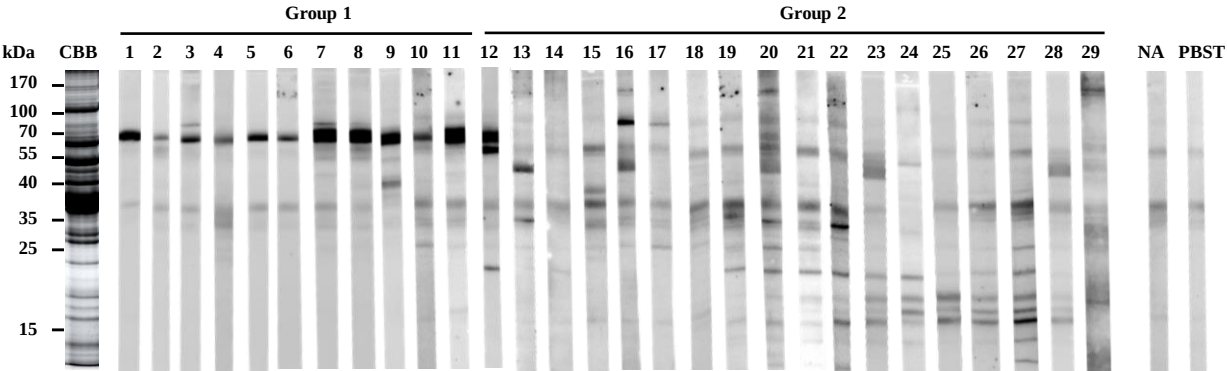
TABLE II. Inhibition of patients' IgE binding to rGal d 7 by rabbit anti-rGal d 7 antibodies*

Patient	OD ₄₅₀ values measured after pre-incubation of rGal d 7 with		% inhibition of IgE binding
	preimmune serum	anti-rGal d 7 serum	
11	1.326	0.293	78%
20	0.619	0.375	39%
21	1.143	0.705	38%
26	0.448	0.314	30%
28	0.439	0.308	30%
30	2.404	0.627	74%
31	1.615	0.360	78%
33	0.326	0.145	55%

*ELISA plate-bound rGal d 7 was pre-incubated with 1/100 diluted rabbit anti-rGal d 7 antibodies or with rabbit preimmune and subsequently exposed to sera from 8 chicken meat allergic patients. IgE binding was measured by ELISA. Mean OD values and percentage inhibition of IgE binding are displayed.

