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A matrix effect in pectin-rich fruits hampers digestion of allergen by pepsin *in vivo* and *in vitro*

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Clinical and Experimental Allergy

Abstract

Background It is a general belief that a food allergen should be stable to gastric digestion. Various acidic plant polysaccharides, including pectin, are ubiquitous in fruit matrixes and can form hydrogels under low-pH conditions.

Objective The purpose of this study was to investigate the effect of hydrogel forming polysaccharide-rich fruit matrixes on *in vivo* gastric and *in vitro* pepsic digestion of fruit allergens.

Methods Fruit extract proteins (kiwi, banana, apple and cherry) and a purified major kiwi allergen Act c 2 were digested with simulated gastric fluid in accordance with the US Pharmacopeia. *In vivo* experiments on kiwi fruit digestion were performed on four healthy non-atopic volunteers by examining the gastric content 1 h after ingestion of kiwi fruit. The Act c 2 and kiwi proteins were detected in immunoblots using monoclonal anti-Act c 2 antibodies and rabbit polyclonal antisera.

Results Crude fruit extracts were resistant to digestion by pepsin when compared with commonly prepared extracts. In the gastric content of all volunteers, following kiwi fruit ingestion and immunoblotting, intact Act c 2 was detected with anti-Act c 2 monoclonal antibodies, while kiwi proteins of higher molecular weights were detected using rabbit polyclonal antisera. Addition of apple fruit pectin (1.5% and 3%) to the purified kiwi allergen was able to protect it from pepsin digestion *in vitro*.

Conclusion The matrix effect in pectin-rich fruits can influence the digestibility of food proteins and thereby the process of allergic sensitization in atopic individuals.

Keywords Act c 2, digestibility assay, food allergy, matrix effect, pectin, thaumatin-like protein

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Introduction

Along with other types of allergic diseases, the prevalence of food allergy is increasing. In order to elicit an IgE response, an allergen must be able to enter the body by crossing the mucosal barrier and then survive *in vivo*, while retaining sufficient structural integrity for a sufficient length of time to interact with the immune system. A major characteristic of many food allergens is their resistance to gastric digestion [1]. It is a general belief that food allergens that sensitize patients via the gastrointestinal tract should be able to survive the harsh conditions of the acidic and proteolytic environment of the stomach to reach the immune system of the intestinal mucosa [2, 3]. Readily digestible food proteins could evoke allergic

symptoms but sensitization is driven through a non-oral route with a cross-reactive contact or inhalant allergens as a result of IgE cross-reactive structures shared by pollen and food allergen sources [4]. Such a food allergen, having the potential only to cause peroral elicitation, is often referred to as an incomplete food allergen [5].

The potential allergenicity of proteins expressed in genetically modified organisms is an important item in their safety assessment [2, 6]. The validity of digestion stability as a criterion for protein allergenicity assessment has recently encountered some criticism, implying the need for standardization of assay protocols, as well as for identifying minimal allergologically relevant stability under standardized assay conditions [7, 8]. An effort has been made to standardize a protocol to assess the

digestibility of proteins using simulated gastric fluid [9]. This assay is therefore designed to test the comparative pepsin resistance of different proteins, and not to mimic their *in vivo* digestion stability.

The stability of proteins to digestion may be altered in the presence of various components that all form part of the food matrix (a matrix effect). Thus, increased stability of the milk allergen, α -lactalbumin, to pepsin digestion in the presence of physiologically relevant amounts of phospholipids was shown [10]. The effects of structural polysaccharides of plant origin, commonly used in the food industry (gum arabic, pectin and xylan), on *in vitro* digestion stability and the pattern of pepsin and trypsin digestion of peanut allergens and β -lactoglobulin have been investigated recently [11–14].

The purpose of this study was to examine the effects of the fruit matrix on *in vitro* pepsin and *in vivo* gastric digestion of fruit allergens.

