RESEARCH ARTICLE

Physicochemical properties and thermal stability of Lep w 1, the major allergen of whiff

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Received: January 29, 2009
Revised: June 19, 2009
Accepted: June 25, 2009

Whiff (*Lepidorhombus whiffiagonis*) is a fish frequently consumed in Spain. Lep w 1, its major allergen, is a calcium-binding β-parvalbumin. The resistance of Lep w 1 to heat denaturation and to digestion were studied by circular dichroism spectroscopy and by *in vitro* gastric digestion systems. Purified Lep w 1 was thermally stable up to 65°C at neutral pH. Calcium depletion resulted in a change of its structure as determined by circular dichroism spectroscopy. A partial loss of structure was also observed at acidic pH; however, the allergen retained its full IgE-binding ability. The partially denatured Lep w 1 was easily digested by pepsin within 2 min. Further, the IgE reactivity of proteins extracted from cooked fish and their stability to proteolysis were analyzed. The extract revealed a higher number of IgE reactive bands than an extract from uncooked fish. IgE binding to these proteins could not be inhibited by an extract from uncooked fish. In contrast to a raw fish extract, the cooked extract showed higher resistance to pepsinolysis. The stability of Lep w 1 to thermal denaturation and digestion explains the high allergenicity of whiff.

Keywords:
Aggregates / Food allergy / Gastric digestion / Thermal stability / Whiff parvalbumin

1 Introduction

Fish is not only an important source of dietary protein but also a common elicitor of food allergies in coastal countries. One of the most allergenic fish species in Spain is the frequently consumed whiff (*Lepidorhombus whiffiagonis*) [1]. Allergic reactions to fish often manifest themselves already in small children with a tendency to persist [2]. In Spain, 18% of food allergic children suffer from fish allergy [3]. Fish consumption may lead to severe symptoms and even to lethal anaphylaxis [1]. The major fish allergen is parvalbumin, a 12 kDa soluble, acidic protein. It belongs to the second largest animal food allergen family, the EF-hand family [4]. Parvalbumins contain three EF-hand motifs, two of which are able to bind calcium with high affinity [5]. The N-terminal region may regulate the binding affinity of the active calcium-binding motifs. Parvalbumins are subdivided into two different phylogenetic lineages, α and β. Many allergenic cross-reactive β-parvalbumins are found in various fish species [6], including whiff.

It has been described that food allergens are usually abundant in the food and structurally stable [7, 8]. Additionally, food allergens must preserve their structure from degradation by digestive enzymes in order to be taken up by the gut and to be presented to the immune system [9]. β-Parvalbumins with bound calcium are remarkably stable [6, 10, 11]. Many studies report a significant resistance to heat, chemical denaturation and proteolytic enzymes [10, 12, 13]. Calcium binding is essential not only for the conformational stability but also for the allergen's immunoreactivity [6, 14].

However, one study questions the proteolytic stability of fish proteins. Untersmayer *et al.* reported the pepsinolysis of raw codfish proteins to small fragments after incubation with simulated gastric fluid (SGF), pH 2 for 1 min [15].

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Abbreviations: CD, circular dichroism; **SGF,** simulated gastric fluid

Physiochemical changes can impair proteolysis during gastrointestinal digestion and alter the way of presentation of the protein to the immune system [16]. Modifications during food processing, such as heat denaturation, are sometimes responsible for an increased allergenic potency of proteins [16]. Processing may also cause interactions between proteins and other food matrix components [9]. Heat-denatured proteins may rearrange their disulphide bonds and form homo- and heteromeric aggregates [17]. Cooked fish extracts were shown to form immunoreactive high-molecular-weight aggregates of denatured proteins [18]. At present, only few data about the physicochemical properties of parvalbumins have been published.

In this study, we aimed at investigating the thermal and gastric stability of native whiff parvalbumin, Lep w 1, by circular dichroism (CD) spectroscopy and *in vitro* gastrointestinal digestion experiments. Ca^{2+} depletion experiments were performed to examine the relevance of bound Ca^{2+} for the protein's conformational stability and IgE reactivity. Further, the digestibility of calcium depleted and undepleted Lep w 1 was compared. Finally, we examined the gastric stability and IgE-binding ability of proteins extracted from cooked fish.

2 Materials and methods

2.1 Patients' sera

Five patients' sera were selected based on the presence of a positive clinical history of type I fish allergy and positive skin-prick tests to whiff and at least one other fish species (Table 1). Whiff-specific IgE antibodies were determined using the Phadia ImmunoCAP system (Phadia, Uppsala, Sweden). All sera contained IgE specific for Lep w 1 as tested by IgE immunoblotting (data not shown).

2.2 Native protein extraction

Fresh whiff filets were purchased from a local market in Madrid, Spain. Two hundred and fifty grams of raw fish muscle was homogenized by grinding in three volumes w/v of 20 mM *Bis*-Tris-HCl, pH 6.5. Proteins were extracted by stirring for 3 h at 4°C. Subsequently, the homogenate was centrifuged (17 000 × *g*, 45 min, 4°C) and the pellet discarded. After removing cellular debris by filtration through Miracloth (Merck Biosciences, Nottingham, UK) and filter papers, the extract of soluble whiff proteins was freeze-dried.

