

Emulsification of Simulated Gastric Fluids Protects Wheat α -Amylase Inhibitor 0.19 Epitopes from Digestion

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Abstract Wheat α -amylase inhibitors (α -AIs) are known anti-nutritional factors, respiratory allergens, and they can sporadically cause food allergy. α -AIs are therefore expected to reach the enteric mucosae in an immunologically active form, but information on their stability to gastric digestion is not available. Resistance to pepsinolysis is nonetheless a key factor for any food allergen. We therefore investigated whether α -AIs could resist pepsin digestion in simulated gastric fluids (SGF) and in emulsified SGF, the latter simulating more realistically the multiphase nature of stomach bolus. Since α -AIs comprise a huge family of proteins, we investigated 0.19 α -AI as a prominent member. The digestion patterns were analyzed by immunoblotting using anti-0.19 polyclonal antibodies and sera from wheat allergic patients sensitized to 0.19 α -AI. The results show that the immune epitopes of α -AI are detectable up to 120 min of digestion in emulsifying conditions. Intra-molecular disulfide bonds and, in particular, emulsification were found to be crucial factors for protein stability. The results show that 0.19 α -AI must be considered a potential food allergen.

Keywords α -Amylase inhibitors · Emulsion · Pepsin · Simulated gastric fluids (SGF) · Wheat allergy

Abbreviations

α -AI α -Amylase inhibitor
SGF Simulated gastric fluids
ESGF Emulsified simulated gastric fluids
SPT Skin prick test

Introduction

The wheat albumin/globulin fraction contains a number of proteins, among which α -amylase/trypsin inhibitors represent a major subset. Depending on their quaternary structure, α -amylase inhibitors (α -AIs) are subdivided in monomeric, dimeric, and tetrameric proteins. Several members of this family have been characterized so far showing up to 98% sequence homology (Feng et al. 1996; Franco et al. 2002; Tatham and Shewry 2008; Salcedo et al. 2004). Interestingly, their inhibition activity toward pests, animal, and human digestive enzymes was shown to be extremely different in affinity and specificity (Silano et al. 1975). For this reason, they have been suggested to act as a defense mechanism that might in principle be transferred to other plants of agronomic interest through genetic engineering (Franco et al. 2002).

Besides their biological activity, α -AIs have been recognized as strong inhalant allergens, capable of eliciting respiratory symptoms to flour that frequently lead to the occupational pathology known as baker's asthma (Gomez et al. 1990; Armentia et al. 1993; Franken et al. 1994; Weiss et al. 1997; Amano et al. 1998; Sanchez-Monge et al. 1992). These polypeptides have been associated also with

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food allergy, including wheat-dependent exercise-induced anaphylaxis and children atopic dermatitis, caused by the ingestion of cooked wheat derivatives (James et al. 1997; Kusaba-Nakayama et al. 2000; Zapatero et al. 2003; Weichel et al. 2006; Pastorello et al. 2007). Differently from inhalant allergens, food allergens must resist food processing and gastrointestinal digestion in order to reach intact the enteric mucosa, sensitize the immune system, and elicit adverse reactions. The only exception to this rule is given by the so-called incomplete food allergens (e.g., Bet v 1 homologues), a group of proteins capable of inducing allergic symptoms mostly limited to the oral cavity as a result of a previous sensitization by cross-reacting inhalant allergens (e.g., pollens). FAO/WHO (2001) published a report detailing how to assess the allergenic potential of genetically modified foods and more in general novel foods. More recently (Alinorm 2003), these safety assessment recommendations were improved by the Codex Alimentarius Commission that outlined a “body-of-evidence approach” in which the determination of protein stability to pepsin digestion is a key parameter to define a complete food allergen. The rationale is that nutritionally safe proteins are considered those that are rapidly digested and cleared; on the contrary, allergens, or fragments thereof carrying immunogenic epitopes, must resist digestive processing. It is widely accepted that this can be evaluated *in vitro* by means of simulated gastric fluids (SGF; Besler et al. 2001; Astwood et al. 1996; Taylor and Lehrer 1996; Burks et al. 1992; Becker 1997; Taylor et al. 1987). Although SGF assays may not actually mimic the complete *in vivo* gastroenteric digestion, the stability of a protein in SGF is believed to be related to the resistance to proteolytic processes that are encountered within the digestive system and/or in the intracellular environment (Goodman et al. 2005; Bannon et al. 2003; Huby et al. 2000; Metcalfe 2005; Dearman et al. 2002).

Burnett and co-workers (Burnett et al. 2002), considering the heterogeneity of a normal meal, showed that well-known allergens, among which the “CM3” wheat α -AI, can absorb to model stomach emulsions. This, in turn, would protect these proteins from pepsin digestion. This finding demonstrates that the multi-phase nature of the bolus can modify qualitatively and quantitatively the digestive process and that protein allergenicity should be investigated in more realistic setups. In order to assess the resistance of wheat α -AIs to pepsin digestion, we chose to investigate the dimeric 0.19 α -AI as a prominent member of the α -AIs family. This protein has indeed been frequently described as inhalant and food allergen (Gomez et al. 1990; Franken et al. 1994; Amano et al. 1998; Weichel et al. 2006), and in addition, it is one of the most abundant proteins in wheat albumin fraction. To compare the effects of the emulsification on pepsinolysis, we treated the 0.19 α -AI by SGF and

emulsified SGF (ESGF), demonstrating that the emulsion protects the protein from digestion. This might help explaining why 0.19 α -AI possesses food allergenic potency.