Methods

Fruit extract preparation

The extracts of various fruits [kiwi (*Actinidia deliciosa*), banana (*Musa* sp.), apple (*Malus domestica*) and cherry (*Prunus avium*)] were prepared according to a previously published protocol [15]. Briefly, the peeled fruits were homogenized 1:1.5 (w/v) in 100 mM ammonium-bicarbonate buffer, pH 8.5, containing 2% polyvinylpyrrolidone (PVPP) and 0.2% NaN_3 in a blender for 1 min. After a 3 h extraction at 4 °C and centrifugation, the extracts were divided into two parts. The first part (designated as crude extract) was lyophilized or stored at –20 °C. The second part of the fruit extract was dialysed extensively against the starting buffer, with or without previous precipitation of proteins with 90% saturation of ammonium sulphate, and lyophilized or stored at –20 °C (designated as dialysed extract). Protein concentrations in the extracts were determined by the Bradford assay [16].

Antisera production

Rabbits were immunized to kiwi proteins [17], and the obtained antisera were purified as described previously [18].

Monoclonal anti-thaumatol-like protein antibody production

Monoclonal antibodies to the kiwi thaumatol-like protein (TLP) were produced by hybridoma technology. Mice were immunized to the purified kiwi TLP and the selected antibodies were proven to be specific in ELISA and immunoblot assay. The details of the antibodies production and characterization will be published elsewhere.

Digestion in the simulated gastric fluid

Proteins from different fruit extracts were digested according to a previously published protocol [19]. Briefly, 280 μg of the protein extracts were dissolved in 60 μL of pre-warmed deionized water. Twenty microlitres of pre-warmed 0.4 M HCl containing 8 g/L of NaCl and 1.28% of pepsin A, 3.64 U/mg of protein (Sigma-Aldrich, Taufkirchen, Germany), were added. Digestion proceeded at 37 °C with continuous shaking and an aliquot (20 μL) of the digest was periodically withdrawn (at 15 min, 1 and 3 h). The digestion was stopped with 6 μL of 0.2 M Na_2CO_3 , and samples were mixed with 6.5 μL of five times sample buffer for SDS PAGE analysis. Fifteen microlitres of each sample was applied per lane (32 μg of proteins per well).

Determination of total sugars in fruit extracts

Total sugars in fruit extract were determined by the phenol-sulphuric acid method [20]. Briefly, 40 μL of fruit extracts or glucose standards were mixed with 20 μL of phenol solution (5%). After 5 min, 100 μL of 98% sulphuric acid was added and the absorbance of the solutions was recorded at 492 nm 15 min later.

Pepsin inhibition experiments

Pepsin activity was determined using a haemoglobin assay [21]. Bovine haemoglobin (80 mL of 2.5% w/v) in deionized water was denatured by adding 20 mL of 0.3 M HCl. Briefly, inhibitors (100 μL of the crude lyophilized fruit extracts or 5 mg of glucose or 5 mg of sucrose) were dissolved in 0.5 mL of denatured bovine haemoglobin solution. After addition of 0.1 mL of pepsin solution (0.4 mg/mL in 0.01 M HCl), a reaction was allowed for 5 min before being stopped by adding 1 mL of 50% trichloroacetic acid. Samples were cleared by centrifugation, and the absorbance of the supernatants was recorded at 405 nm. The negative control for every inhibitor represented the degree of spontaneous haemoglobin degradation in the presence of the specific fruit extract lyophilizate and was conducted as described, but with addition of 0.1 mL of 0.01 M HCl instead of the enzyme solution. Each negative control served as a specific blank for absorbance measurements. Positive control and positive control blank were conducted as described, but without the addition of any inhibitor.

Digestion of bovine serum albumin in the presence of glucose or sucrose

Bovine serum albumin (BSA) (ICN Galenika, Belgrade, Serbia), 0.04 mg, was dissolved in 40 μL of pre-warmed simulated gastric fluid (SGF) containing 52 mg/mL of glucose or sucrose. Digestion proceeded and was stopped

as described above. Fifteen microlitres of each sample was applied per lane.