Figure 1

A



B

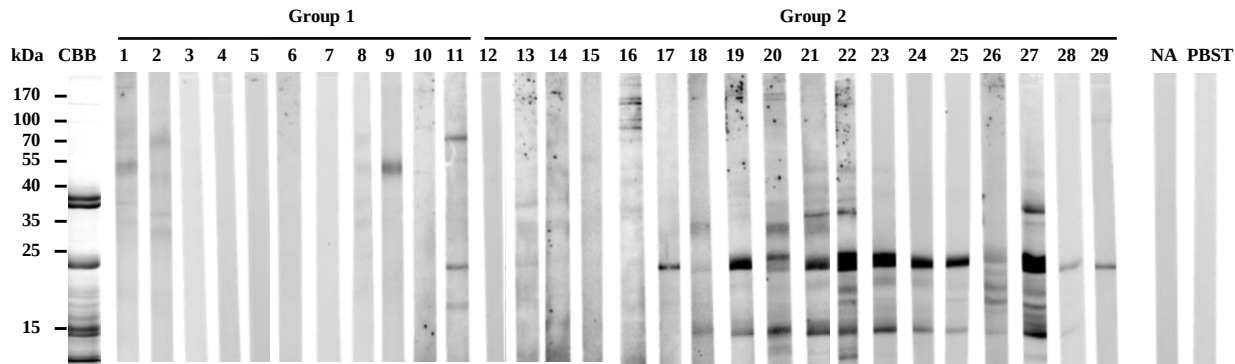


Figure 2

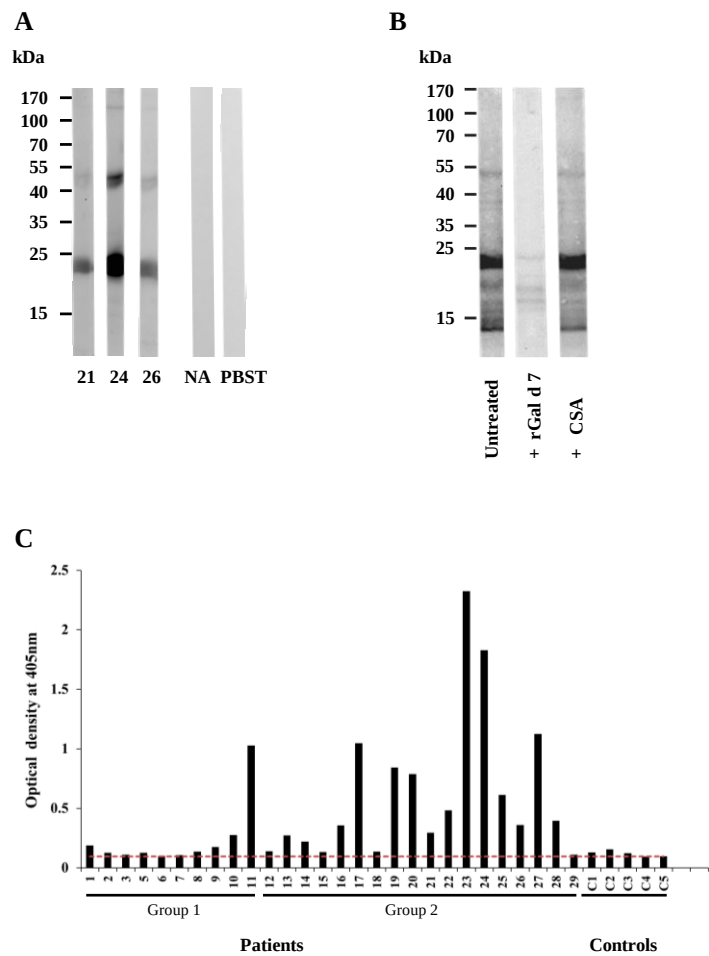


Figure 3

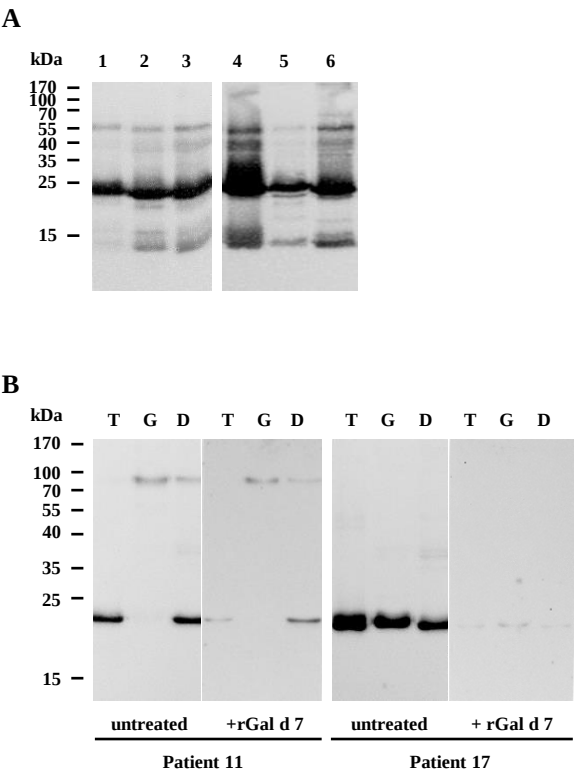
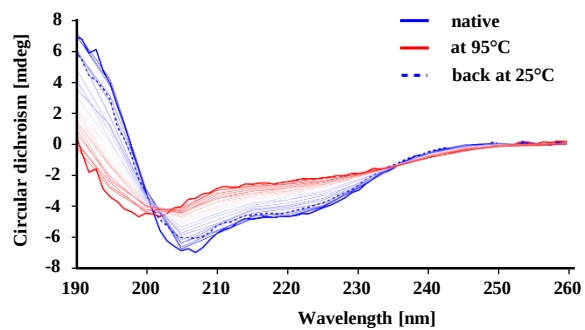
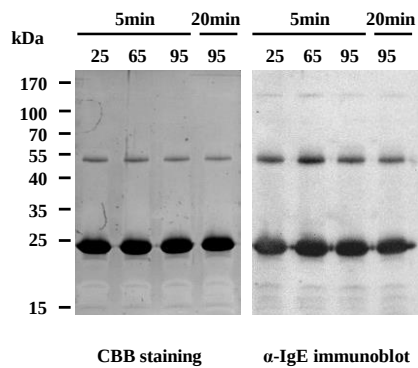


