

In Vivo Digestion of a Thaumatin-Like Kiwifruit Protein in Rats

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Abstract Food allergens must exhibit sufficient gastrointestinal stability to reach the intestinal mucosa where absorption and sensitization can occur. Therefore, investigation of protein stability within the gastrointestinal tract may provide a prospective test for the allergenic potential of novel proteins. The aim of this work was to examine the effect of the fruit matrix and purified pectin on the digestion in vivo of kiwifruit allergens in the rat gastrointestinal system. The major kiwi allergen, Act d 2, was quantified in several compartments of the gastrointestinal tract by a monoclonal antibody-based ELISA. Protein intactness was demonstrated by immunoblot. Under conditions of complex food digestion in vivo, a pepsin-labile protein survived passage from the stomach to the caecum during a 3-h period. Decay of Act d 2 in the rat gut exhibited an exponential pattern. Ingestion of kiwifruit was followed by a decrease in both total and specific pepsin activity. When purified, Act d 2 allergen was consumed together with pure apple pectin; both gastric acidity as well as specific and total pepsin activity declined and thus protected 23% of the ingested allergen from digestion for 90 min. In conclusion, ingestion of pectin-rich fruits and particularly pectin

supplements may have a protective action on pepsin-labile allergens and prolong their survival in both gastric and duodenal juices, enabling efficient uptake and presentation to the immune system.

Keywords Act d 2 · *Actinidia deliciosa* · Allergen · In vivo digestion · Kiwifruit · Thaumatin-like protein

Abbreviations

GIT Gastrointestinal tract
SGF Simulated gastric fluid
TLP Thaumatin-like protein

Introduction

Food allergy affects 4–6% of children and 1–3% of adults in Europe [23] and 6–8% of children and 4% of adults in the USA [15].

Based on their potential to trigger specific IgE antibody production, food allergens are divided into two classes. In addition to their ability to cross-link IgE, class 1 or complete allergens are the primary source of sensitization. Many class 1 allergens are low-molecular-weight glycoproteins with acidic isoelectric points, and most of them are highly abundant in food. These proteins are usually resistant to proteases, heat, and denaturants, which preserves them from degradation during food preparation and digestion, thereby enabling direct oral gastrointestinal sensitization [1, 2].

Class 2 or incomplete food allergens are postulated to lack sensitizing capacity. These proteins have the potential to elicit symptoms only after primary sensitization with cross-reactive inhalative allergens and are therefore termed nonsensitizing elicitors. Examples are protein homologues

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of Bet v 1, the major birch pollen allergen. They are present in fruits and vegetables and their susceptibility to pepsin digestion has been demonstrated. This might explain why local but not systemic symptoms are frequently triggered after ingestion of Bet v 1 homologues [34]. A similar observation was noted for the digestion of stable versus labile allergens of kiwifruit as labile allergens more often bind IgE from patients with localized symptoms to kiwifruit ingestion—the so-called oral allergy syndrome [21].

Thus, there is a general hypothesis that food allergens must exhibit sufficient gastrointestinal stability to reach the intestinal mucosa where absorption and sensitization can occur. Therefore, an investigation of the stability of proteins within the gastrointestinal tract (GIT) may provide prospective testing for allergenic potential as a parameter to distinguish food allergens from non-allergens. Various in vitro digestion models have been developed to assess the stability of food allergens during digestion [25, 35], but systematic evaluation of the stability of food allergens that are active via the GIT is currently made using traditional pepsin digestibility models [19, 32].

Special attention has been focused on genetically modified crops, which should be carefully tested before entering the market, including a detailed analysis of allergenic risks from novel proteins [17, 20]. Protein stability to digestion, as predicted by a simulated gastric fluid assay in vitro, is currently used as one element in the risk assessment process [14, 19].

Although correlation between resistance to pepsin digestion and allergenic potential was proposed long ago [1], the relationship is not absolute and it is still a matter of scientific dispute [9, 10]. In addition, more evidence is accumulating about the protective effects of the food matrix on pepsin and pancreatin digestion of proteins. Thus, the food matrix may contain substances able to hamper enzymatic digestion by making physical obstacles to enzyme action. Those described so far are lipids, e.g., phosphatidylcholine [8, 24] secreted in the stomach and also abundant in milk, as well as polysaccharides, e.g., pectin in fruit matrices [28, 29].