Digestion of Act c 2 in the presence of fruit pectin

Kiwi fruit TLP, Act c 2, was isolated from kiwi fruit extract according to Gavrovic-Jankulovic et al. [15] and lyophilized. TLP (0.02 mg) was dissolved in 20 µL of deionized water or 20 µL of 3% or 6% pectin (Fluka, Schmeiz, Germany) suspension in deionized water. Ten microlitres of 10 mM CaCl₂ containing 600 mg/mL of glucose was added to the pectin-TLP mixture. After intensive vortexing, the mixtures were warmed to 37 °C, and 10 µL of pre-warmed 0.4 M HCl containing 8 g/L of NaCl and 1.28% of pepsin A, 3.64 U/mg of protein (Sigma-Aldrich) were added. The molar ratio of TLP: pepsin was 1 : 6.5. Digestion proceeded at 37 °C with continuous shaking. After 1 h, the digestion was stopped with 12 µL of 0.2 M Na₂CO₃, and samples were mixed with 13 µL of five times the sample buffer for SDS PAGE analysis. A pepsin control and pepsin-3% pectin control were set up in the same manner but with no addition of TLP. A TLP-3% pectin control was set with addition of deionized water instead of the pepsin solution. Fifteen microlitres of each sample was applied per lane.

Experiments on kiwi fruit digestion in vivo

The study was carried out with the approval of the Research Ethics Committee of the University Clinical Children's Hospital, Belgrade, Serbia. Subject provided informed written consent.

Four healthy non-atopic volunteers were asked to ingest 100 g of kiwi fruit. After 1 h, a 10 mL sample of their gastric fluid was taken and digestion was stopped by adding 3 mL of 0.2 M NaHCO₃. Gastric fluid taken from two healthy non-atopic volunteers who did not ingest kiwi fruit was used as a negative control. The samples were kept frozen at -20 °C until homogenized and prepared for SDS-PAGE (by mixing with five times sample buffer) and immunoblot analysis. Twenty microlitres of each sample was applied per lane.

Immunoblot experiments

All digested samples were resolved on the polyacrylamide (PAA) gel and transferred onto a nitrocellulose membrane (Millipore, Bedford, MA, USA, 0.45 µm pore size) by electroblotting. The nitrocellulose membranes were blocked with 1% BSA in Tris-buffered saline (TBS) containing 0.05% Tween 20, (TTBS), pH 7.6, for 1 h at room temperature. After blocking, the membranes were incubated with the pool of three monoclonal anti-TLP antibodies diluted 1 : 100 in 0.25% BSA in TTBS for 2 h at room temperature. The blots were incubated for 2 h with

1 : 10 000 diluted monoclonal anti-mouse IgG antibody labelled with alkaline phosphatase (Sigma-Aldrich) and visualized using nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) as a substrate.

The gastric fluid samples were resolved by SDS PAGE and transferred on to a nitrocellulose membrane. The samples were probed with anti-TLP antibodies, or with a pool of rabbit antisera raised against kiwi fruit proteins in 1 : 50 000 dilution. The rabbit IgG was detected using goat anti-rabbit IgG-labelled alkaline phosphatase (Sigma-Aldrich) and the same substrate (BCIP/NBT) as above.

Results

Commonly prepared fruit extracts were rapidly digested by simulated gastric fluid

We prepared extracts of kiwi, apple, cherry and banana fruits according to the common protocols, which include extensive dialysis and/or ammonium sulphate precipitation to partly isolate the protein fraction. When low-molecular-weight components of the fruit matrix were removed, almost all proteins from the kiwi fruit, apple, cherry and banana extracts were digested within the first 15 min of exposure (Fig. 1, DE) regardless of the method of extract preparation. The exceptions were a highly resistant protein fraction of 15 kDa in banana extract and protein fractions of 24 kDa in apple and cherry. They might correspond to the molecular weights of TLPs of these fruits [22, 23] and 20 kDa in banana extract [24], which we also believe could be banana TLP. The TLP are important fruit allergens and, resistance to digestion has been shown for some of them [25]. Contrary to cherry, apple and banana TLPs, which are stable proteins, kiwi TLP is rapidly digested in the SGF [15, 19]. The 24 kDa band corresponding to the kiwi TLP (Fig. 1, DE) also disappeared from the digestion mixture within the first few minutes of pepsin exposure.

The results of SGF digested ammonium-sulphate-precipitated extracts are not shown as these are essentially the same as those presented in Fig. 1 (DE).