Figure 4

A



B



C

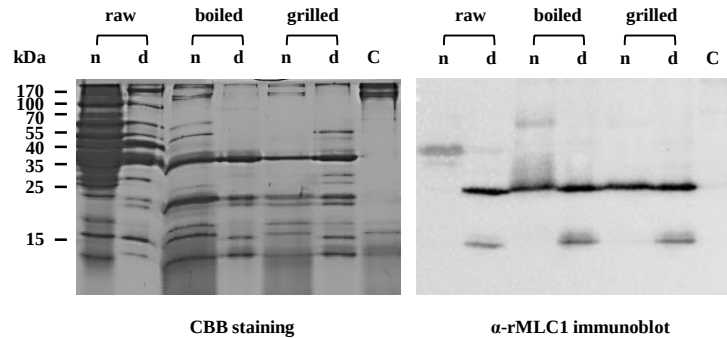


Figure 5

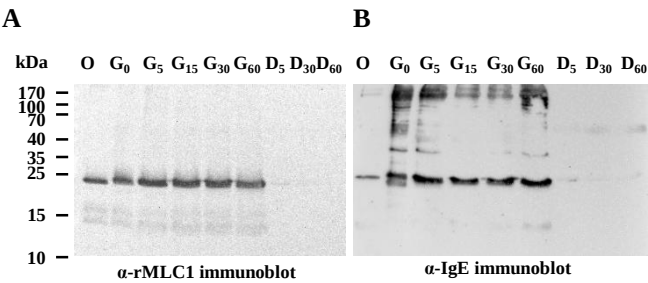


TABLE E1. Peptides of myosin light chains identified by Nano LC-ESI MS/MS after tryptic digestion of IgE reactive proteins*

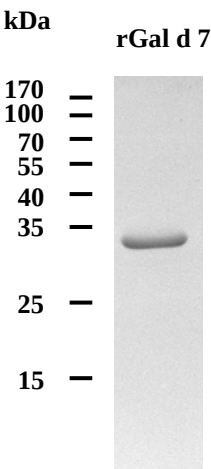
Peptide sequence	Experimental peptide mass	AA position in MLC 1f
<i>KPAAAAAPAPAPAPAPAPAKPK</i>	2126.2882	9-32
<i>EPAIDLK</i>	783.5854	33-39
<i>SIKIEFSK</i>	949.5854	40-47
<i>EAFLLFDR</i>	1008.5054	55-62
<i>ALGQNPTNAEINK</i>	1367.4654	79-91
<i>ILGNPSKEEMNAK</i>	1428.3454	92-104
<i>EGNGTVMGAELR</i>	1247.5054	140-151
<i>HVLATLGEK</i>	965.6454	152-160
<i>MTEEEVEELMK</i>	1397.3654	161-171

*Peptides only identified in the 23 kDa protein band are shown in italics.
AA, amino acid.