Materials and Methods

Pepsin (E.C. 3.4.23.1) from hog stomach was from Sigma (Milan, Italy; product no. P6887; activity 3,300 U/mg of solid). Human salivary α -amylase (E.C. 3.2.1.1) was from Sigma (product no. A1031; activity 46 U/mg of solid. One unit releases 1.0 mg of maltose from starch in 3 min at pH 6.9 at 20 °C). All other chemicals, if not differently stated, were from Sigma.

Sample Preparation

Dimeric 0.19 α -AI was purified by gel filtration and reversed-phase high-performance liquid chromatography (RP-HPLC) as previously described (Zoccatelli et al. 2007). Briefly, salt-soluble protein fraction was obtained by extracting 100 g of commercially available wheat flour in 500 mL phosphate-buffered saline (PBS; 20 mM Na-phosphate buffer, 150 mM NaCl, pH 7.2) for 1 h at room temperature. After centrifugation (9,000×g for 30 min at 4 °C), the supernatant was filtered and subjected to protein precipitation with ammonium sulfate at 50% saturation. The pellet was resuspended, dialyzed against distilled water, and lyophilized. Thirty milligrams of crude preparation was dissolved in 2 mL of 100 mM ammonium acetate buffer, pH 6.8, and separated by gel filtration on Sephadex G-100 (110×2.5 cm). The peak corresponding to approximately 25–30 kDa was collected and lyophilized. Several gel filtrations were performed in order to accumulate a protein amount sufficient for all the digestion analyses. The lyophilized sample was solubilized in 0.1% TFA (trifluoroacetic acid) and subjected to reverse-phase HPLC chromatography performed with a Beckman System Gold on a Vydac C4 column (10×250 mm, particle size 5 μ m). Absorbance was monitored at 214 nm. Run parameters were as follows: flow 4 mL/min, linear gradient from 15% acetonitrile (ACN) in 0.1% TFA to 35% ACN in 0.1% TFA in 60 min, and temperature 40 °C. The peak corresponding to 0.19 α -AI was lyophilized. Protein concentration was measured by bicinchoninic acid assay (Pierce, Cheshire, UK) using bovine serum albumin (BSA) as standard.

Simulated Gastric Fluid

Two hundred milligrams of 0.19 α -AI was solubilized in 1 mL of acid solution containing 0.084 N HCl and 35 mM NaCl, pH 1.2 as described by Thomas et al. (2004) and preheated to 37 °C. After addition of pepsin (from a 2-mg/mL

stock solution) in a 1:30 enzyme/protein ratio, the tube was gently vortexed and incubated at 37 °C in a shaking bath. Aliquots of 150 µL were collected at 0, 5, 10, 30, 60, and 120 min.

The digestion was stopped by addition of 50 µL of a 200-mM NaHCO₃ (pH 11) buffer to each sample. An aliquot was removed from the tube before the addition of pepsin and incubated for 120 min in the same conditions. This sample was labeled as undigested (“Und”).

Emulsified Simulated Gastric Fluid

Two hundred milligrams of 0.19 α-AI was solubilized in 0.730 mL of acid solution and mixed with 0.270 mL of *n*-hexadecane at 2.7:1 ratio. The mixture was sonicated with a Microson™ XL-2000 (Qsonica, LLC., Newtown, CT, USA) for 10 s at 4 W (RMS) in order to obtain the emulsion. Once confirmed that pH was 1.2, the emulsion was preheated at 37 °C. After addition of pepsin (1:30 ratio), the tube was vortexed and incubated at 37 °C in a shaking bath. Sampling was performed as described for the SGF protocol.

SDS-PAGE

Samples collected from the simulated digestions were immediately mixed with 2× loading buffer (62.5 mM Tris–HCl pH 6.8, 1.3% (w/v) sodium dodecyl sulfate (SDS), and 10% (w/v) glycerol) and heated at 100 °C for 5 min. The proteins were separated in 16% SDS–polyacrylamide gel electrophoresis (PAGE; Laemmli 1970) by means of a Mini Protean III apparatus (Bio-Rad, Milan, Italy) in 25 mM Tris–192 mM glycine, 0.1% SDS (pH 8.4) under reducing (4% β-mercaptoethanol) or non-reducing conditions. The gels were stained with Coomassie R250.

Urea-PAGE

Electrophoresis in the presence of 8 M urea (U-PAGE) was performed as described previously (Zoccatelli et al. 2007). The running gel (7% polyacrylamide) contained 0.25% gelatinized starch from potato. Samples collected from the simulated digestions were diluted with 2 volumes of 8 M urea as loading buffer. The running buffer was 25 mM Tris–192 mM glycine.

Anti-zymogram

The protocol is detailed in Zoccatelli et al. (2007). Briefly, after U-PAGE, the gels were rinsed in distilled water for 15 min and equilibrated for further 15 min in PBS. After discharging the buffer, human salivary amylase was solubilized in 25 mL of PBS and incubated with the gel at 37 °C for 45 min (final amylase activity 2 U/mL). The

presence of α-AIs in the gel inhibits starch degradation by the enzyme. The inhibition activity was visualized by dipping the gel in iodine solution: dark brown bands, therefore, appear onto a transparent background.

Antibody Production

An immune serum specific to 0.19 α-AI was raised in rabbits (New Zealand White, Charles River). One subcutaneous injection of 0.19 α-AI (200 µg) emulsified in complete Freund’s adjuvant was followed by three injections every 10 days with the same protein amount in incomplete adjuvant. Serum was obtained from blood drawn after 7 days after the final injection. Experiments with rabbits were carried out according to the ethical regulations established by the European Directive 86/609/EEC.