Non-dialysed crude fruit extracts were highly resistant to simulated gastric fluid digestion

When the crude extracts were exposed to the SGF conditions, and the aliquots were withdrawn at the same time points as in Fig. 1 (DE), clearly different digestion patterns were observed (Fig. 1, CE). Apparently, the non-dialysed crude fruit extracts were resistant to pepsin digestion that was driven for up to 3 h. The presence of different components of the matrix that are otherwise removed by extensive dialysis hampered the pepsin digestion under the acidic conditions of the simulated gastric fluid. We

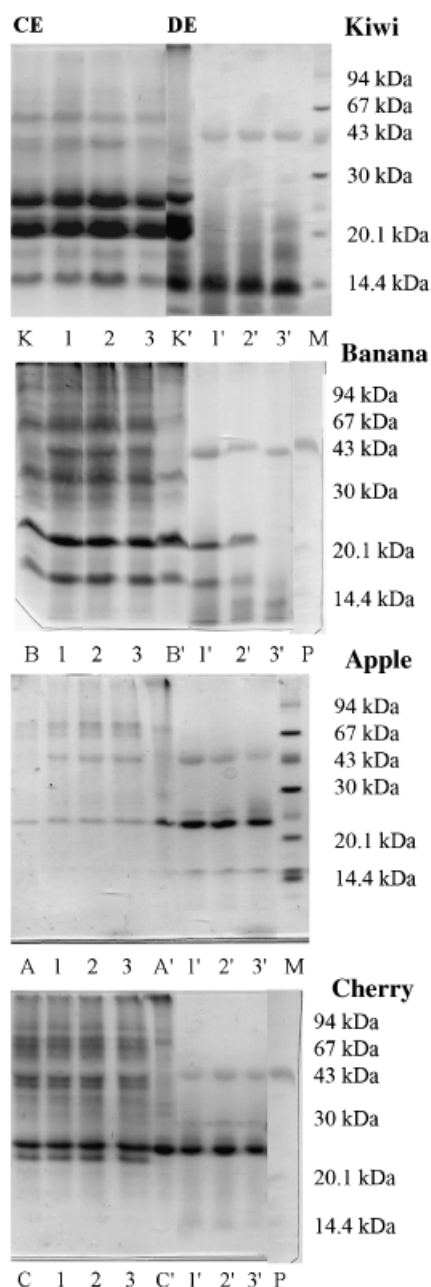


Fig. 1. Digestibility of crude (CE) and dialysed (DE) fruit extracts proteins in simulated gastric fluid. K, kiwi fruit; B, banana; A, apple; C, cherry; P, pepsin control; M, molecular weight markers; kinetics of digestion: 1–15 min, 2–1 h, 3–3 h.

also observed that the band sharpness in SDS PAGE was less pronounced in the presence of the matrix, probably because of the higher viscosity of the non-dialysed samples and the presence of pectin that made clearly visible hydrogels in acidic solutions of SGF. We could not see any clear difference in the protein pattern of the undigested samples (K, C, B and A) when compared with samples exposed to the SGF digestion for 3 h (K3, C3, B3 and A3) (Fig. 1, CE). We conducted a further study in order

to investigate the nature of pepsin inhibition in the matrixes of the investigated fruits.

There was no low molecular specific pepsin inhibitor in the fruit extracts

The inhibition experiments were conducted in a standard haemoglobin assay and showed that the presence of a very specific pepsin inhibitor in the fruit extracts was unlikely. In the presence of various fruit extracts, levels of the total pepsin activities were within the error range of 100% (99 ± 1 for kiwi fruit, 105 ± 7 for banana, 90 ± 15 for apple and $99 \pm 10\%$ for cherry extract).

The resistance to digestion was not due to high contents of mono and disaccharides in the kiwi fruit extract

The total sugar content in crude fruit extracts was very high compared with the dialysed fruit extract and total protein content (Table 1).

Residual amounts of sugars after dialysis were considerable for all the investigated fruit extracts, up to 7.4% (banana extract). Sugar/protein ratios in the crude extracts were about 295/1 with the exception of kiwi fruit extract, where it was 87/1, as a consequence of the high total protein content. Glucose and sucrose, added to pepsin in a quantity equivalent to that for sugars in the total kiwi fruit extract, slightly reduced pepsin activity (19 ± 3 and $25 \pm 4\%$, respectively) in the standard haemoglobin assay, probably because of the higher viscosity of the solution.