Significant efforts have been made toward improving in vitro digestibility assays in order to evaluate protein stability in more physiologically relevant conditions, including those that occur during life, i.e., infant versus adult in vitro digestion models [8, 35].

Kiwifruit (*Actinidia deliciosa*) has been recognized as a food allergen source for almost 30 years. Since the early 1960s when kiwifruit became available on the world market, its growing popularity has been accompanied by an increasing number of reports of allergic reactions. Besides actinidin (Act d 1) [27] and kiwellin (Act d 5) [31], a thaumatin-like protein (TLP, Act d 2) [11] is considered to be one of the most relevant allergens in kiwifruit.

TLPs belong to the PR-5 family and are described as having allergenic properties in fruits [5]. When the digestibility of kiwi, banana, apple, and sour cherry TLPs were compared in vitro, kiwi TLP was the only gastric labile protein, being readily digested by pepsin within several minutes [28].

The aim of this work was to investigate the effect of the fruit matrix on the digestion of kiwifruit allergens in the GIT of rats. The major kiwi allergen, Act d 2, was quantified throughout the tract by a monoclonal antibody-based ELISA. Protein intactness was demonstrated by immunoblot. Under conditions of complex food digestion in vivo, the pepsin-labile protein survived passage through the GIT of rats to the caecum in the course of 3 h. The presence of pectin was an important but not sufficient factor for the survival of the labile allergen.

Material and Methods

Kiwifruit Homogenate Preparation

Kiwifruit homogenate was prepared as previously described [29]. Peeled kiwifruit (*A. deliciosa*) was homogenized in a blender for 2 min.

Isolation of Act d 2

Kiwifruit extract was prepared according to Gavrovic-Jankulovic et al. [12]. Protein concentration was determined by the method of Bradford [4]. A thaumatin-like protein (Act d 2) was isolated using a combination of anion exchange chromatography on QAE-Sephadex and anion exchange HPLC as described previously [13]. Purity of the isolated allergen was estimated using SDS-PAGE, and the protein was lyophilized and stored at -20°C .

Animals

All procedures involving animals and their care were approved by the Animal Ethics Committee of the Faculty of Pharmacy of the University of Belgrade (AEC no. 3/09).

Eight-week-old male Wistar rats were obtained from the Military Technical Institute farm (Belgrade, Serbia). During a 5-day acclimatization period, they were housed three to four in standard cages at $22 \pm 2^{\circ}\text{C}$ with a 12:12-h light/dark cycle and free access to food pellets and water.

Gavage Protocol and Gastrointestinal Organ Preparation for Biochemical Analysis

Thirty rats were randomly assigned to five groups, each comprising six animals. They were fasted for 24 h, but with

free access to water prior to gavage. On the day of the experiment, conscious animals were gavaged (110-mm bulb-ended needle) either with 2 mL of kiwifruit homogenate (containing 0.135 mg mL^{-1} of Act d 2, as determined by ELISA; experimental groups) or 2 mL of tap water (control group). Other treatments applied are described in “Statistical Analysis.”

Rats were killed by an intraperitoneal injection of sodium pentobarbital (Nembutal, Abbott Laboratories, Chicago, IL, USA) at 150 mg kg^{-1} at different times after gavage: experimental groups after 15, 90, 180, and 300 min and the control group after 15 min. A central incision was made into the abdominal wall and the stomach, duodenum, and caecum were excised. The initial part of the small intestine, approximately 2 cm long, was considered to be the proximal part of the duodenum. The organ content was then completely emptied and transferred to previously weighed plastic tubes, weighed again, and stored at -80°C until required. The samples of stomach content were neutralized with 0.5 mL of 0.3 M sodium carbonate prior to freezing.

Animal Handling and Feeding for the Pectin Protection Experiment

The influence of pectin on gastric Act d 2 digestion was investigated in 12 rats previously fasted for 24 h: Each of six animals from the first group was gavaged with 0.3 mg Act d 2 in distilled water (1 mL) and tap water (1 mL), while rats in the second group received 0.3 mg mL^{-1} Act d 2 in a 2% suspension HM pectin in distilled water and 1 mL of tap water in the same way. Animals were killed 90 min after treatment, and the stomach content was collected as previously described.

Protein Extracts from Different Gastrointestinal Compartments

All the gastrointestinal samples were weighed using a standard laboratory balance, followed by immediate extraction of proteins.