IgG Immunoblotting

Proteins separated by SDS-PAGE were electroblotted onto polyvinylidene fluoride (PVDF) membranes (Millipore, Milan, Italy) at constant voltage (50 V for 150 min) and immunodetected with the anti-0.19 α-AI immune serum raised in rabbits, diluted 1:5,000 in blocking solution (3%, w/v of skimmed dry milk and 0.05% Tween-20 in PBS) for 2 h. After washings, an alkaline phosphatase-conjugated anti-rabbit IgG (clone RG-96, Sigma) was added at a dilution of 1:5,000. Sigma-Fast BCIP/NBT (Sigma) substrate was used to detect bound IgG.

Patients

Two patients allergic to wheat were recruited. Patient A was a 48-year-old male reporting asthma and rhinitis after wheat flour inhalation. Positive skin prick test (SPT) was observed for wheat, grass, mugwort, and dust mites. His serum showed high total IgE (1,910 kU_A/L) and wheat-specific IgE (>100 kU_A/L) titers. Patient B was a 30-year-old woman displaying an uncommon clinical picture: she reports respiratory symptoms (asthma) upon flour inhalation and adverse reactions (vomiting, abdominal pain, and diarrhea) after eating wheat-derived foodstuffs. Positive SPT was observed for wheat, apple, celery, and different pollens (hazel, grass, mugwort, and birch). Total IgE was 327 kU_A/L and wheat-specific IgE was 42 kU_A/L.

IgE immunoblotting

The sera of the patients A and B (named serum A and serum B accordingly) were used to probe the PVDF membranes overnight at 1:20 dilution in blocking solution. Bound IgE was detected by a horseradish peroxidase (HRP)-conjugated anti-human IgE at 1:2,000 dilution

(Southern Biotech, Birmingham, AL, USA) and chemiluminescence reaction. ECL (GE Healthcare Life Sciences, Uppsala, Sweden) and a Chemidoc XRS apparatus (Bio-Rad) were used to this purpose.

ELISA

ELISA inhibition was performed on Maxisorb 96 well-microplates (Nunc A/S, Roskilde, Denmark); 0.19 α -AI (50 ng per well) was non-covalently coated in PBS for 30 min at RT. After washing with PBS, 0.05% Tween 20, the wells were blocked with PTB (PBS, 0.05% Tween 20, 1% BSA) for 30 min at RT.

Inhibition Curve. To define the operating dilutions of the digestion products, we first established inhibition curves incubating for 1 h the rabbit anti-0.19 immune serum and serum A (diluted respectively at 1:1,000 and 1:50 in PTB) with the purified 0.19 α -AI (from 1 to 0.0001 μ g/mL) as inhibitor of antibody binding. The amount of serum B was insufficient to carry out further ELISA experiments. Bound IgE was detected by incubation with an unlabelled mouse monoclonal anti-human IgE antibody (1:500, Southern Biotech) for 1 h followed by an HRP-conjugated anti-mouse IgG antibody (1:2,000, Sigma) for 1 h, both diluted in PTB. Bound IgG was detected by incubation with an

HRP-conjugated anti-rabbit IgG antibody (1:2,000, clone RG-96, Sigma) in PTB. Tetramethylbenzidine (Sigma) was used as chromogenic substrate. Absorbance was monitored at 450 nm by a Power Wave microplate reader (Bio-Tek).

Analysis of Digestion Products. Based on the above analysis, the 0.19 α -AI concentration giving nearly 80% inhibition was selected (0.1 μ g/mL). Protein samples treated with SGF and ESGF were diluted to this protein concentration and used as inhibitor of antibody binding in the same conditions as described above. To calculate the percentage of antibody binding, two controls were included: one performed by omitting serum (considered as the 100% inhibition), the other excluding the inhibitor (= 0% inhibition). All analyses were carried out in triplicate.

Results

Digestion in SGF/ESGF

Two different profiles of hydrolysis were obtained depending on whether 0.19 α -AI was subjected to proteolysis in SGF or ESGF. By SDS-PAGE under reducing conditions, SGF treatment (Fig. 1a) caused the complete loss of the protein (represented by the band at $\sim$ 14 kDa) after 60 min