To replicate a preparation method frequently used for whiff in Spain, 60 g of raw fish was heated to 100°C in 600 mL double-distilled H_2O for 10 min. Subsequently, the cooked fish was homogenized in 1.6 volumes w/v of double-distilled H_2O and extracted following the procedure for fish filets. The extract was stored at 4°C until further use.

2.3 Purification of Lep w 1, the whiff parvalbumin

The freeze-dried protein extract was suspended in 20 mM *Bis*-Tris-HCl, pH 6.5, and its protein content was determined by using the BCA Protein Assay Reagent Kit (PIERCE, Rockford, Ireland). The extract was treated with 0.1% Biocryl BPA-1000 (Supelco, Bellefonte, PA, USA) for 10 min at room temperature with stirring. Precipitates were pelleted at 17 000 × *g*, 30 min at 4°C, and the supernatant was loaded onto a DEAE Sepharose Fast Flow column (GE Healthcare, Chalfont St. Giles, Great Britain) pre-equilibrated in 20 mM *Bis*-Tris-HCl, pH 6.5. Bound proteins were eluted by a linear gradient from 0 to 25% of 20 mM Bis-Tris-HCl, pH 6.5, 1 M NaCl. Fractions were collected and analyzed by 15% SDS-PAGE and immunoblotting using a rabbit polyclonal anti-Gad m 1 antibody (Tepnel Biosystems, Deeside, Flintshire, UK). Fractions containing parvalbumin

Table 1. Patients' characteristics and results of IgE ELISA inhibition assays. ELISA plates were coated with purified Lep w 1 and inhibited with native and treated Lep w 1 samples

Patients	Symptoms[b)]	CAP[c)] whiff (kU/L)	IgE blot Lep w 1	Results of IgE ELISA inhibition assay (% inhibition)[a)]					
				Inhibitor					
				Untreated pH 7.0	Cooked pH 7.0	EGTA pH 7.0	Untreated pH 2.5	Cooked pH 2.5	EGTA pH 2.5
#1	AE, A	48.5	+	100	100	100	96	98	99
#2	AN	10.2	+	100	100	100	91	89	99
#3	AN	5.9	+	100	100	100	100	100	100
#4	U, AE	19.9	+	97	100	100	87	87	90
#5	AE	6.06	+	nd	nd	nd	nd	nd	nd

A: asthma, AE: angioedema, AN: anaphylaxis, U: urticaria.
a) nd: not done.
b) After fish ingestion.
c) CAP: capsulated hydrophobic carrier polymer (kU/L).

were subjected to gel filtration chromatography on a HiPrep 16/60 Sephacryl S-200 High Resolution column (GE Healthcare). The whiff parvalbumin, designated Lep w 1.0101 by the IUIS allergen nomenclature sub-committee, was separated from higher molecular weight proteins and eluted from the column as one peak. The fractions were analyzed by SDS-PAGE and the purified parvalbumin detected by a polyclonal anti-Gad m 1 antibody (Tepnel BioSystems).

2.4 N-terminal sequencing

The N-terminus of Lep w 1 was obtained as described for Gad m 1 [6].

2.5 Structure modelling

The structure of *L. whiffiagonis* parvalbumin (Lep w 1) was modelled using the Swiss-Model server (http://swissmodel.expasy.org [19]). The structure of carp parvalbumin (PDB code 5cpv [20]) was used as the template. Visualization of the structure was carried out using DeepView/Swiss-PdbViewer 4.0 [19].

2.6 CD spectroscopy

Far ultraviolet CD spectra of native and unfolded Lep w 1 (unfolding induced by heat, Ca^{2+}-depletion and at pH 2.5) were recorded with a JASCO J-810 spectropolarimeter (Jasco, Essex, UK) at 20°C in aqueous solutions. Protein samples were dissolved in 10 mM K-phosphate buffers pH 7.0 and 2.5 and were diluted to 0.1 μg/μL (as determined by the BCA Protein Assay Reagent Kit, Pierce) and measured in quartz cuvettes (Hellma, Müllheim, Baden, Germany) of 0.1 cm path length. CD between 190 and 260 nm was monitored at 0.5 nm intervals. Each spectrum was obtained by averaging three individual runs and corrected by subtraction of the solvent spectrum obtained under identical conditions. The results were expressed as mean residue ellipticity. The temperature dependence of the CD at 222 nm was measured at heating rates of 2°C/min, full spectra were recorded from 25 to 95°C at intervals of 10°C, and the reversibility of the heat-induced unfolding transition was assayed by measuring the spectrum of the sample cooled down in a single step to 25°C.