Gut contents were buffered with 1.5 M Tris, pH 8.8 (200 μL), and the mixture resuspended in 1 mL 200 mM Tris, pH 8.0, sonicated for 5 min, and cleared by centrifugation at $10,000\times g$. Duodenal samples were resuspended in 200 μL 200 mM Tris, pH 8.0, sonicated for 5 min, and cleared by centrifugation at $10,000\times g$. Caecal samples were resuspended in 1 mL 200 mM Tris, pH 8.0, sonicated for 5 min, and cleared by centrifugation. All supernatants were kept frozen at -80°C until analyzed by ELISA. One tenth of the total volume of each extract was taken to make pools for SDS-PAGE and immunoblot analysis of the content from each gastrointestinal region.

Electrophoresis

Pooled gastrointestinal extracts from five groups of rats were mixed with fivefold concentrated sample buffer for SDS-PAGE and resolved on 12% polyacrylamide (PAA) gel according to the method of Laemmli. Forty microliters of each sample was applied per lane.

Detection of Act d 2 on Immunoblots

In order to detect intact Act d 2 or Act d 2 fragments in the gastrointestinal samples of rats fed with kiwi homogenate, the proteins were resolved on PAA gel and transferred to a nitrocellulose membrane (0.45 μm , Serva) by electroblotting. Monoclonal anti-TLP antibody BG7 was affinity-purified and labeled with biotin [13]. The nitrocellulose membranes were cut into strips (region 14–25 kDa) and blocked with 1% bovine serum albumin (BSA) in Tris-buffered saline (TBS) containing 0.05% Tween 20 (TTBS), pH 7.6, for 1 h at room temperature (RT). After blocking, the duodenal and caecal strips were incubated with mAb BG7 ($0.1 \text{ }\mu\text{g mL}^{-1}$) for 2 h at RT, while biotin-labeled mAb7 was used for the gastric samples. Appropriate controls were set up by incubation in TTBS. After washing with TTBS, the strips were incubated in alkaline phosphatase-labeled anti-mouse IgG (1:30,000, Jackson ImmunoResearch Laboratories) or streptavidin-AP (1:5 000, Sigma-Aldrich) for 2 h at RT and visualized using nitroblue tetrazolium (NBT, Serva) and 5-bromo-4-chloro-3-indolyl phosphate (Serva) as substrates.

Quantification of Act d 2 by Monoclonal Antibody ELISA

Act d 2 was quantified in gastrointestinal samples from control rats and those fed with kiwi homogenate using a highly specific monoclonal antibody-based sandwich ELISA according to [13]. Briefly, MaxiSorp ELISA plates (Sigma-Aldrich) were coated with affinity-purified mAb BG148 ($5 \text{ }\mu\text{g mL}^{-1}$, 100 μL per well) in 15 mM Na_2CO_3 , 35 mM NaHCO_3 , pH 9.5, overnight at 4°C . After washing with PBS containing 0.01% Tween 20 (TPBS) and blocking for 1 h at 37°C with 1% BSA in TPBS, tenfold dilutions of Act d 2 (100 ng mL^{-1}) or the gastrointestinal extracts (100- to 10,000-fold dilutions) in 0.1% BSA in TPBS were added to the plate and incubated for 2 h at 37°C . The bound Act d 2 was detected using biotin-labeled capturing mAb BG7 ($5 \text{ }\mu\text{g mL}^{-1}$, 100 μL per well). After 2 h at 37°C , the plate was subsequently incubated with 100 μL streptavidin-alkaline phosphatase (1:5,000, Sigma-Aldrich) for 1 h. After incubation with substrate solution (1 mg mL^{-1} 4-nitro-phenylphosphate, Sigma-Aldrich), the absorbance at 405 nm was recorded.

The concentration of Act d 2 in the gastrointestinal compartments of rats was expressed as milligrams of Act d 2 per kilogram of compartment content.

Determination of pH in Gastric Contents

In order to explore the effect of pectin or kiwifruit matrix at the prevailing gastric pH, four groups of six rats were gavaged with 2 mL of either water or 0.15 mgmL⁻¹ aqueous solution of Act d 2 or 0.15 mgmL⁻¹ Act d 2 in 2% pectin suspension in water or homogenized kiwifruit. The rats were killed 90 min thereafter and their stomach content taken and weighed as described previously, without addition of neutralizing buffer.