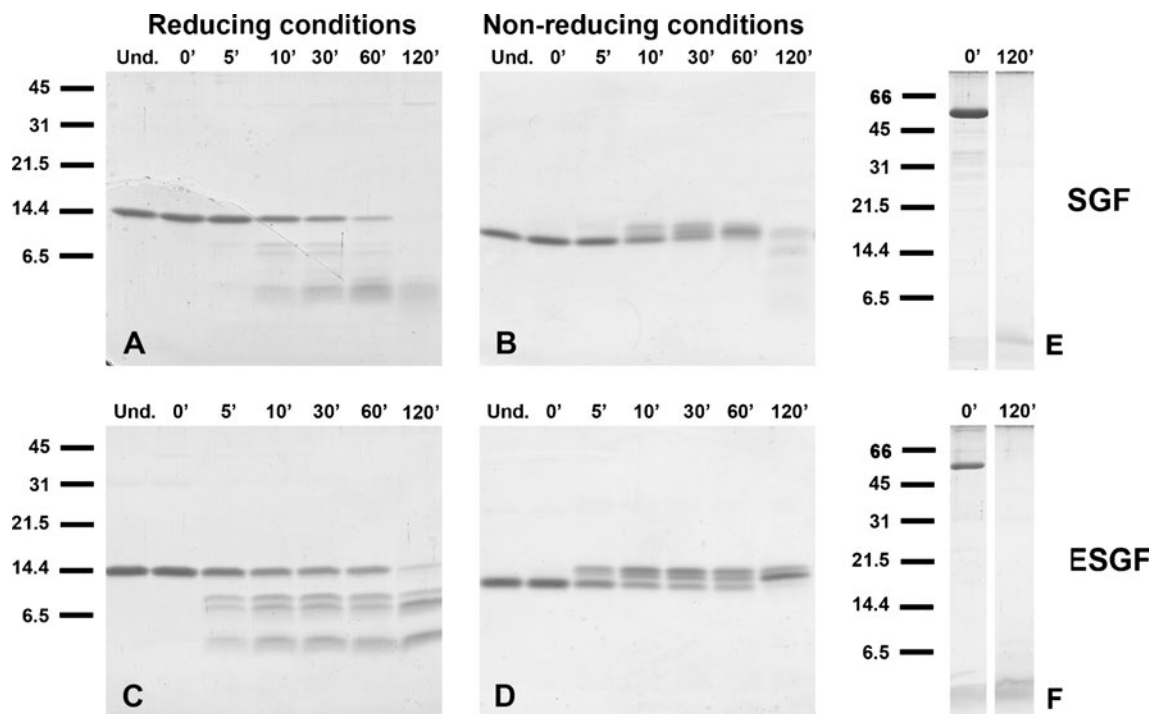


Fig. 1 SDS-PAGE (Coomassie staining) under reducing conditions (**a** and **c**) and non-reducing conditions (**b** and **d**) of the digestion products of 0.19 α -AI in SGF (**a** and **b**) and in ESGF (**c** and **d**). *Und.* sample treated for 120 min without pepsin. 0', 5', 10', 30', 60', 120'

digestion time points in minutes. **e, f** SDS-PAGE analyses (Coomassie staining) under reducing conditions of the digestion products at 0' and 120' min of α -amylase, respectively, in SGF and ESGF

of digestion, while a group of smearing peptides with mass around 4 kDa was still appreciable. These peptides persisted in the gel up to 120 min of treatment, the maximum duration of our experiments, even if a gradual decrease of the staining was evident. The profile obtained by ESGF was quite different (Fig. 1c): The digestion trend was not progressive in time as observed for SGF treatment but characterized by a rapid hydrolysis of the α -AI within a few minutes, with a corresponding appearance of two main fragments besides the smearing peptides. The fragments were observed also in Fig. 1a, but they were less appreciable. This pattern remained almost unaltered at later treatment times, with only a small decrease of the band corresponding to the main protein. In particular, the released fragments seemed to be unaffected by pepsin for the whole duration of the assay. This result is not due to pepsin inactivation, for example, as a consequence of the emulsification step, since a control protein (i.e., α -amylase from *Aspergillus oryzae*) treated with pepsin in ESGF in the same conditions as for 0.19 α -AI was digested giving only very small peptides at 120 min (see Fig. 1f). Similar results were obtained treating the control proteins in SGF (Fig. 1e).

The analysis carried out under non-reducing conditions (Fig. 1b) of the α -AI treated with SGF showed that within the first 5–10 min two major fragments with electrophoretic mobilities slightly lower than those of the undigested protein gradually formed. At 30–60 min, the band of the

wheat α -AI totally disappeared and no lower molecular mass fragments were still observable. Even the treatment with ESGF, once analyzed under non-reducing conditions (Fig. 1d), showed the appearance of these two upper bands, but in this case, they began to form immediately persisting for the whole duration of the experiment.

IgG Immunoblotting with Anti-0.19 Immune Serum

The analysis of the reduced samples collected from both SGF and ESGF treatments by IgG immunoblotting (Fig. 2a, c) showed that some low molecular mass fragments observed in Fig. 1a, c could not be detected. This is not surprising because, even if polyclonal antibodies were used, certain fragments might not harbor recognizable epitopes. Interestingly, under reducing conditions a 12-kDa fragment was detected in ESGF-treated samples only (Fig. 2c) and not in those treated by SGF (Fig. 2a). Under emulsified conditions (Fig. 2c, d), an appreciable amount of protein and derived peptides could be detected up to 120 min, suggesting that immune epitopes of 0.19 α -AI might actually reach the small intestine being resistant to peptic digestion for such long times.

IgE Immunoblotting with Human Sera

To investigate the fate of IgE epitopes of 0.19 α -AI subjected to digestion treatments, we used the sera of clinically

Fig. 2 SDS-PAGE under reducing conditions (a and c) and non-reducing conditions (b and d) of the digestion products of 0.19 α -AI in SGF (a and b) and in ESGF (c and d). Immunoblotting developed with anti-0.19 immune serum. Und. sample treated for 120 min without pepsin. 0', 5', 10', 30', 60', 120' digestion time points in minutes