2.7 IgE ELISA inhibition assay

To evaluate the recognition of the treated proteins by patients' serum IgE, Covalink NH plates (Nunc, Roskilde, Denmark) were used. The assay was analyzed in duplicates. Samples were heat-treated by incubation for 10 min at 95°C. For calcium depletion, 5 mM EGTA were added to the protein solution and subsequently incubated for 1 h. Purified Lep w 1 (2 μg/mL diluted in 50 mM KPO_4, pH 8.2) was covalently bound to activated (1.25% v/v glutaraldehyde in 50 mM KPO_4, pH 8.5, overnight, 37°C) microtiter plates overnight at 4°C. Plates were saturated with 1 M ethanolamine, pH 8.0, and subsequently, non-specific binding sites blocked by Tris-buffered saline, 0.5% Tween-20 (TBST), 3% w/v BSA. Four individual patients' sera (diluted in TBST, 0.25% w/v BSA) were preincubated with 50 μg/mL untreated and treated proteins for 3 h and transferred to ELISA plates. Bound IgE was detected with alkaline phosphatase-conjugated mouse anti-human IgE antibody (BD-Biosciences Pharmingen, San Diego, CA, USA) and developed with a p-nitrophenyl phosphate substrate (Sigma-Aldrich, St. Louis, MO, USA). Buffer and sera of three non-allergic subjects were used as negative controls. Percent inhibition is given as reduction of bound IgE compared to the controls where no inhibitor protein was added.

2.8 SDS-PAGE and immunoblotting

Whiff protein extract and Lep w 1 were separated by SDS-PAGE under reducing conditions. The purity of Lep w 1 was assessed by Coomassie staining. For immunoblotting, proteins were transferred to a nitrocellulose membrane and whiff parvalbumin was detected by a rabbit polyclonal anti-Gad m 1 antibody (Tepnel) as previously described for Gad m 1 [6]. To determine the IgE reactivity of patients' sera to undigested and digested proteins, blotted proteins were incubated with a serum pool of four fish allergic patients and bound IgE was detected with an alkaline phosphatase-conjugated mouse anti-human IgE antibody (BD-Biosciences Pharmingen) and developed with a 5-bromo-4-chloro-3-indolyl phosphate/*p*-nitroblue tetrazolium chloride reagent solution.

2.9 *In vitro* gastric and duodenal digestion

In vitro gastric (phase I) and duodenal (phase II) digestions were performed as described by Moreno *et al.* [21]. Digestions of purified Lep w 1 with or without bound Ca^{2+} were performed. For calcium depletion, 5 mM EGTA in KPO_4 buffer, pH 7.5, was added and the protein solution incubated for at least 1 h. Half a milligram of Lep w 1 (2 mg/mL) with or without EGTA treatment was dialyzed against SGF (0.15 M NaCl, pH 2.5) for 3 h at room temperature and diluted with SGF to 0.15 mg/mL. The pH was adjusted to 2.5 with 1 M HCl. A solution of porcine pepsin (3.2 mg/mL in SGF, Sigma-Aldrich; 4230 U/mg) was added at an approximately physiological ratio of enzyme:substrate (1:20, w/w). The digestion was carried out at 37°C and aliquots were taken from the single digestion mixture at 0, 2, 5, 15, 30, 60 and 120 min for further analysis. The reaction was

stopped by raising the pH to 7.5 by the addition of 1 M NaOH. Uncooked or cooked protein or protein extract dialyzed against SGF, 0.15 M NaCl, pH 2.5, was used as negative controls.

In vitro duodenal digestion was performed as previously described [21] using 120 min *in vitro* gastric digesta as a starting material. Intestinal digestion was carried out at 37°C, with shaking for 120 min using a 125 mM bile salt mixture containing sodium taurocholate, glycodeoxycholate (Sigma-Aldrich), 9.2 mM $CaCl_2$ and 25mM *Bis*-TRIS-HCl, pH 6.5 mM. A solution of trypsin (Sigma-Aldrich, 12 400 U/mg) and α-chymotrypsin (Sigma-Aldrich, 52 U/mg) was added at approximately physiological ratios of protein:trypsin:chymotrypsin = 1:400:100 w/w/w. Aliquots were taken at 2, 5, 15, 30, 60 and 120 min for analysis. The digestion was stopped by adding a solution of a Bowman–Birk trypsin–chymotrypsin inhibitor from soybean (0.25 mg/mL in water; Sigma-Aldrich). For control of gastric and duodenal enzymes, α-lactalbumin was digested under identical conditions. Samples without added enzymes were used as negative controls.