Then, we set up an experiment of simulated gastric digestion of BSA in the presence of mono and disaccharides in quantities that mimicked the conditions for the crude kiwi extract (Table 1). However, the digestion kinetics for BSA with added glucose or sucrose was the same as that for BSA alone, as judged by SDS PAGE (results not shown). The BSA was fully digested within 5 min, despite the presence of monosaccharides (glucose) or disaccharides (sucrose) in quantities that mimic their content in kiwi fruit extract. We may conclude that the viscosity phenomena due to the high content of sugars cannot explain the resistance to pepsin digestion of crude fruit extracts in the SGF assays conducted above (Fig. 1, CE).

The fruit pectin protected kiwi thaumatin-like protein from proteolytic digestion in vivo

Finally, we investigated the possibility that the presence of pectin in the fruit matrixes hampered digestion.

We set up an *in vitro* digestion experiment with purified kiwi TLP. This allergen is normally rapidly digested, but the addition of commercial apple pectin with 150 mg/mL of glucose as a cosolute was able to partly protect it from pepsin digestion (Fig. 2). In the presence of 1.5% and 3%

Table 1. Total sugar and total protein content in kiwi, banana, apple and cherry crude and dialyzed extracts

Fruit extract	Total sugar content (mg/mL)			Total protein content (mg/mL)		Sugar/protein mass ratio in the crude extracts
	Crude extracts	Dialyzed extracts	Percent of residual sugars after dialysis	Crude extracts	Dialyzed extracts	
Kiwi fruit	52	1.8	3.5	0.60	0.38	87/1
Banana	65	2.7	4.2	0.24	0.18	270/1
Apple	133	9.9	7.4	0.45	0.35	295/1
Cherry	56	1.2	2.1	0.19	0.09	295/1

of pectin, the digestion proceeds slower when compared with that for the pure protein alone. Intact TLP molecules (of the same molecular weight as undigested TLP and able to recognize the monoclonal antibodies raised against kiwi TLP) were detected in the presence of pectin after 1 h of digestion.

The fruit matrix influenced digestion of kiwi proteins in vivo

We obtained a partial protection when pectin was added to the purified kiwi TLP in the *in vitro* assay. Then, we sought to investigate whether a similar phenomenon occurs during *in vivo* digestion. Four non-atopic volunteers participated in the study. After allowing the *in vivo* digestion to proceed for one hour, we took a sample of their gastric fluid and analysed it by SDS PAGE and immunoblot.

Different enzymes of the gastrointestinal tract (pepsin and amylase, as indicated by the molecular weights) were the proteins most abundant in the SDS PAGE of the gastric fluid taken from non-atopic volunteers. There were individual differences in the dilution of the samples (lanes 1–4 in Fig. 4a). The typical kiwi protein pattern, due to dilution in the gastric fluid, could not be observed. Without any concentrating treatment of the sample, we probed the resolved proteins with the monoclonal anti-TLP antibodies. Monoclonal antibodies used in this study were highly specific for Act c 2, recognizing only a band that corresponds to the Act c 2 in the immunoblot of a crude

kiwi fruit extract (Fig. 3). It was possible to detect intact TLP bands of different intensity in the immunoblot of the gastric content of four non-atopic volunteers 1 h after ingestion of the kiwi fruit (Fig. 4b). Differences in the TLP band intensity could arise from two possibilities: dilution during fruit digestion differed between individuals, and/or in some of them, hydrolysis took place to a certain extent. In the control samples (lanes C1 and C2 in Fig. 4b), no bands were detected. Nevertheless, we demonstrated the presence of intact TLP *in vivo* in gastric fluid from all healthy volunteers 1 h after the fruit had been ingested, which is longer than its stability in our *in vitro* assay and other similar studies [19].

When probing the resolved proteins with the antisera against kiwi proteins, we obtained bands of various molecular weights, ranging from 94 to 20 kDa. The different band intensities and distribution in the kiwi extract were probably due to different extraction conditions. The very acidic environment of the gut might promote extraction of proteins that are less abundant when made in aqueous extraction systems (Fig. 4c).