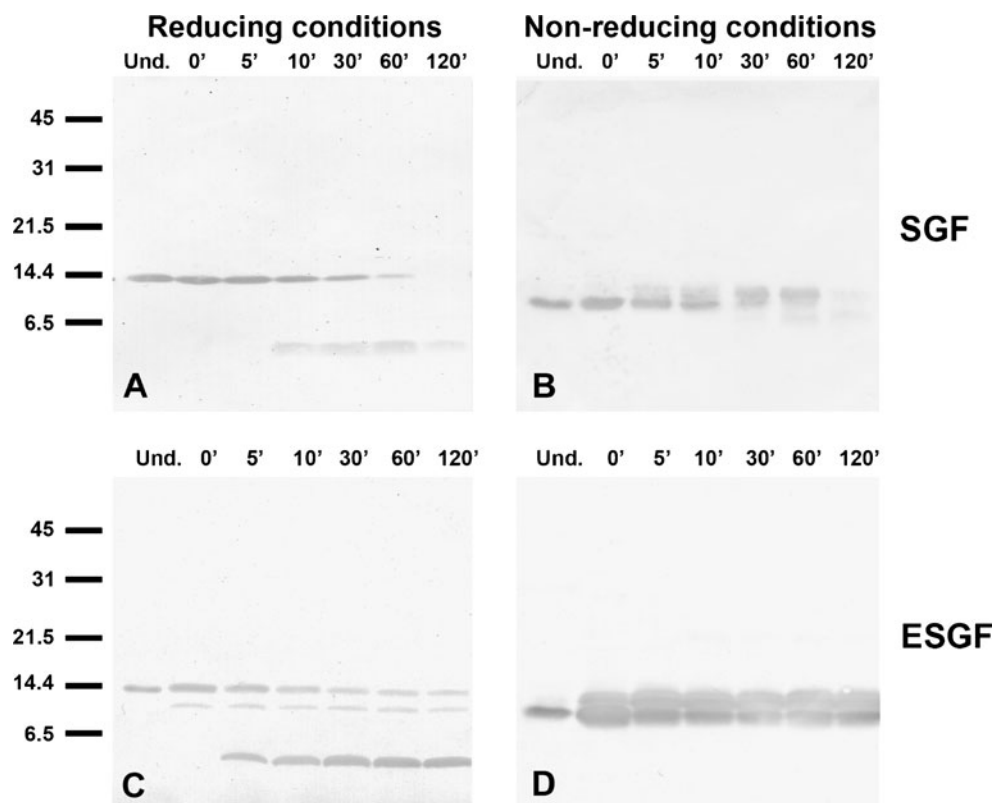
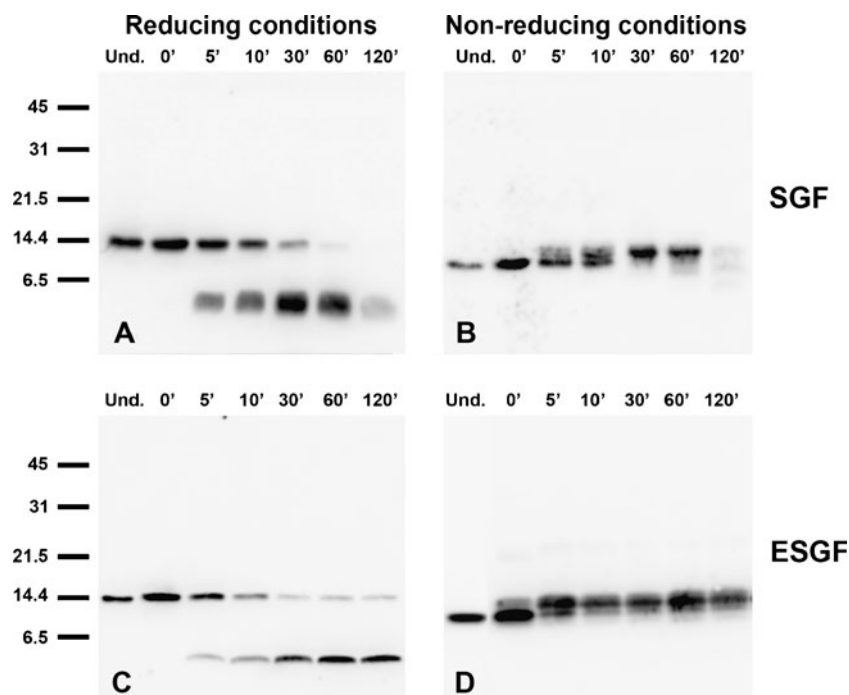


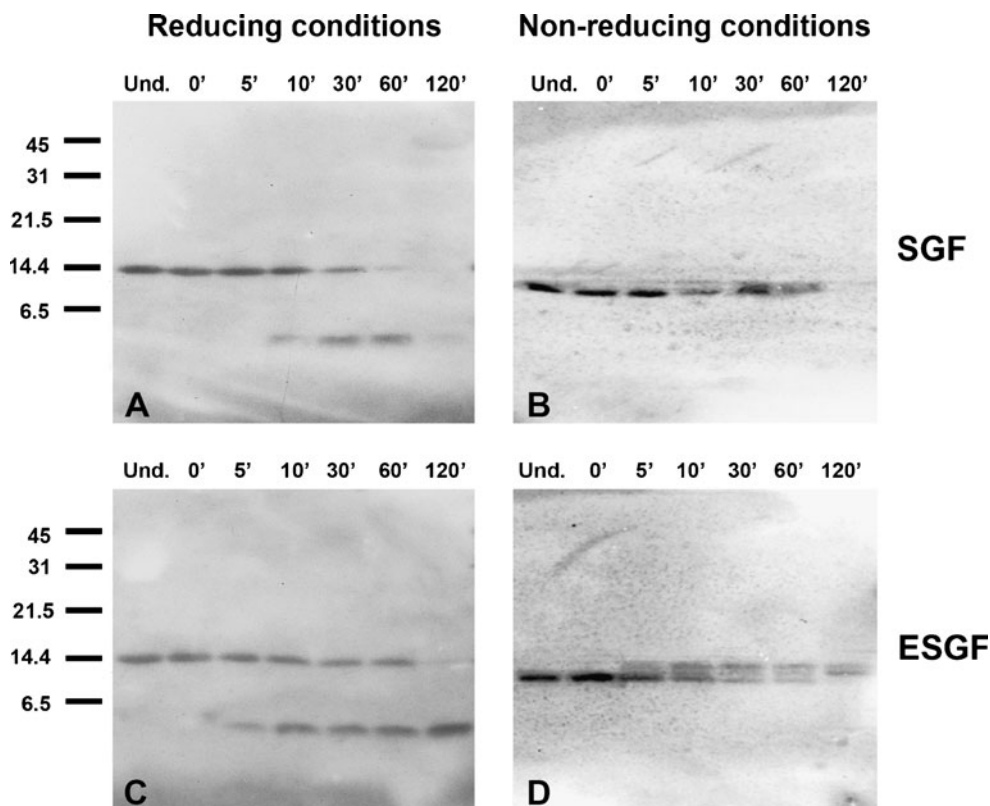
Fig. 3 SDS-PAGE under reducing conditions (**a** and **c**) and non-reducing conditions (**b** and **d**) of the digestion products of 0.19 α -AI in SGF (**a** and **b**) and in ESGF (**c** and **d**). IgE immunoblotting developed with serum A. *Und.* sample treated for 120 min without pepsin. 0', 5', 10', 30', 60', 120' digestion time points in minutes



characterized patients (see “Materials and Methods”). While the IgE immunodetection patterns of the samples collected from SGF treatment almost overlapped those observed with the anti-0.19 polyclonal antibodies, some differences could

instead be observed for the samples subjected to ESGF treatment. In particular, the 12-kDa fragment released after pepsin attack and detected in Fig. 2c was not recognized by the IgE of both patients (Figs. 3c and 4c). This indicates