3 Results

3.1 Extraction and purification

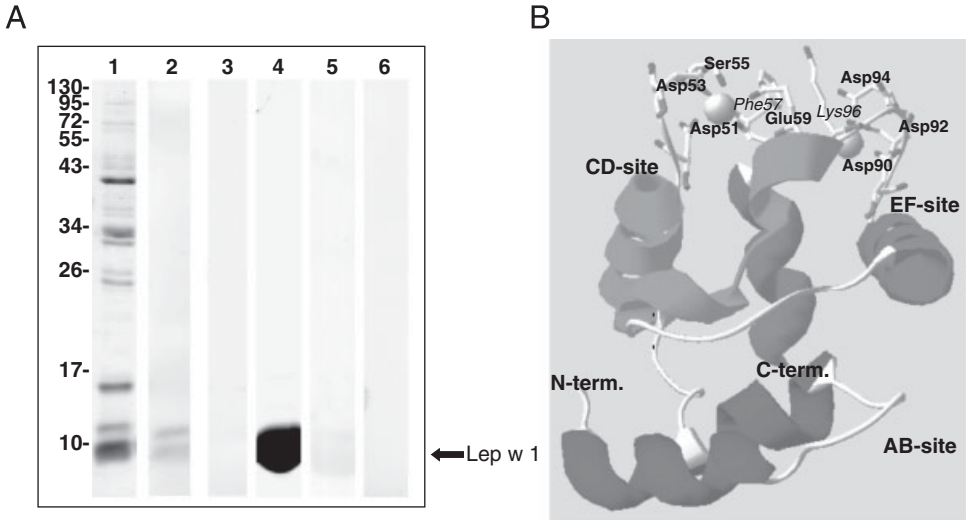
Whiff parvalbumin was abundant in the protein extract as visualized by Coomassie staining and immunoblotting using an anti-Gad m 1 antibody (Fig. 1A, lanes 1, 2). Thirty milligrams of one isoform, Lep w 1.0101, was obtained from approximately 100 g fish filet. Coomassie staining of the purified parvalbumin showed only one band at 11 kDa (Fig. 1A, lane 4). The identity was confirmed by immunoblotting using a polyclonal anti-Gad m 1 antibody (Fig. 1A, lane 5) and N-terminal sequencing. The initiating methionine was cleaved off, resulting in the first five amino acid residues TFAGL with an *N*-acetyl modification of the N-terminal threonine.

3.2 Structure modelling

On the basis of the conserved EF-hand motifs of parvalbumins, a model of Lep w 1 (Acc. No. AM904681) was constructed using the structure of the calcium-bound carp parvalbumin (PDB Acc. No. 5cpv) as template (Fig. 1B). The sequence alignment used for building the Lep w 1 model is shown in Fig. 1C. This alignment exhibits 69% amino acid sequence identity. The N-terminal calcium-binding loop showed four conserved amino acid residues (Asp 51, Asp 53, Ser 55 and Glu 59) and the backbone oxygen of Phe 57 as binding partners. Four conserved interaction partners of the molecule with calcium were found at the C-terminal loop (EF loop) (Asp 90, Asp 92, Asp 94 and the backbone oxygen of Lys 96) (Fig. 1B).

3.3 Stability studies by CD

The extent of secondary structural changes induced in Lep w 1 at different pH values and temperatures was measured by



C

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Lepw1   TFAG-LDAAEIKAALDGCAAADSFDYKKFFGACGLAKKSAEEVKAAFNKIDQDESGFIEE  59
5cpv    AFAGVLNDADIAAALEACKAADSFNHKAFFAKVGLTSKSADDVKKAFAIIDQDKSGFIEE  60
             *: *:* ***:.* *****::* **.  **:.***::** **  ****:******

Lepw1   DELKLFLQNFSASARALTDKETANFLKAGDVDGDGKIGIEEFTDLVRSK  108
5cpv    DELKLFLQNFKADARALTDGETKTFLKAGDSDGDGKIGVDEFTALVKA-  108
        **********.*.****** ** .****** *******::*** **:
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Figure 1. (A) Purification and characterization of whiff parvalbumin. Lane 1: 50 μg whiff protein extract detected by Coomassie staining (lane 1) and immunoblotting (lane 4) with an anti-Gad m 1 antibody; lane 4: Coomassie staining of 20 μg purified Lep w 1; lane 5: detection of Lep w 1 by an anti-Gad m 1 antibody; lanes 5 and 6: negative controls of anti-Gad m 1 immunoblots. (B) Secondary structure of modelled Lep w 1 (Acc. No. AM904681). Binding partners of calcium are indicated. Bold: bidentate ligation; italic: monodentate ligation; spheres: calcium. (C) Sequence alignment for building the Lep w 1 model. Ca^{2+}-binding sites are underlined; bold: bidentate cation-binding partners, italic: monodentate-binding partners.

far-UV CD. The CD spectrum at pH 7.0 showed characteristic double minima at 208 and 222 nm, and a maximum at 190 nm (Fig. 2A, black line). The secondary structure of the native protein was highly resistant to thermal unfolding. Temperatures higher than 65°C were reached before Lep w 1 started to unfold (Fig. 2C, black line). Upon cooling, the denaturation was reversible (Fig. 2A, black dotted line). In contrast, acidification to pH 2.5 changed the overall structure of the protein without unfolding it. These changes are reflected by a deeper minimum at 208 nm and a shallower one at 222 nm as well as a slight shift of the intersection with the *x*-axis to a lower wavelength, while retaining the maximum at 190 nm (Fig. 2A, grey solid line). These structural changes were even more pronounced after heating to 95°C (Fig. 2A, grey dotted line). The thermal denaturation curve at pH 2.5 showed a continuous increase in ellipticity without a clear melting point (Fig. 2C, grey line).

Conformational changes and structural stability were further tested by Ca^{2+} depletion using EGTA at pH 7.0 and 2.5. The calcium-depleted Lep w 1 showed no minimum at 222 nm. Two nearly identical spectra of the untreated and heat-treated calcium-depleted protein at pH 7.0 were obtained (Fig. 2B, dotted black and grey line). The negative ellipticity at 222 nm and the maximum at 190 nm were remarkably diminished. The thermal denaturation curve of the calcium-depleted protein showed a loss of structure already at room temperature (Fig. 2C, dotted line). At pH 2.5, the spectrum of Lep w 1 obtained after EGTA treatment (data not shown) was superimposable to the spectrum without EGTA (Fig. 2A, grey line).