An aliquot (0.20 g) of gastric content was resuspended in 10 mM KCl (1 mL) and shaken at 500 rpm for 5 min at RT. The samples were cleared by centrifugation at 10,000×g for 3 min. The pH value of supernatants was determined using a standard laboratory pH meter.

Pepsin Activity Determination Using a Hemoglobin Assay

Pepsin activity in stomach contents was measured by a standard Sigma hemoglobin assay at pH 2.00. Specifically, pepsin activity was also measured at the pH found for each rat ("true" pH) as described above. The buffers used for adjusting pH values were: 0.3 M HCl (for pH 2.00 as proposed by Sigma), 0.3 M glycine (for pH values 2.00–3.0), and 0.3 M sodium citrate or acetate (for pH values 3.00–5.00).

Briefly, 100 µL of gastric contents resuspended in 10 mM KCl and 100 µL of appropriate buffer were added to the tubes. Tubes were then filled with 2.5% bovine hemoglobin in deionized water (400 µL) for the determination of pepsin activity. The reaction was stopped after exactly 10 min by adding 5% trichloroacetic acid (TCA) in deionized water (1 mL). After centrifugation at 10,000×g for 20 min, the absorbance at 280 nm was recorded in supernatants.

Blanks were set up as described, except that the enzyme solution was added after the TCA solution.

One unit (U) is defined as a change of absorbance by 0.001 per minute of reaction time in a cuvette with 1 cm of light pathway.

Statistical Analysis

The data are expressed as group medians or means, as indicated in the figures and table. Mann–Whitney *U* test was used for comparisons between groups (differences were considered significant at $p < 0.05$). For ELISA measurements of Act d 2 concentration during transit through the GIT, the statistical significance of differences was estimated by the non-parametric Kruskal–Wallis ANOVA followed by a post hoc Mann–Whitney *U* test for comparisons between groups ($*p < 0.05$, $**p < 0.01$, $***p < 0.001$). STATISTICA software, version 7.1, was used for these calculations.

Results

Quantification of Act d 2 in Gastric, Duodenal, and Caecal Contents of Rats Fed with Fruit Homogenate

Passage of Act d 2 through the rats' GIT was followed for 5 h using a monoclonal antibody-based ELISA (Fig. 1). The concentration of allergen was estimated as milligrams of Act d 2 per kilogram GIT content as weighed after extraction. After 15 min in the gut, the allergen concentration in the stomach peaked at around 89 mgkg⁻¹, while the maximal concentration in the duodenum of about 14 mg kg⁻¹ was noted after 90 min. The concentration peak in the caecum of 7 mgkg⁻¹ of caecum content also occurred at 90 min.

It was possible to estimate the extent of survival through the rat gut as the whole gut contents were taken for analysis. Act d 2 pepsinolysis in the rat gut followed an exponential (pseudo-first-order) pattern of decay for the first 3 h of digestion (Fig. 2).

Detection of Act d 2 in Gastric, Duodenal, and Caecal Contents of Rats Fed with Fruit Homogenate by Immunoblot

In order to confirm intactness of the allergen, GIT contents from rats fed with kiwifruit homogenate were prepared for SDS-PAGE and immunoblotting. In the same compartments, most protein detected by the monoclonal antibody was intact (M_w around 24 kDa), although fragments of molecular weight 14–18 kDa were also present (Fig. 3).

Influence of Kiwifruit Homogenate on Gastric Acidity and Pepsin Activity

Ingestion of kiwifruit homogenate increased the mass of rat gut content, suggesting slower gastric emptying, but the tendency for gastric acidity to decrease was not statistically significant (Table 1). Nevertheless, both total and specific pepsin activity fell (Fig. 4). To avoid small variations in pepsin activity at different pH values, separate assays were performed at the pH found in each animal's stomach (Fig. 4). Regardless of the pH during the assay, both specific and total pepsin activity decreased significantly in the presence of fruit homogenate.