Fig. 4 SDS-PAGE under reducing conditions (**a** and **c**) and non-reducing conditions (**b** and **d**) of the digestion products of 0.19 α -AI in SGF (**a** and **b**) and in ESGF (**c** and **d**). IgE immunoblotting developed with serum B. *Und.* sample treated for 120 min without pepsin. 0', 5', 10', 30', 60', 120' digestion time points in minutes



that no linear allergenic epitopes are harbored in this peptide. Similarly, under non-reducing conditions, the band of the main protein detected until 120 min in Fig. 2d decreased drastically after 5 min of treatment as shown in Figs. 3d and 4d. The data confirm that (a) IgE epitopes of 0.19 α -AI can reach the enteric mucosae and (b) at least some of the epitopes recognized by the rabbit immune serum and the two human sera are different.

ELISA

We performed ELISA inhibition assays by coating the microplates with 0.19 α -AI and inhibiting the antibody

binding with the same 0.19 α -AI. The results are depicted in Fig. 5a, c and show the typical sigmoid curve. The concentration 0.1 μ g/mL (indicated by the arrow) was taken as the value giving nearly 80% inhibition of the binding as described in “Materials and Methods.” The SGF and ESGF samples were diluted to this concentration and used as inhibitors in the subsequent ELISA assays. Figure 5b, d shows that the SGF digestion products of 0.19 α -AI gradually lost their capacity of inhibiting antibody binding. On the contrary, the inhibition capacity of ESGF digestion products remained constant, indicating that the epitopes were still immunologically active.

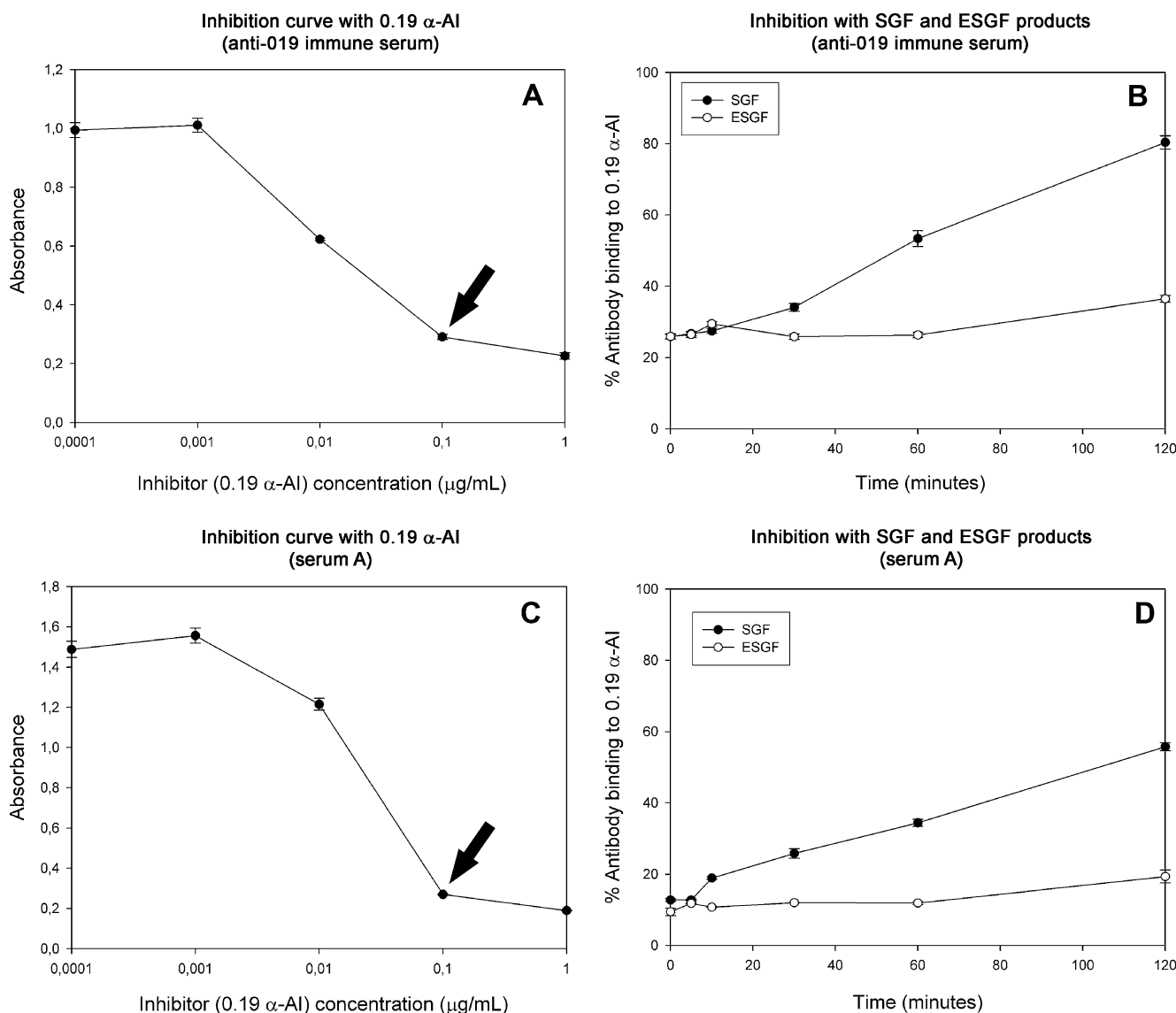


Fig. 5 ELISA inhibition experiments performed by coating 0.19 α -AI. **a, c** The inhibition curves carried out using purified 0.19 α -AI as inhibitor of the binding of respectively the anti-0.19 immune serum and serum A. The inhibitor concentration giving 80% inhibition is