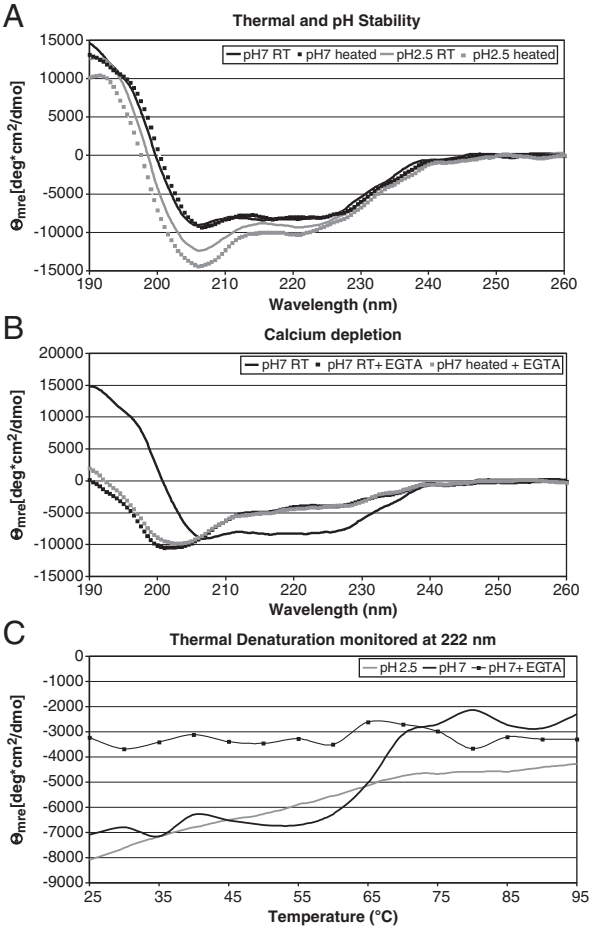

Figure 2. Stability of Lep w 1 studied by CD: (A) CD spectrum of thermally untreated (solid line) and heated (dotted line) protein at neutral (black) and acidic (grey) pH. (B) CD spectrum of calcium-depleted protein (+EGTA) before and after thermal treatment; black: calcium-bound, black dotted: calcium-depleted, grey dotted: calcium-depleted and cooked protein. (C) Thermal unfolding of Lep w 1 monitored at 222 nm wavelength. Black: pH 7.0; dotted: calcium-depleted (+EGTA) and grey: pH 2.5. RT: room temperature.

3.4 IgE reactivity of EGTA and heat treated Lep w 1

Pretreated proteins were further tested by IgE ELISA inhibition assays. Four individual fish allergic patients' sera containing IgE specific for Lep w 1 were used. Preincubation of the sera with thermal and EGTA-treated proteins showed similar inhibitions of IgE binding to immobilized native Lep w 1 (approximately 90–100%) (Table 1).

3.5 *In vitro* gastric digestion of purified parvalbumin

Calcium-bound and depleted parvalbumins were evaluated individually for gastric digestibility in SGF (Fig. 3). Five seconds after adding pepsin, most of the uncooked Lep w 1 was degraded without and with adding EGTA (Fig. 3, lanes 2 and 4). Two fragments of lower molecular weight of approximately 4.5 kDa were still visible at the earliest time point of the digestion in Coomassie staining (Fig. 3, lanes 2 and 4). Both fragments were completely digested by pepsin within 2 min (Fig. 3, lanes 3 and 5). α-Lactalbumin, a

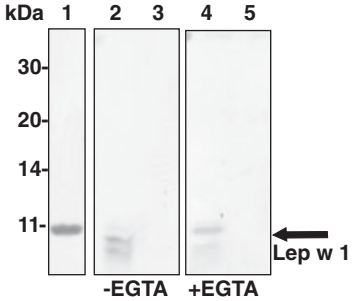

Figure 3. Coomassie staining of *in vitro* gastric digestion of uncooked whiff parvalbumin calcium-bound (−EGTA) or calcium-depleted (+EGTA). Lane 1: undigested Lep w 1 in SGF, pH 2.5; lanes 2 and 4: 5 s after adding pepsin; lanes 3 and 5: 2 min gastric digestion.

positive control, was degraded within 120 min as published previously [22] (data not shown).

3.6 IgE binding to uncooked and cooked fish extracts

The IgE-binding protein patterns of uncooked and cooked fish extracts were compared by IgE immunoblotting (serum pool of ♯1, 2 and 4). The protein extract of thermally treated fish revealed a higher number of IgE reactive bands than the extract of uncooked fish (Figs. 4A and B, lane 1). Several high-molecular-weight bands ranging from 55 kDa to about 130 kDa were observed in the cooked fish extract (Fig. 4B, lane 1). On the other hand, a double band of about 40 kDa appeared only in the uncooked fish extract (Fig. 4A, lane 1). An 11-kDa protein band corresponding to Lep w 1 was detected in both extracts. The immunoreactivity of the extracts was analyzed by IgE inhibition experiments. IgE binding to the high-molecular-weight proteins formed after cooking could not be inhibited by the extract of uncooked fish (Fig. 4B, lanes 2 and 3). In contrast, cooked extract showed 100% inhibition of IgE binding to uncooked proteins (Fig. 4B, lanes 2).