Discussion

The assay of the *in vitro* digestion of proteins in the simulated gastric fluid was not meant to mimic precisely the fate of proteins *in vivo* but rather to evaluate the susceptibility of the protein to pepsin digestion under fixed conditions *in vitro*. We applied the same standardized assay to study the susceptibility of the proteins

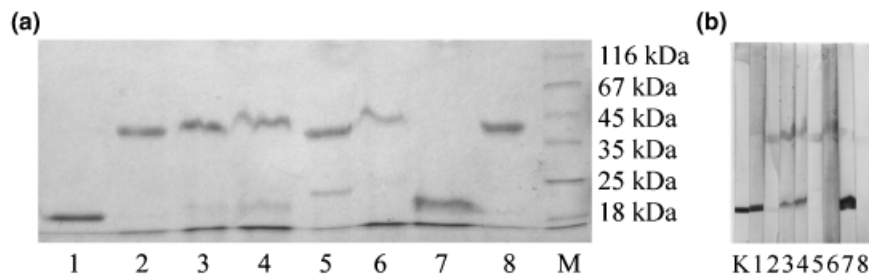


Fig. 2. Influence of pectin on kiwi thaumatin-like protein (TLP) digestability in simulated gastric fluid (SGF). (a) SDS PAGE, (b) Kiwi TLP detection in immunoblot. 1, TLP control; 2, TLP in SGF; 3, TLP-1.5% pectin in SGF; 4, TLP-3% pectin in SGF; 5, SGF, 6–3% pectin in SGF; 7, TLP-3% pectin; 8, SGF control; M, protein standards; K, kiwi extract.

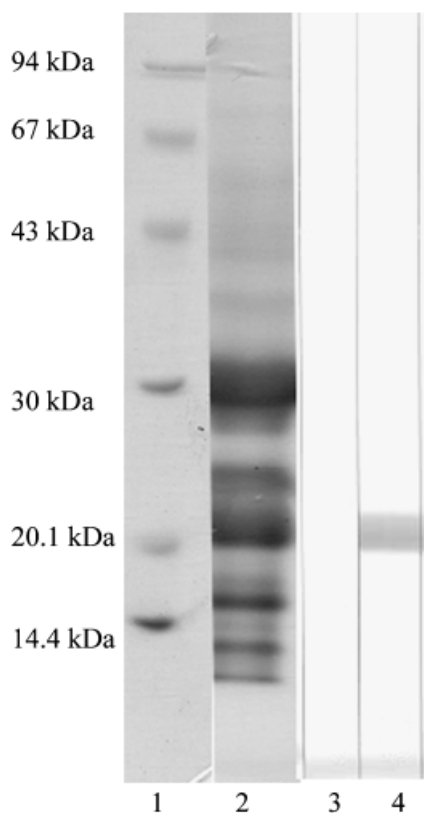


Fig. 3. Specificity of monoclonal anti-Act c 2 antibodies determined in immunoblot. 1, protein standards; 2, SDS PAGE of kiwi fruit proteins (10 µg/mL); 3, Kiwi fruit extract control immunoblot (anti-mouse IgG labelled with alkaline phosphatase was used as a secondary antibody, but incubation step with monoclonal anti-Act c 2 antibodies was omitted); 4, immunoblot of kiwi fruit proteins developed with monoclonal Act c 2 antibodies (10 µg/mL of kiwi fruit proteins were applied per lane); anti-mouse IgG labelled with alkaline phosphatase was used as a secondary antibody.

tested in our study to the pepsin digestion in the presence of fruit matrix, pectin and viscosity phenomena due to a high sugar content with the same purpose.

An influence of increased viscosity in a sample or pH changes in gastric acid resulting from the presence of polysaccharides on gastric digestion was observed by other researchers in animals [26–30]. We obtained partial protection when pectin was added to the purified kiwi TLP *in vitro*. The full protection observed in the fruit matrix during the *in vitro* assay (Fig. 1, CE) on crude fruit extracts is probably a combination of all the hampering components (including high viscosity and the presence of other proteins in the mixture), but the major contributing factor seemed to be pectin.

The presence of gel-forming polysaccharides (pectin) provides a physical obstacle to pepsin and protein substrate mobility in the reaction mixture. Pectin forms gels in the conditions present during *in vivo* and *in vitro* pepsin digestion. During digestion of fruits, the high content of

sugars lowers the activity of water, necessary for gel formation at low pH values [31]. In addition, calcium ions normally present in drinking water also enhance the stability of pectin gels. Formation of a structural pectin network in synergism with other possible matrix-protective effects could delay and hamper gastric digestion of kiwi allergens for 1 h *in vivo* (Fig. 4).