indicated with an arrow. **b, d** The inhibition assays carried out using SGF and ESGF digestion products (diluted to 0.1 μ g/mL protein concentration) as inhibitors of the binding of the same sera

0.19 α -AI Inhibition Activity Test

The principle of this assay is that a polypeptide capable of inhibiting amylase activity protects the starch present in the gel from hydrolysis. The detection is achieved by staining the residual starch with an iodine solution. Since reducing agents could destroy the biological activity of α -AIs, the anti-zymographic assays were performed exclusively under non-reducing conditions. On the other hand, the activity of α -AIs was totally restored after removal of urea from the gels (Fig. 6). The results show that a fraction of the α -AI maintains biological activity up to 120 min of peptic digestion in ESGF (Fig. 6b) following the trend described above (see Fig. 1c, d): an almost instantaneous hydrolysis of a protein fraction (at 0 min) and the persistence of the remaining fraction for the whole duration of the assay. In agreement with the data shown in Figs. 1a and 2a, the inhibition band of α -AI disappeared in 30–60 min of SGF treatment (Fig. 6a).

Discussion

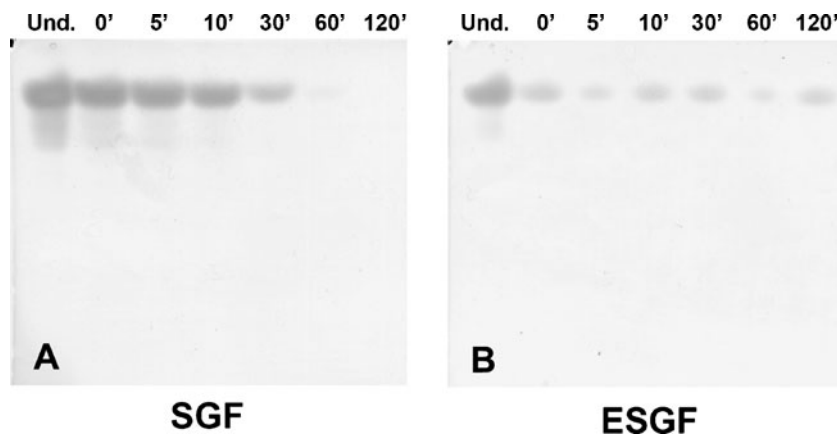
The 0.19 α -AI was digested with pepsin in HCl solution (SGF) or in the same solution emulsified with *n*-hexadecane (ESGF). The fact that human gastric fluids are in an emulsified form is well-known (Armand et al. 1996). The use of *n*-hexadecane to produce physiological-like model stomach emulsions has been already reported (Burnett et al. 2002) and takes advantage from the totally inert and lipophilic character of this molecule. The pepsin/protein ratio used in our experiments was empirically determined to obtain appropriate and visible digestion kinetics, and this allowed us to compare the fate of the protein under the two digestion conditions. The value, set at 1:30, is the same used by other authors (Schmidt et al. 1995; Vassilopoulou et al. 2006; Moreno et al. 2005a), and it is also close to that reported for human digestion *in vivo* (Moreno et al. 2005a).

The results show that there is a dramatic difference between the electrophoretic separation of 0.19 α -AI and related fragments performed under reducing and non-reducing conditions. This can be explained by taking into account the typical conserved motif of intra-molecular disulphide bridges present in wheat α -AIs, a characteristic shared by other low molecular mass proteins belonging to the prolamin superfamily such as non-specific lipid transfer proteins, 2S albumins and indolines (Mills et al. 2004). The fragments can be held together by the presence of disulfide bonds and only after reduction they can separate. The presence of disulfide bonds could further explain the apparent molecular mass increase in SDS-PAGE shown in the gels run under non-reducing conditions. Indeed, the partially hydrolyzed proteins would raise their effective radius as a consequence of a partial unfolding of the structure, hence determining a lower mobility in SDS-PAGE.

The results also show that the 0.19 α -AI is more stable to pepsin digestion in ESGF than in SGF. It is well-known that the stable conformation of a protein, the small dimensions, the good water solubility, and above all its amphiphilicity are ideal biochemical characteristics for a protein to behave as a good emulsifier for an oil-in-water (o/w) emulsion. These characteristics are shared also by 0.19 α -AI; in particular, the balance in hydrophobic and hydrophilic residues is evident by the observation of the hydrophobicity plot (Kyte and Doolittle 1982; Fig. 7). Indeed, the plot shows a marked amphiphilicity of 0.19 α -AI with the hydrophobic minimum (approximately from Asp₆₂ to Gly₇₈) closed to the absolute maximum (from Lys₈₉ to Ser₁₀₈).