Acidity and Pepsin Activity in the Stomach of Rats Fed with Pectin

To investigate the direct influence of plant polysaccharide pectin on the gastric content and its pH, the animals were fed with purified allergen, Act d 2, alone and in the presence of purified commercial apple fruit pectin. There

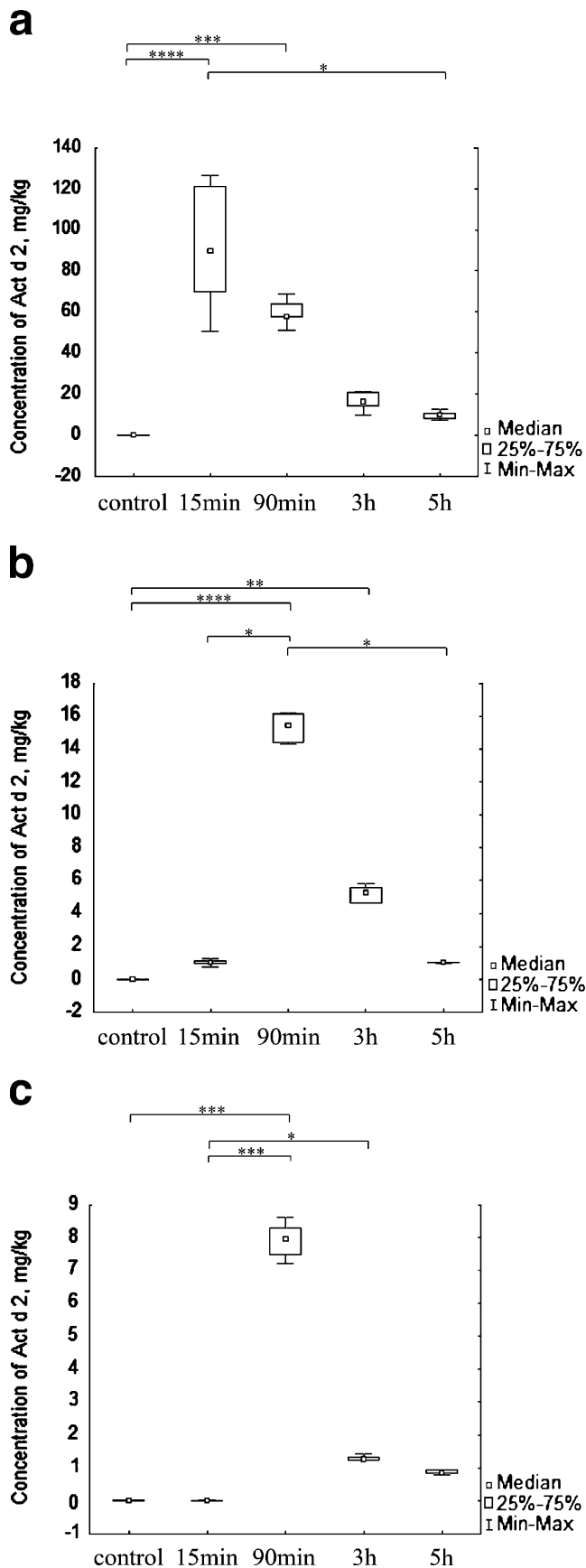


Fig. 1 Concentrations of Act d 2 determined by ELISA in stomach (a), duodenum (b), and caecum (c) of rats gavaged with kiwifruit homogenate. Differences were evaluated statistically by the non-parametric Kruskal–Wallis ANOVA (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$)

was a dramatic increase in gut content, accompanied by a statistically significant increase in pH (Table 1). As with ingestion of fruit homogenate, both total and specific pepsin activity decreased in the presence of pectin. In this experiment, these findings can also be partially attributed to the direct effect of pectin on gastric acidity, with consequent inhibition of pepsin activity (Fig. 4).

Total pepsin activity following ingestion of the protein (Act d 2) was higher than in the control group, which received water.

Quantification of Act d 2 in the Stomach Content of Rats Fed with Purified Act d 2 and Pectin

About 23% of the Act d 2 given to rats as a gavage with pectin was retained in the stomach 90 min later. At the same time, however, Act d 2 was not detected in duodenal and caecal contents. In animals that received Act d 2 without pectin, the protein was not found in any gastrointestinal compartment tested, implying complete Act d 2 digestion by pepsin within 90 min.