Thus, cases of monosensitization to kiwi fruit, whose prevalence is increasing worldwide, are being described more often in the literature [32, 33]. Formation of a protective network of pectin-rich matrixes could enable structurally unstable food allergens to behave as complete food allergens. One such example may be actinidin (Act c 1, the major allergen of kiwi fruit, also promptly digested in *in vitro* SGF (Fig. 1, DE), for which no known homologues among inhalant and contact allergens have been found so far [3, 34].

The protective effect of pectin on digestion in the intestine was not investigated in this study. Pectin hydrogels formed in an acidic environment slowly dissolve in the basic environment of the intestine (pH 7.2) when large amounts of sodium-bicarbonate are secreted into the lumen. It is not very likely that a pectin network, once dissolved, can delay further proteolytic digestion in the intestine in the same manner. However, viscosity or weak local electrostatic and hydrogen bonding phenomena cannot be fully excluded [14]. Studies conducted on the influence of different polysaccharides on peanut allergen digestibility showing that the presence of gum arabic, pectin and xylans affects the size of peanut peptides obtained by two-step hydrolysis (pepsin followed by trypsin and chymotrypsin digestion) [11] support this. No influence of these polysaccharides on the peptin digestion of peanut allergens was observed, as peanut allergens are stable under acidic conditions and resistant to peptin digestion [35–38]. The effect of polysaccharides on β-lactoglobulin hydrolysis by pepsin under conditions mimicking gastric digestion has also been studied [11–13]. Changes in nitrogen release indicated that pepsin digestion in the presence of pectin was lower than in the control sample. The effect of polysaccharides on digestibility was explained by reduction of pepsin activity, protein-pectin complex formation and other phenomena, such as viscosity.

It is noteworthy that there is a close correlation of the results obtained *in vivo* and *in vitro*. The *in vitro* stability was a good indication for the *in vivo* stability to digestion in this study. To the best of our knowledge, this is the first report on the existence of a matrix effect in pectin-rich food extracts on the digestion stability of fruit allergens. We demonstrated a protective effect of the fruit matrix, mainly pectin, on the *in vitro* digestion of crude extracts of various fruits (kiwi, banana, cherry and apple). Moreover, using monoclonal antibodies raised against the major allergen of kiwi fruit (Act c 2), we demonstrated

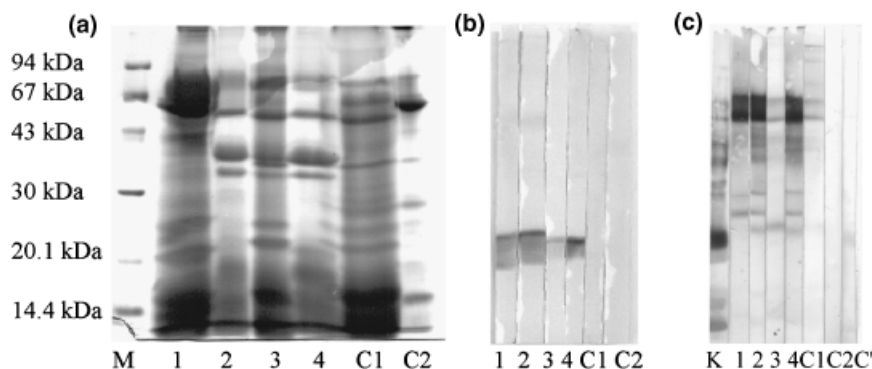


Fig. 4. *In vivo* digestibility of kiwi fruit proteins. (a) SDS PAGE; (b) immunoblot developed with anti-thaumatococcus protein monoclonal antibodies; (c) immunoblot developed with anti-kiwi fruit extract polyclonal sera. Gastric content of 4 volunteers that ingested kiwi fruit (1–4) and two controls (C1–2). M, protein standards; K, kiwi extract; C', kiwi extract control.

the presence of intact, otherwise readily digestible, kiwi fruit allergen in the gastric content of healthy volunteers, 1 h after kiwi fruit ingestion.

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