Figure E1

MLC 1f	1	MAPKKDVKKP	AAAAAPAPAPAPAPAPAPAKP	KEPAIDLKSIKIEFSKE	QQDDFKEAFL	LD	61	
MLC 3f	1	-----MSFSPDEINDFKEAFL					LD	19
MLC 1f	62	RTGDAKITLSQVGDIV	RALGQNPTNAEINKI	LGNPSKEEMNAKKIT	FEFLPMLQAA	ANNK	122	
MLC 3f	20	RTGDAKITLSQVGDIV	RALGQNPTNAEINKI	LGNPSKEEMNAKKIT	FEFLPMLQAA	ANNK	80	
MLC 1f	123	DQGTTFEDFVEGLRVF	DKEGNGTVMGAELRHV	LATLGEKMT	EEEEVEELMK	QGEDSNGCINYE	183	
MLC 3f	81	DQGTTFEDFVEGLRVF	DKEGNGTVMGAELRHV	LATLGEKMT	EEEEVEELMK	QGEDSNGCINYE	141	
MLC 1f	184	AFVKHIMSV					192	
MLC 3f	142	AFVKHIMSV					150	

Figure E2



Online Repository**Gal d 7 - a major allergen in primary chicken meat allergy**

Christoph Klug, MSc^{a, *}, Wolfgang Hemmer, PhD^b, Patricia Román-Carrasco, MSc^a, Margit Focke, PhD^c, Santiago Quirce MD, PhD^d, Teresa Boyano-Martínez, MD^d, Erwin Gaubitzer, PhD^e, Herbert Wank, PhD^a, Ines Swoboda, PhD^a

^aBiotechnology Section, FH Campus Wien, University of Applied Sciences, Campus Vienna Biocenter, Vienna, Austria; ^bFAZ - Floridsdorf Allergy Center, Vienna, Austria; ^c Division of Immunopathology, Department of Pathophysiology and Allergy Research, Center for Pathophysiology, Infectiology and Immunology, Medical University of Vienna, Vienna, Austria; ^dDepartment of Allergy, Hospital La Paz Institute for Health Research (IdiPAZ), Madrid, Spain; ^eMax F. Perutz Laboratories, Department of Structural Biology and Biomolecular Chemistry, University of Vienna, Vienna, Austria.

*Christoph Klug, MSc, is currently affiliated with MacroArray Diagnostics GmbH, Vienna, Austria.

Corresponding author:

Ines Swoboda, PhD, Biotechnology Section, FH Campus Wien, University of Applied Sciences, Campus Vienna Biocenter, Helmut-Qualtinger-Gasse 2, A-1030 Vienna, Austria. Phone: +43-1-6066877-3515, Fax: +43-1-6066877-3509, E-mail: ines.swoboda@fh-campuswien.ac.at.

METHODS

Patients' sera

All patients reported clinical symptoms (oral allergy syndrome, vomiting, gastric cramping, diarrhea, urticaria, angioedema, dyspnea, conjunctivitis) after the ingestion of poultry meat and had elevated levels of chicken meat specific IgE as measured by ImmunoCAP (Thermo Fisher, Uppsala, Sweden). Skin prick tests with chicken meat extracts were always positive (see Table I). Patients were classified as primary or secondary chicken meat allergic based on *in vitro* measurements of specific IgE reactivity to chicken meat, egg white, egg yolk and budgerigar feathers (determined by ImmunoCAP) and to the major egg white (Gal d 1, Gal d 2, Gal d 3) and egg yolk (Gal d 5) allergens (determined by ImmunoCAP ISAC¹¹²). All individuals with elevated IgE levels to Gal d 5 (chicken serum albumin) were classified as secondary chicken meat allergic patients (patients 1-11 in Table I). In these patients, specific IgE levels to egg yolk were higher than those to chicken meat, and most of the patients (8 out of 11) had also specific IgEs to egg white allergens, suggesting a primary egg allergy (egg-bird syndrome). In addition, all the secondary meat allergic patients showed strong IgE reactivity to budgerigar feathers. In some of these patients, high IgE levels to budgerigar feathers without specific IgE to the major egg white allergens suggested a primary sensitization to bird feathers indicative of the bird-egg syndrome (e.g., patients 4, 6, 11 in Table I). In contrast, individuals lacking IgE reactivity to Gal d 5 were classified as primary chicken meat allergic patients (patients 12-33 in Table I). In the majority of these patients (18 out of 22) the IgE scores for egg yolk were either negative or at least significantly lower than the IgE scores for chicken meat. Exceptions were only those few patients with a concomitant egg white allergy, as indicated by high IgE levels to egg white and to at least one of the major egg white allergens (e.g., patients 15, 16, 17, 20 in Table I). Among the primary chicken meat allergic patients the IgE levels to budgerigar feathers were always low.