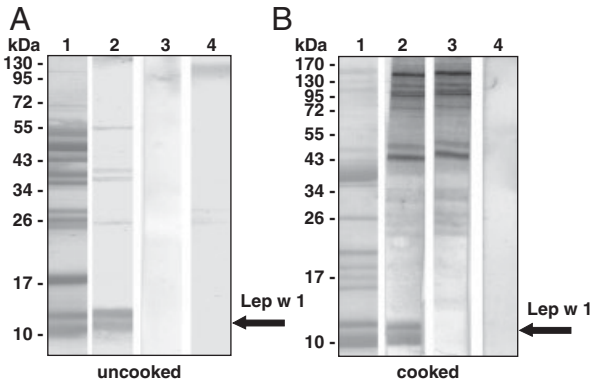

Figure 4. IgE immunoblot inhibition assay using cooked and uncooked whiff protein extract. (A) Lane 1: CBB staining of uncooked extract; lane 2: IgE immunoblot of uncooked extract; lane 3: inhibition of IgE binding from fish allergic patients' sera to uncooked extract with cooked extract and lane 4: normal human sera. (B) Lane 1: CBB staining of cooked extract; lane 2: IgE immunoblot of cooked extract; lane 3: inhibition of IgE binding from fish allergic patients' sera to cooked extract with uncooked extract; lane 4: normal human sera; Lep w 1 is indicated by arrows.

3.7 *In vitro* gastric digestion of uncooked and cooked fish extracts

To assess whether the heating of fish proteins affected the digestion rate, the degradation of proteins from uncooked and cooked fish by pepsinolyis was evaluated. By *in vitro* gastric digestion, uncooked fish extract (Fig. 5A, lane 1) was degraded to small fragments still visible on Coomassie stained protein gels after 15 min (Fig. 5A). In contrast to raw fish extract, the cooked extract (Fig. 5B, lane 2) showed higher resistance to gastric proteolysis. Cooked extract precipitated in SGF at pH 2.5, and subsequent *in vitro* gastric digestion gave rise to a complex mixture of proteins immediately after adding pepsin (Fig. 5B, lane 5''). Some of them were still visible at 120 min after starting the digestion reaction (Fig. 5B). In detail, Lep w 1 (11 kDa band) started degrading immediately after adding the enzyme. As digestion proceeded, the protein with a molecular weight of 130 kDa showed a partial breakdown and disappeared after 60 min (Fig. 5B). Fragments of another abundant protein at approximately 34 kDa were still detected by Coomassie staining after 2 h of pepsinolysis. Additionally, one protein band at 24 kDa (Fig. 5B, lane 2), absent in uncooked fish (Fig. 5B, lane 1), was only degraded after 5 min. A turbidity of the sample was observed during all 120 min of incubation time. After raising the pH to 6.5, mimicking the transfer to

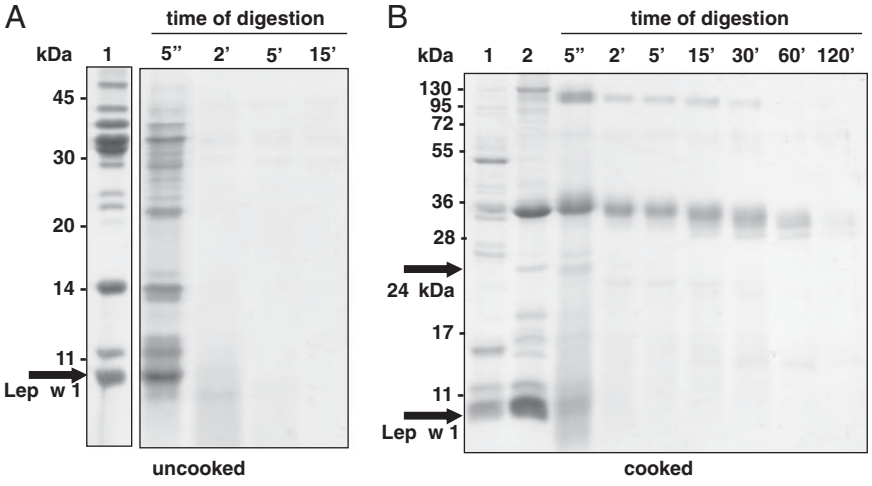

Figure 5. Coomassie staining of *in vitro* gastric digestion of uncooked and cooked fish protein extracts. (A) Uncooked extract. Lane 1: raw fish extract in SGF, pH 2.5; lanes 5″, 2′, 5′ and 15′: seconds or minutes of digestion, respectively. (B) Cooked fish extract. Lane 1: raw extract in SGF, pH 2.5; lane 2: cooked extract in SGF, pH 2.5; lanes 5″, 2′, 5′, 15′, 30′, 60′ and 120′: seconds or minutes digestion, respectively. The Lep w 1 dimer is indicated by an arrow.