Discussion

In this study, breakdown of a major kiwifruit allergen was monitored throughout the gastrointestinal tract. Animals were gavaged with kiwifruit homogenate and Act d 2 was detected at various time points in three compartments of the gut (stomach, duodenum, and caecum), implying passage of intact Act d 2 down to the lowest compartments of the gastrointestinal tract. The concentration of allergen peaked

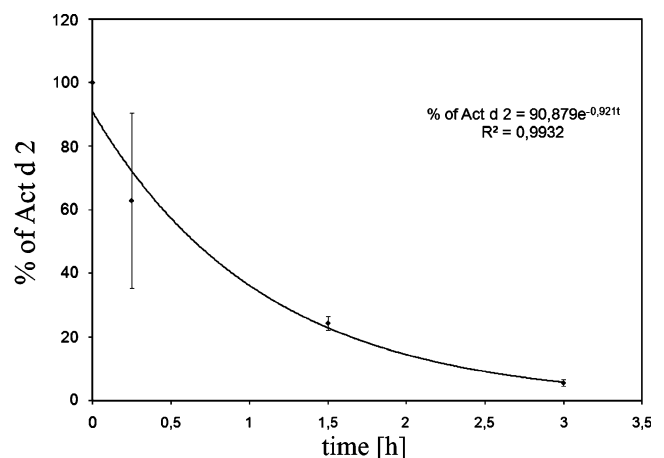
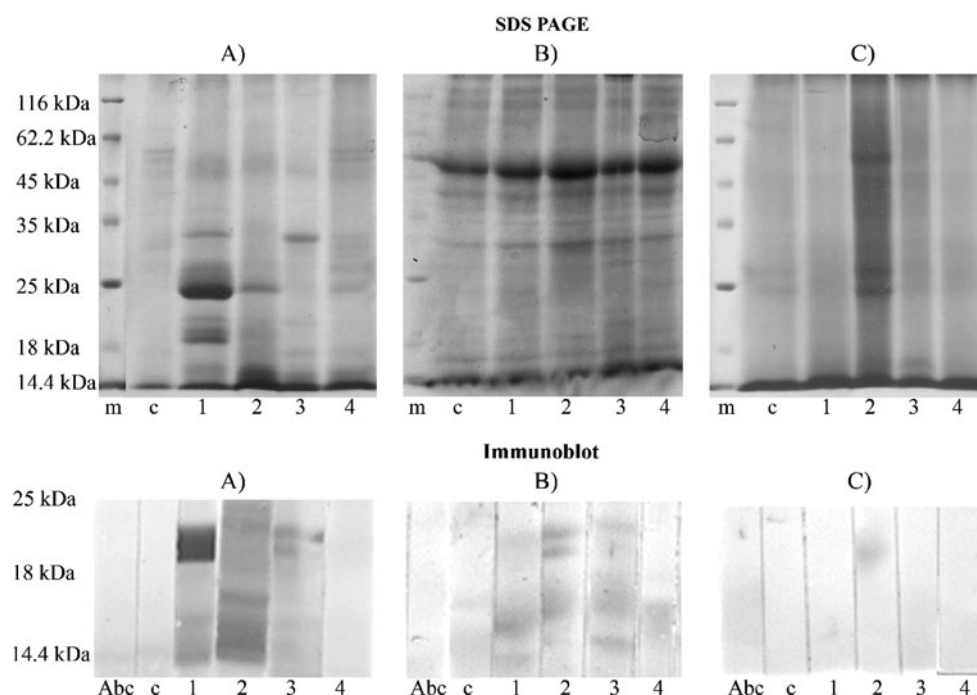


Fig. 2 Act d 2 survival in the gut of rats. Degradation followed an exponential pattern for the first 3 h of digestion

Fig. 3 Rat gastric (a), duodenal (b), and caecal contents (c) were analyzed by SDS-PAGE and immunoblot using antiAct d 2 monoclonal antibodies at various time points after fruit ingestion: 0 min—control group not gavaged with kiwifruit (c), 15 min (1), 90 min (2), 3 h (3), and 5 h (4). *M* molecular weight markers, *Abc* control, no primary antibody included



at 15 min in the stomach and at 90 min in the duodenum and caecum.

When analyzed in a standard *in vitro* digestibility assay, Act d 2 is degraded by pepsin completely within 8 min [11]. *In vitro* assay conditions involve a high pepsin concentration and a very low pH for the enzymatic reaction (1.2). However, when kiwifruit's acidic polysaccharide (pectin) is added, Act d 2 digestion by pepsin was slowed down significantly under the same conditions [28]. Similarly, herein, we have demonstrated that in rats gavaged with purified allergen and apple pectin, a substantial quantity of Act d 2 was retained in the stomach, but it was not detected in the duodenum and caecum 90 min later. The protein was completely digested in animals fed with Act d 2 only. The presence of pectin hampered the digestion of Act c 1, another major allergen of kiwifruit, both by pepsin and subsequent *in vitro* pancreatin digestion, up to its gel breakdown point [29].