Preparation of protein extracts

Breasts, legs and wings from chicken, breast from turkey, and legs from goose and duck were purchased at local butcher stores and meat markets. The muscle tissues were always cut into small pieces with a surgical blade, frozen in liquid nitrogen and ground to a fine powder with mortar and pestle. Five gram of the homogenized meats were extracted with 50 ml phosphate buffered saline (PBS) pH 7.5 overnight under continuous shaking. After centrifugation of the homogenates (30 min, 4500 rpm), the supernatants were filtered through sterile filters (0.45 μ m) and stored at a -20°C. For extraction of proteins insoluble in aqueous buffers, the pellets obtained after centrifugation of the homogenates were resuspended in PBS containing 4 M urea for 10 min under vigorous shaking, followed by centrifugation (10 min, 13000 rpm). The urea protein extracts were also filtered and stored at -20°C. For cooked and grilled meat extracts, the meat was either cooked in boiling water (5 g in 200 ml) or grilled in a frying pan without additives for 20 minutes before extraction. Both methods (boiling in water and grilling) were used, since it is known that heat is transferred differently during these cooking processes. During boiling in water temperatures of a maximum of 100°C are reached in the whole piece of meat, during grilling much higher temperatures are reached on the surface of the meat (>140°C), whereas the inside temperatures are much lower. These differences in the transfer of heat might also effect the stability and the structure of allergens. Proteins present in the cooking water were concentrated using centrifugal filter units (Merck Millipore, Billerica, Massachusetts) prior to storage at -20°C. All extraction steps were performed at 4°C. The protein concentrations of the extracts were determined by the method of Bradford^{E1} with bovine serum albumin (BSA) as a standard.

Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE), immunoblot and inhibition immunoblot experiments

Protein extracts (~20 μ g/cm gel) were separated on 12% or 15% SDS polyacrylamide separating gels with 5% stacking gels under reducing conditions as described by Laemmli^{E2}. Proteins were either visualized by Coomassie Brilliant Blue (CBB) staining or transferred onto nitrocellulose membranes (GE healthcare, Little Chalfont, UK) for immunoblot experiments^{E3}. The membranes were cut into strips,

washed and blocked with PBS containing 0.5% Tween 20 (PBST) before incubating them with sera of meat allergic patients (diluted 1:10 with PBST) for 14 to 20 hours at 4°C. For control purposes, strips were incubated with sera of non-allergic individuals and with PBST only. Bound IgE antibodies were detected with an alkaline phosphatase labeled mouse anti-human IgE antibody (BD Biosciences, Franklin Lakes, NJ) diluted 1:5000 with PBST using Lumi-Phos (Lumigen, Southfield, MI) on a FluorChem E Protein simple device (Biozym Scientific GmbH, Hessisch-Oldendorf, Germany).

For analysis whether recombinant Gal d 7 (rGal d 7) displays IgE reactivity comparable to its natural counterpart, IgE inhibition immunoblot experiments were performed. For this, nitrocellulose strips containing chicken meat protein extracts were exposed to individual sera (diluted 1:10 in PBST) or to a pool of 5 sera (sera 24 - 28; each serum diluted 1:50 in PBST), which had been preincubated with rGal d 7 or, for control purposes, with purified chicken serum albumin (Sigma-Aldrich, St. Louis, MO) (50 µg/ml per diluted serum). Bound IgE antibodies were detected as described above for the immunoblots.