Similar results were obtained by Gilbert et al. (2003) for CM3 α -AI, a wheat tetrameric inhibitor subunit already identified as an allergen (Armentia et al. 1993; Kusaba-Nakayama et al. 2000; Zapatero et al. 2003; Pastorello et al. 2007). The hydrophobic regions of α -AI can interact with the oil phase and stabilize the emulsion by reducing the surface tension. It is tempting to speculate that the

Fig. 6 U-PAGE separation and anti-zymogram of the digestion products of 0.19 α -AIs in SGF (a) and in ESGF (b). Und. sample treated for 120 min without pepsin. 0', 5', 10', 30', 60', 120' digestion time points in minutes



0.19 Inhibitor (P01085)

10	20	30	40
SGP ~~W~~ MCYPGQ	AFQVPALPAC	RPLLRLQCNG	SQVPEAVLRD
50	60	70	80
CCQQLAHISE	WCRCGALYSM	LDSMYKEHGA	QEGQAGTGAF
90	100	110	120
PRCRREVVKL	TAASITAVCR	LPIVVDASGD	GAYVCKDVAA

YPDA

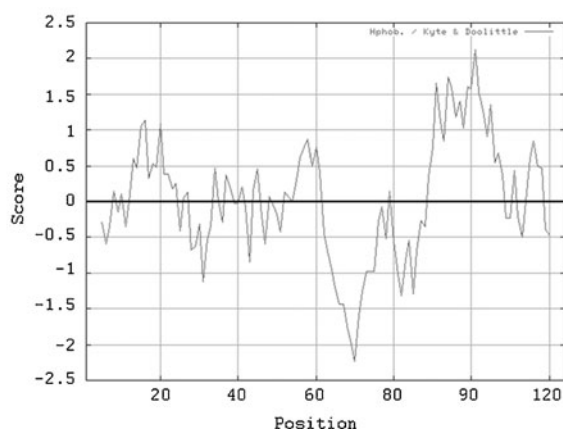


Fig. 7 Hydrophobicity plot of 0.19 α -AI (entry no. P01085) calculated by Kyte and Doolittle (1982) method (<http://www.expasy.ch/tools/protscale.html>)

hydrophobic portions of 0.19 α -AI might therefore be protected from the action of pepsin, and this would explain the higher resistance of α -AIs in ESGF as shown in Fig. 1c, d. It is worth noting that this behavior has already been observed for the major milk allergen α -lactalbumin by Moreno et al. (2005b).

Since it is known that the fragments of an allergen may retain their immune reactivity (Matsuda et al. 1983; Sen et al. 2002; Diaz-Perales et al. 2003), we investigated whether the peptides originated by SGF and ESGF treatments could be recognized by anti-0.19 antibodies. The immune epitopes can be detected up to 120 min of digestion in ESGF. Interestingly, the 12-kDa fragment was not detected by human IgE, and this suggests that the allergenic epitopes might be confined to one of the two terminals of the protein, even if the present data do not allow us to discriminate between the C- or the N-terminal. However, previous studies demonstrated that one linear epitope of the monomeric 0.28 α -AI is harbored in the sequence spanning residues 9–26, suggesting also in the case of 0.19 that the epitope might be located in the same region. The human sera used in this work come from two patients that display different allergic reactions, the first (A) suffers from baker's asthma, while the second (B) suffers from both asthma and gastrointestinal symptoms. Despite this clinical difference, the binding patterns of the sera are almost identical, indicating that the IgE of asthmatic patients recognize α -

AI fragments resulting from pepsin activity. However, the study of the prevalence of IgE binding of 0.19 from the different clinically selected populations is far from our scope. What we aim to report here is that 0.19 α -AI, depending of the physiochemical conditions, shows different kinetics of digestion that end, in the case of ESGF, with the persistence of the protein. These findings and the fact that the derived fragments are bound by the IgE of two selected patients confirm that some epitopes endure pepsinolysis.

In order to evaluate the presence of conformational epitopes that might have been denatured in SDS-PAGE analyses and of small immunoreactive peptides that can be lost during electrophoresis, we performed competitive ELISA experiments using the samples collected along SGF and ESGF treatments as inhibitors of antibody binding. For this purpose, we utilized the anti-0.19 immune serum and serum A. Figure 5b, d shows that the SGF digestion product of the last time point is characterized by a lower inhibition capacity (expressed as 60% increase of antibody binding), proving that not only the fragments generated from 0.19 α -AI possess lower immune reactivity if compared to the undigested protein but also that a part of the immune epitopes is retained. The most important result is that ESGF digestion products maintain their inhibition capacity demonstrating that even after 120 min of pepsin activity the emulsified 0.19 α -AI and the related fragments conserve IgE and IgG epitopes.

Besides their role in the pathogenesis of allergic diseases, wheat α -AIs are considered anti-nutritional factors hampering the activity of exogenous amylases, among which those secreted by human salivary and pancreatic glands (Buonocore and Silano 1986). In order to evaluate the anti-nutritional potential of the 0.19 α -AI subjected to SGF/ESGF treatments, we applied a recent anti-zymographic method based on protein separation by U-PAGE in a starch-containing gel (Zoccatelli et al. 2007). The biochemical activity of 0.19 α -AI is preserved up to 120 min of treatment in ESGF, and this demonstrates that the protein can indeed inhibit human digestive enzymes.

Our results show that 0.19 α -AI is characterized by a significant stability to simulated gastric fluids under experimental conditions that better approximate the physiological digestion of a bolus in the stomach. The higher resistance of α -AIs in ESGF is likely to depend upon their capacity to be absorbed at the water/oil interface, and in fact α -AIs are known to behave as emulsifying agents. This would in turn make the protein less accessible to pepsin and hence resistant to proteolysis for times comparable to those of the human gastric digestion process. This might help explaining why members of this protein family behave as food allergens (James et al. 1997; Kusaba-Nakayama et al. 2000; Zapatero et al. 2003; Weichel et al. 2006; Pastorello et al. 2007).

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