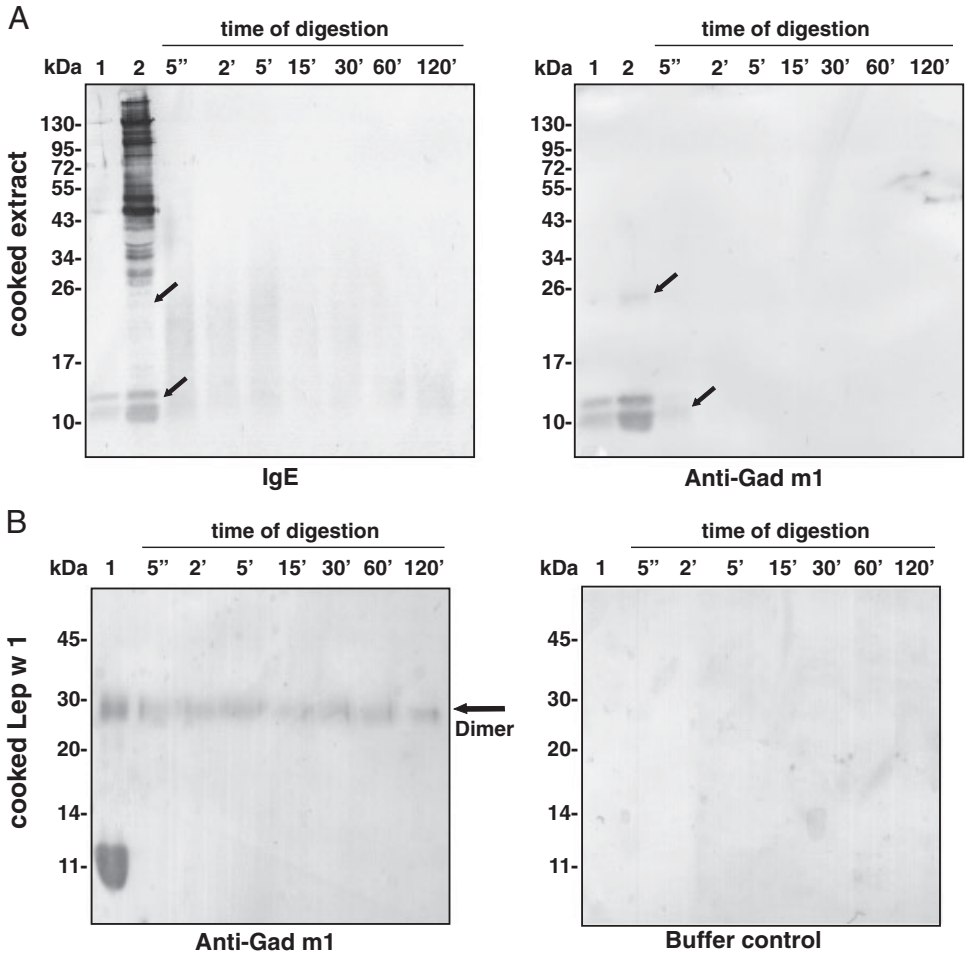

Figure 6. (A) Immunoblot of cooked fish extract after *in vitro* gastric digestion. Left: IgE immunoblot using a pool of fish allergic patients' sera no.1, 2, 3 and 5. Right: Detection of whiff parvalbumin by an anti-Gad m 1 antibody. Lane 1 native and lane 2 cooked fish proteins in SGF, pH 2.5 (50 μg each). Monomeric and dimeric Lep w 1 are indicated by arrows. (B) Anti-Gad m 1 immunoblot (left) and buffer control (right) of cooked Lep w 1. Lane 1: Cooked Lep w 1 in SGF, pH 2.5 (2.5 μg). Lanes 5″, 2′, 5′, 15′, 30′, 60′ and 120′: seconds or minutes digestion, respectively. Dimeric Lep w 1 is indicated by an arrow.

the duodenum, precipitates were dissolved and completely degraded after adding duodenal digestion enzymes. After 5 s of duodenal digestion, no protein fragments were detectable by Coomassie staining (data not shown).

The digested uncooked and cooked protein extracts were tested for IgE reactivity by immunoblotting. A pool of patients' sera no. 1, 2, 3 and 5 was used. The immunoblot of uncooked protein extract showed that the immunoreactivity was lost after 15 min (data not shown). In contrast, patients' serum IgE bound to fragmented proteins of cooked extract even at 120 min (Fig. 6A, left). The IgE binding decreased as the proteins were degraded. In addition, weak IgE binding to a 24 kDa protein was detected in cooked extract. No IgE bound to the abundant 34 and 130 kDa proteins after adding pepsin. To evaluate the duration of complete digestion of Lep w 1, the anti-Gad m 1 antibody was used for immunoblotting. A double band at 11 kDa corresponding to whiff parvalbumin was detected in uncooked (Fig. 6A, right, lane 1) and cooked extract (Fig. 6A, right, lane 2). Additionally, the anti-Gad m 1 antibody recognized a 24 kDa protein corresponding to the dimeric parvalbumin in the non-digested cooked extract (Fig. 6A, right, lane 2), but failed to detect the Lep w 1 monomer as early as 2 min after the start of digestion in cooked (Fig. 6A, right) and uncooked extract (data not shown). The digestion of cooked purified Lep w 1 was analyzed by immunoblotting with anti-Gad m 1 (Fig. 6B, left). A pronounced band at 11 kDa corresponding to the Lep w 1 monomer and a 24 kDa protein band were detected in the undigested control (Fig. 6B, left, lane 1). After 5 s of digestion, the monomer of Lep w 1 was no longer visible (Fig. 6B, left, lane 5″), whereas the dimer only showed a partial degradation after 5 s, but was still recognized after 120 min.