For IgE inhibition immunoblots carried out to analyze whether rGal d 7 displays cross-reactivity to myosin light chains from other poultry species, nitrocellulose strips containing extracts from turkey, goose and duck were exposed to 1/10 diluted sera that had been preincubated with rGal d 7 (50 µg/ml per diluted serum) or, for control purposes, with buffer only. In these experiments patients' sera and the secondary antibody were diluted in PBST containing 0.1% human serum albumin (HSA). Bound IgE was detected with a horseradish peroxidase (HRPO) labeled anti-human IgE antibody (0.5 µg/ml Southern Biotech, Birmingham, AL) as a secondary antibody using the SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific) on the FluorChem E Protein simple device.

For specific detection of Gal d 7 the blots were incubated with the rabbit antisera generated against rGal d 7 (in a 1:30000 dilution) with a HRPO labeled goat anti-rabbit secondary antibody (Vector laboratories, Burlingame, CA) diluted 1:10000. Detection of bound IgG was carried out with a SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific) on the FluorChem E Protein simple device.

Liquid chromatography ion trap mass spectrometry (Nano LC-ESI MS/MS) and Matrix-assisted laser desorption/ionization time-of-flight (MALDI-ToF) mass spectrometry

For identification of meat allergens, LC-ESI MS/MS analyses were performed as described by Hemmer et al.^{E4}. For this, protein bands of interest were excised from SDS-PAGE gels and digested using an In-Gel Tryptic Digestion Kit (Pierce, Rockford, IL). The LC-ESI MS/MS data were acquired using an UltiMate 3000TM (Dionex, Vienna, Austria) Nano-LC system coupled to a Bruker HCT Ultra ESI ion trap (Bruker Daltonics, Bremen, Germany) and an online nanospray source. Analysis was carried out on a C8 PepMap100 (3mm, 100Å) separation column using a binary solvent gradient from 5% to 80% acetonitrile. During elution, mass spectra were continuously recorded in a mass/charge range from 300 to 3000. Data processing was carried out using DataAnalysisTM 3.4 and the generated peak lists were searched against the protein databank NCBI using the database search engine MASCOT (Matrix Science).

The molecular weight of the recombinant protein was determined by Matrix-assisted laser desorption/ionization time-of-flight (MALDI-ToF) mass spectrometry on a Microflex mass spectrometer (Bruker). For this, the recombinant protein was dissolved in 10% acetonitrile (0.1% trifluoroacetic acid), and dihydroxybenzoic acid (dissolved in 60% acetonitrile, 0.1% trifluoroacetic acid) was used as a matrix. For sample preparation, a 1:1 mixture of protein and matrix solution was deposited onto the target and air-dried.

cDNA cloning of chicken myosin light chain 1 (Gal d 7) into the bacterial expression vector pET-17b

For cDNA cloning of the chicken myosin light chain, fresh chicken breast muscle tissue (a kind gift by Prof. Nimpf, Max F. Perutz Laboratories, University of Vienna) was immediately frozen in liquid nitrogen and then ground to a fine powder. Total RNA was extracted using the TRIFAST reagent (PeqLab, Erlangen, Germany). cDNA synthesis was performed with the Revert Aid Minus RT kit (Promega, Madison, WI) by reverse transcription PCR (RT-PCR) using the following specific primers,

which were based on the published sequence of chicken myosin light chain (UniProt accession number P02604): Primer MLC1_FW (GGT GGT CAT ATG GCA CCT AAG AAG GAC GTT AAG AAA) and primer MLC1_RV (GGT GGT GAA TTC CGG TCA GTG ATG ATG ATG ATG ATG GAC AGA CAT GAT GTG CTT TAC). Primer MLC1_RV added the coding sequence of a 6x Histidine purification tag (underlined) and an *EcoRI* recognition site (italics) at the 3'-end of the cDNA clone, whereas primer MLC1_FW introduced a *NdeI* recognition site (italics) at the 5'-end. The obtained cDNA coding for chicken myosin light chain, designated Gal d 7 by the International Union of Immunological Societies allergen nomenclature subcommittee, was subcloned into the *NdeI/EcoRI* sites of the expression vector pET-17b (Novagen, Darmstadt, Germany) and the Gal d 7-construct was verified by sequencing (Microsynth, Balgach, Switzerland).