Based on these previous observations, large amounts of intact Act d 2 in several rat gastrointestinal compartments, including the caecum, 3 h after ingestion of fruit homog-

enate, may be explained by a pectin-gelling protection effect, as observed in previous *in vitro* experiments. A significant change in gastric volume and pepsin activity following fruit ingestion was observed *in vivo*, implying additional mechanisms of protection by the food matrix besides gel formation (in the acidic conditions of the stomach) by natural highly methoxylated fruit pectin. Thus, the observed decrease in pepsin total and specific activity, the delayed gastric emptying, as well as a tendency for the gastric pH to increase 90 min following ingestion of kiwi homogenate can be attributed to an enterogastric reflex which inhibits both gastric secretion and emptying in the later phases of digestion.

Nevertheless, the *in vivo* decay of Act d 2 in the rat gut followed an exponential (the pseudo-first-order) pattern, as previously described for pepsinolysis of various allergenic and non-allergenic proteins *in vitro* [16, 18, 30].

By focusing on the pectin protection effect, our data show that a single component of the fruit matrix, although the major contributor, cannot account for the observed results, especially the survival of the allergen downstream

Table 1 Influence of Act d 2, kiwifruit homogenate, and pectin ingestion on gut mass and gastric pH

	pH value of gastric content (mean $\pm$ SD)	Gut content mass 90min after ingestion of the fruit (g)	No. of animals
Control	2.2 $\pm$ 0.1	0.3 $\pm$ 0.2	6
Kiwi homogenate	2.5 $\pm$ 0.4	0.9 $\pm$ 0.4*	6
Act d 2	2.3 $\pm$ 0.2	0.6 $\pm$ 0.1*	6
Act d 2+pectin	4.6 $\pm$ 0.1*	7.6 $\pm$ 1.1*	6

* $p < 0.05$ (compared to control group values, Mann–Whitney *U* test)