4 Discussion

Food proteins that can sensitize and elicit allergic reactions are characteristically structurally stable to thermal treatments and gastrointestinal digestion. Thus, the intact allergen can be presented to the gut immune system. Besides the stability, interactions with the food matrix and physicochemical modifications induced by food processing are also responsible for the allergenicity of proteins [9].

The β-parvalbumin of whiff, Lep w 1, was stable to thermal treatment up to 65°C, but only when calcium was bound as a ligand and the tests were performed at neutral pH. In contrast to natural Gad m 1 [6], Lep w 1 lost its structure completely when calcium was depleted from the protein. In line with an earlier study of Gad m 1, Lep w 1 underwent a structural change at pH 2.5, resulting in a loss of the broad minimum observed at 222 nm, (Fig. 2A). As the glutamate residues became uncharged at acidic conditions [6], the calcium ion of the EF-loop closer to the surface [20] was lost, only the calcium of the CD-site persisted due to the stronger binding of five binding partners. Interestingly, the structural change at pH 2.5 did not influence the IgE-binding ability of the protein tested by an IgE ELISA inhibition assay (Table 1). All treated proteins showed similar percentages of inhibition of approximately 90–100%. This is in contrast to one report that indicated a much stronger IgE binding to the calcium-bound form of carp parvalbumin as opposed to the calcium-depleted protein [14]. It was suggested that the binding sites of parvalbumin-specific IgE were distant from the calcium-binding loop, and only the conformational changes after calcium depletion impaired the IgE-binding ability [23].

The CD measurements showed a partial denaturation of the protein at pH 2.5, which could explain the efficient pepsinolyis of Lep w 1 within seconds in *in vitro* gastric digestion assays. No difference of gastric stability was observed between the EGTA treated and untreated parvalbumin as the chelator was inactive in the acidic SGF, pH 2.5 (Fig. 3).

It is interesting to note that whiff parvalbumin, although a major allergen, is completely degraded by pepsin within seconds. Therefore, we examined the role of processing. As only thermally treated whiff is consumed in Spain, we extracted proteins from boiled and raw whiff filet and determined their IgE reactivity. IgE binding to various proteins within the high-molecular-weight range was only observed in the cooked fish extract (Fig. 4) as described previously for Indian fish species [24]. The IgE reactivity to these proteins could not be inhibited by raw fish extract (Fig. 4B). These findings were in accordance with an already published report of Bernhisel-Broadbent *et al.* [18], where immunoreactive high molecular weight protein aggregates were formed from cooked protein extracts of tuna, salmon, cod and flounder. Subsequently, we examined the digestibility of the whole fish protein extract compared with uncooked fish and determined the IgE-binding capacity of the resulting digestion fragments by IgE immunoblotting. IgE from fish allergic patients' sera could detect fragments after 15 min of gastric digestion of raw fish extract (data not shown), which is in accordance with a digestion study of raw cod protein extract, using a gastric tablet from Fédération International Pharmaceutique [15]. In contrast, cooked fish extract fragments were still recognized by patients' IgE after more than 120 min of digestion by pepsin (Fig. 6A). As one report detected aggregates or polymers of parvalbumins by an anti-parvalbumin antibody in raw cod extracts depending on storage duration [25], we tested our cooked protein extract for parvalbumin oligomers with an anti-Gad m 1 antibody. The antibody failed to recognize high-molecular protein bands in cooked fish, but detected a protein at 24 kDa (Fig. 6B). Such a molecular weight is characteristic for a parvalbumin dimer [6, 26]. The protein was also detected by immunoblotting of cooked purified Lep w 1. The monomeric Lep w 1 was digested after 5 s, whereas the dimer was still detected after 120 min using the anti-Gad m 1 antibody. Because of the low parvalbumin content of the cooked total extract, we could not detect the parvalbumin dimer by fish allergic patients' IgE and the anti-Gad m 1 antibody.

We observed that, despite the remarkable stability to heating, Lep w 1 was easily digested using physiological gastric conditions. Additionally, food processing such as cooking could generate dimers that were partially stable towards gastric digestion. It is likely that the observation of stable parvalbumin dimers and the formation of protein aggregates after cooking explain the high allergenicity of this fish. However, to explain the role of food allergens, the importance of the food matrix should also be considered.

The support by the Austrian Academic Exchange Service (ÖAD) under "Acciones Integradas 2007-2008" project no. ES 01/2007, by Acciones Integradas Hispano Austriacas project no. HU2006-2008 and by the Austrian Science Fund grant SFB F1802 is gratefully acknowledged. The authors thank Nina Balazs for her excellent technical assistance.

The authors have declared no conflict of interest.

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