Enzyme linked immuno sorbent assay (ELISA) and competition ELISA experiments

Polystyrene microtiter plates (Nunc, Thermo Fisher Scientific) were coated with 4 µg/ml of rGal d 7 in 100 mM sodium bicarbonate buffer (pH 9.6) overnight at 4°C. After washing with TBST (Tris buffered saline, 0.5% v/v Tween 20) unspecific antibody binding sites were blocked with TBST for 2.5 h at 37°C. Wells were then incubated at 4°C overnight with sera of poultry meat sensitized patients (diluted 1:5 in TBST). Control wells were incubated with the serum of a non-allergic individual or sera of patients allergic to allergen sources unrelated to poultry meat. Bound IgE antibodies were detected with an alkaline phosphatase labeled monoclonal mouse anti-human IgE antibody (BD Biosciences) diluted 1:2000 in TBST. Alkaline phosphatase substrate (Sigma-Aldrich) was added to the wells and the plates were developed in the dark. The resulting absorbance was measured at 405nm in an ELISA reader (Thermo Fisher Scientific). All experiments were conducted in duplicates and results were expressed as mean values. Values were considered positive when exceeding the two fold standard deviation of the buffer controls.

In ELISA competition experiments, the capacity of rabbit antisera generated against rGal d 7 to inhibit the binding of patients' IgE to rGal d 7 was analyzed: for this, microtiter plate bound rGal d 7 (1

$\mu\text{g}/\mu\text{l}$) was incubated overnight at 4°C with either the anti-rGal d 7 antiserum or the corresponding preimmune serum, diluted 1:100 in TBST, before being exposed to patients' sera (diluted 1:5 in TBST). Bound IgE antibodies were detected with HRPO labeled goat anti-human IgE antibodies (1:2500 diluted in TBST; Kirkegaard & Perry Laboratories) and the color reaction was started by addition of 1.7 mM 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (Sigma-Aldrich) in 60 mM citric acid, 77 mM $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, and 3 mM H_2O_2 . OD levels were measured in an ELISA reader at 405 nm. The percentage of inhibition of IgE binding achieved by pre-incubation with the anti-rGal d 7 rabbit antiserum was calculated as follows: percent inhibition of IgE binding = $100 - (\text{OD}_s/\text{OD}_p) \times 100$, where OD_s and OD_p represent the extinction coefficients after pre-incubation with the rabbit serum and the preimmune serum, respectively.

Simulated gastric and intestinal digestion of chicken meat

In order to evaluate the stability of Gal d 7 to gastrointestinal digestion, cooked chicken meat was subjected to stimulated *in vitro* digestion following the harmonized INFOGEST method published by Minekus et al.^{E5} with minor modifications. Briefly, 5g of cooked meat were minced, mixed with 4 ml of 1.25x simulated salivary fluid electrolyte stock solution (SSF; 15.1 mM KCl, 3.7 mM KH_2PO_4 , 13.6 mM NaHCO_3 , 0.15 mM $\text{MgCl}_2(\text{H}_2\text{O})_6$, 0.06 mM $(\text{NH}_4)_2\text{CO}_3$), 25 μl of 0.3 M CaCl_2 and 5.475 ml of H_2O . After incubation for 5 min at 37°C under continuous shaking, a sample of 500 μl was drawn for SDS-PAGE and immunoblotting (sample O, representing the starting point of the digestion process). Then 0.5 ml human salivary α -amylase solution (1500 U/ml in SSF electrolyte stock solution; α -amylase from human saliva, 852 U/mg protein, Sigma) were added to a final concentration of 75 U/ml α -amylase. This oral bolus mix was incubated for 2 min at 37°C under continuous shaking. Subsequently, 3.9 ml of 1.25x simulated gastric fluid electrolyte stock solution (SGF; 6.9 mM KCl, 0.9 mM KH_2PO_4 , 25 mM NaHCO_3 , 47.2 mM NaCl, 0.1 mM $\text{MgCl}_2(\text{H}_2\text{O})_6$, 0.5 mM $(\text{NH}_4)_2\text{CO}_3$) and 5 μl of 0.3 M CaCl_2 were added and the pH was lowered to 3 by the addition of 1.9 ml 1M HCl and 3.195 ml H_2O and another 500 μl sample (sample G0) was taken. The gastric digestion phase was initiated by the addition of 1 ml pre-warmed porcine pepsin stock solution (38000 U/ml in SGF electrolyte stock