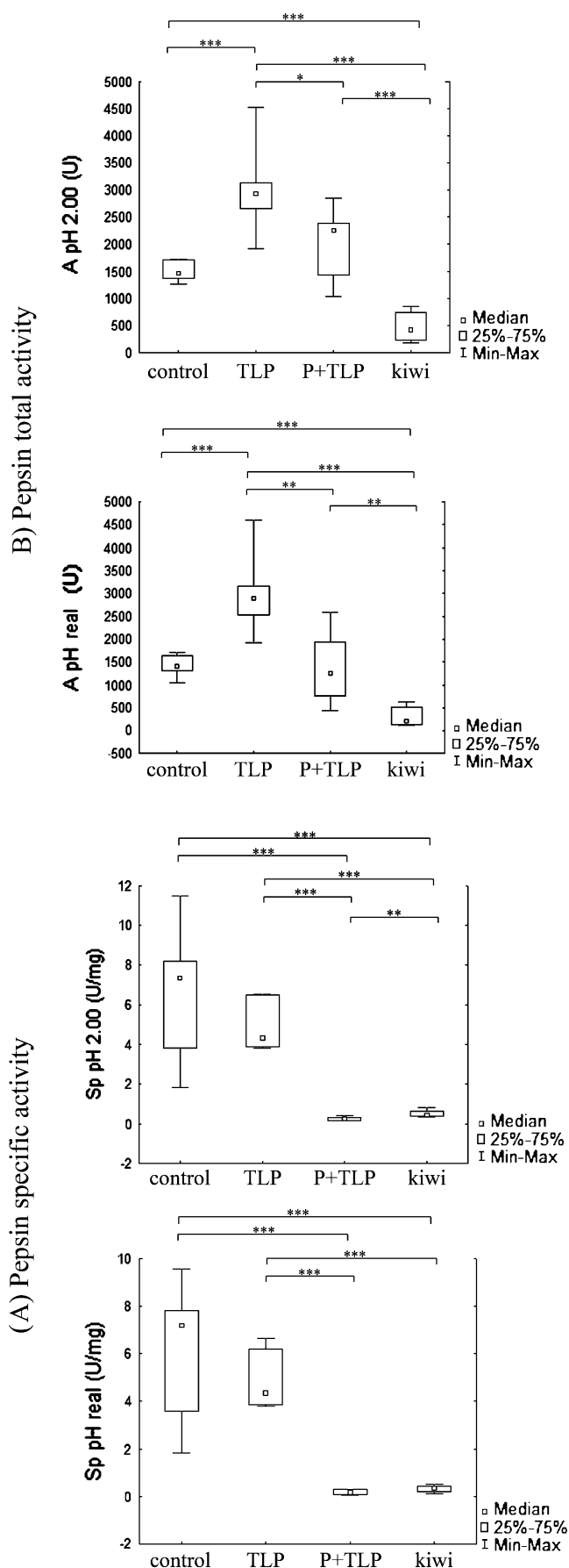


Fig. 4 Total (a) and specific (b) pepsin activity in the gut content of animals gavaged with water only (control), Act d 2 (TLP), apple pectin, and Act d 2 (P+TLP) or kiwifruit homogenate (kiwi) 90 min later. * $p < 0.05$ in Mann–Whitney U test

in the gut. The changes observed in gastric volume and pepsin activity when apple pectin was used in combination with Act d 2 were higher than for the fruit matrix, but this was unable to prevent digestion in the duodenum, probably due to the rapid breakdown of the pectin gel together with dilution leading to pectin hydrogel collapse in the basic conditions induced by the secretion of sodium bicarbonate. It can be concluded that other components of the very complex food matrix assisted allergen survival in vivo. The major impact of apple pectin ingestion was on gastric pH. An increase in pH of gastric fluid from 1.5 to 2.5 was recently shown to reduce pepsin breakdown of kiwifruit allergens significantly [21]. Both Act d 1 and Act d 2 survived longer when digested at pH 2 in vitro, which approximates physiological conditions [7]. Protein structure can also be influenced by pH, which modifies accessibility to cleavage sites, so contributing to changes in proteolysis patterns which may increase the risk of sensitization against food allergens [26]. On the other hand, partial survival of the allergen in the kiwifruit matrix and its presence in the caecum emphasize the importance of investigation of food allergen stability to digestion in vivo.

Although a correlation between resistance to pepsin digestion and allergenic potential has been proposed, the relationship is not absolute. There are examples of proteins in food that are not digestible and do not elicit food allergy [10]. Likewise, it cannot be concluded that allergenic food proteins are necessarily more resistant to digestion. Some studies have shown a protective effect of various components of the food matrix, including phospholipids, such as phosphatidylcholine, on both gastric and duodenal digestion of food allergens in vitro [22, 24, 33].

The relative abundance of the allergen in food should be another factor to be considered together with its structural stability since abundance may influence the amount of allergen that survives gastrointestinal digestion. Gastric digestion substantially decreases the potential of food proteins to bind IgE, which increases the threshold dose of allergens required to elicit symptoms in patients with food allergy. The major allergens found in foods making up a large proportion of the human diet, such as milk, eggs, fish, or potatoes, are all highly abundant, comprising 20–60% of the protein in the original food. In addition, a recent study showed a significant difference in the digestibility of major allergens in infant and adult gastrointestinal digestion models [8]. This raised awareness about alterations in the digestive tract milieu experienced during a lifetime, either physiologically in the very young and the elderly or as a

result of gastrointestinal pathologies, which may have an impact on the food allergy sensitization process.

Moreover, components of the food matrix may prevent digestion of food proteins, thus increasing the quantity of allergen surviving gastrointestinal digestion to the threshold dose required to elicit symptoms in patients with food allergy. The concentration of Act d 2 in kiwifruits and various food products [13] was estimated to be up to 1 mg g⁻¹ of food. In our study, up to 63% of ingested Act d 2 survived in the rat stomach for 15 min, implying that intact allergen would be able to reach the lower gut.

An important consequence of our findings is that ingestion of pectin-rich fruits and pectin supplements will protect pepsin-labile allergens and prolong their survival both in gastric and duodenal juices. Actinidin, a major kiwifruit allergen, has no known homologue among respiratory allergens and could be an example of a pepsin-labile protein able to sensitize patients, both due to its high abundance [3, 12] in kiwifruit and the protective effect of the kiwifruit matrix. Its major role in monosensitization to kiwifruit has recently been clinically demonstrated [6].

In conclusion, digestibility studies aimed at mimicking in vivo conditions should be further improved toward more physiologically relevant in vitro gastrointestinal digestion models, reflecting the complexity of the food matrix as well as variations in the gastric milieu following food ingestion or during the lifetime.

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Conflict of Interest The authors declare no conflict of interest.

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