solution; pepsin from porcine gastric mucosa 3200–4500 U/mg protein, Sigma) to a final pepsin concentration of 2000 U/ml. Samples of 500 µl were drawn after 5, 15, 30 and 60 min of continuous shaking at 37°C and pepsin activity in the aliquots was inactivated by raising the pH to 7 with 250 µl 1M NaHCO₃ and snap freezing in liquid nitrogen. For the intestinal digestion phase, the remaining 17 ml of gastric chyme were mixed with 12.6 ml of 1.25x simulated intestinal fluid electrolyte stock solution (SIF; 6.8 mM KCl, 0.8 mM KH₂PO₄, 85 mM NaHCO₃, 38.4 mM NaCl, 0.33 mM MgCl₂(H₂O)₆), 1 ml pancreatin solution (3400 U/ml in SIF electrolyte stock solution; pancreatin from porcine pancreas, Sigma), 1 ml fresh bile solution (10mM final concentration, Sigma), 152 µl of 0.3 M CaCl₂, 0.7 ml of 1M NaHCO₃ and 1.548 ml H₂O. Samples of 500 µl were drawn after 5, 15 and 30 min of continuous shaking at 37°C and enzyme activity in the aliquots was inactivated by the addition of 5 mM pepabloc trypsin inhibitor (Sigma) and snap freezing in liquid nitrogen. For protein analysis of the samples, solid particles in the aliquots were sedimented by centrifugation (5 min, 16000rpm) and the supernatants were analyzed by SDS-PAGE and immunoblotting.

RESULTS

Identification of myosin light chain as an IgE reactive protein by mass spectrometry analysis

The 23 kDa and 13 kDa proteins of the cooked chicken meat extract were excised from the Coomassie stained gel and subjected to trypsin digestion, HPLC separation and LC-MS/MS analysis. Peptides of both the 23 kDa and 13 kDa proteins were identified as originating from the essential light chains of myosin, MLC 1f (UniProt accession number P02604) and MLC 3f (UniProt accession number P02605). Table E1 lists the identified peptides and Fig E1 shows their position in the protein sequences of MLC 1f and MLC 3f. While the peptides identified in the 23 kDa protein band covered almost the entire MLC 1f sequence, peptides identified in the 13 kDa protein band were only localized to the sequence common to both essential light chain variants. This suggested that the 23 kDa protein band represents MLC 1f and the 13 kDa protein band represents MLC 3f and that both proteins contribute to the IgE reactivity of chicken meat extract.

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FIGURE LEGENDS

FIG E1. Amino acid sequence alignment of the two myosin essential light chain variants MLC 1f (UniProt accession number P0204) and MLC 3f (accession number P02605) present in fast twitch chicken skeletal muscle. Black lines above the MLC 1f sequence indicate peptides identified by peptide mass fingerprinting.

FIG E2. Assessment of purity of rGal d 7. Coomassie brilliant blue stained SDS-PAGE of purified recombinant Gal d 7. Molecular weights (kDa) are indicated in the left margin.