



Multiplex comparison of the digestibility of allergenic and non-allergenic proteins in rice grains by *in vitro* digestion



Gang-hua Lang, Yukari Kagiya, Kazumi Kitta *

National Food Research Institute, National Agriculture and Food Research Organization, 2-1-12, Kannondai, Tsukuba, Ibaraki 305-8642, Japan

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ABSTRACT

To evaluate the digestibility of rice allergenic and nonallergenic proteins under the influence of the rice grain matrix, rice powder was subjected to *in vitro* digestion by simulated gastric fluid (SGF) and simulated intestinal fluid (SIF). Rice proteins were extracted from the liquid and the solid phases and analysed by SDS–PAGE, and rice allergenic proteins were detected by a multiplex immunodetection method. The digestion of soluble proteins was carried out in both liquid and solid phases, while that of insoluble proteins only occurred in the solid phase. In SGF digestion, rice proteins were more quickly digested at pH 1.2 than at pH 2.0 or 2.5. Moreover, the digestibility of five kinds of rice allergenic proteins was influenced by pH level, heat processing, starch matrix, solubility, and protein properties, on a case-by-case basis. On the other hand, all detected rice allergenic proteins and non-allergenic proteins were rapidly digested in SIF.

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1. Introduction

Rice is consumed as a staple food by more than half of the world's population (Kato, Katayama, Matsubara, Omi, & Matsuda, 2000). Rice proteins account for 5–12% of dried endosperm by weight (Villareal & Juliano, 1978), and most of them are seed-storage proteins (SSPs). Rice SSPs consist of 80% or more of glutelin, 2–8% prolamin, 4–15% globulin, and 1–5% albumin (Houston, Iwasaki, Mohammad, & Chen, 1968). Rice SSPs accumulate in two types of protein bodies (PBs); PB-I contains prolamin and PB-II is composed of glutelin and globulin (Tanaka, Sugimoto, Ogawa, & Kasai, 1980). The protein component is important in determining the texture, pasting properties and sensory characteristics of rice. Moreover, the digestibility of rice proteins is closely related to their nutritional value and allergenicity. Several allergenic proteins, with molecular masses of 60, 33, 26, 14–16 and 9 kDa, have been isolated from rice proteins, based on reactivity with IgE antibodies from patients showing hypersensitive reactions to rice ingestion. These rice allergens have been identified as granule-bound starch synthetase (GBSSI), glyoxalase 1 (Gly1), α -globulin, and α -amylase/trypsin inhibitor family proteins and nonspecific lipid transfer protein-1 (nsLTP1). So far, variations of most of these allergens

have been generally considered in the assessment of genetically modified (GM) rice (Satoh, Nakamura, Komatsu, Oshima, & Teshima, 2011; Takagi et al., 2005).

A major characteristic of many food allergens is their resistance to gastric digestion (Lehrer, Ayuso, Reese, Fu, & Gendel, 2002; Mills, Jenkins, Alcocer, & Shewry, 2004; Taylor & Hefle, 2002; Taylor et al., 2002). Food allergens are therefore generally resistant to pepsin digestion, and digestibility analysis has been regarded as one of the appropriate methods for evaluating the allergenic potential of proteins, including expressed foreign proteins in GM organisms (Spök et al., 2005; Taylor & Hefle, 2002). The use of the pepsinolysis test with simulated gastric fluid (SGF), to distinguish food allergens from non-allergens, was initially described by Astwood, Leach, and Fuchs (1996). Although there were several exceptions (Fu, Abbott, & Hatzos, 2002; Tanaka et al., 2002), pepsinolysis assay has commonly been applied as a biological measure of protein stability, and pepsin resistance has generally been considered important evidence of allergenic potential (EFSA, 2007; Mandalari et al., 2009). The digestibility of protein in SGF has been reported to be influenced by many factors, such as pH, the relative amounts of enzyme to test protein and matrix (Astwood et al., 1996; Kamata, Otsuka, Sato, & Shibasaki, 1982). A standardised protocol has been requested to avoid the variability of the pepsinolysis test. In response, Mandalari et al. (2009) designed a multi-laboratory evaluation of the digestibility of β -casein and β -lactoglobulin, and proved that low-protease digestion assay was reproducible among different laboratories.

* Corresponding author. Tel./fax: +81 29 838 7369.

E-mail address: kaz@affrc.go.jp (K. Kitta).

The digestibility of food protein is also an important parameter for nutritional value assessment. Storage proteins in the grains of both sorghum and maize have long been subjected to *in vitro* digestion analyses (Duodu, Taylor, Belton, & Hamaker, 2003; Mertz et al., 1984). Numerous factors, including the natures of the proteins themselves, their organisation within the grains, interaction between protein and non-protein components and cooking, have been suggested to influence the digestibility of sorghum proteins. The digestibility of grain proteins has been compared among rice cultivars both *in vivo* and *in vitro* (Boisen, Duldulao, Mendoza, & Juliano, 2001; Zhang et al., 2010). These studies showed that true protein digestibilities were different between indica and japonica rices in rats, although they were similar in humans. Moreover, with *in vitro* digestion analyses, similar protein digestibilities were found between indica and japonica cultivars.

Although protein digestibility analysis has been widely applied in allergenicity assessment and nutritional value evaluation, thus far, the approaches applied to determine digestibility have varied among research areas. For the evaluation of allergenicity potential, recombinant protein or extracted natural proteins were subjected to digestion analyses (Fu et al., 2002; Herman, Schafer, Korjagin, & Ernest, 2003) while, in the research field of nutritional value assessment, grain powder was digested and only the proteins remaining in the solid phase were subjected to further analyses (this is termed the residue method) (Mertz et al., 1984; Nunes, Correia, Barros, & Delgadillo, 2004; Zhang et al., 2010). Moreover, the pH of digestion assays with pepsin or SGF varied, ranging from a pH of 1.2 (Herman et al., 2003; Nunes et al., 2004; Ofori-Anti et al., 2008) to a pH of 2.0 (Ofori-Anti et al., 2008) and even to a pH of 2.5 (Astwood et al., 1996; Mandalari et al., 2009).

To our knowledge, less is known about the solubility of rice allergenic and non-allergenic proteins in SGF and SIF, and the digestibility of rice allergenic proteins has not been subjected to a multiplex comparison with the influence of rice matrix. In order to simultaneously compare the digestibility of rice proteins, including allergenic and non-allergenic proteins, in both the liquid and the solid phases of SGF and SIF digestion mixtures, as well as to evaluate the influences of pH, heat processing and the grain matrix on the digestion efficiency of rice proteins, we digested proteins in unheated and heat-processed rice grain powder with SGF and SIF. The digested proteins were extracted from the liquid and the solid phases of the digestion mixture and analysed using the SDS–PAGE-based method. Furthermore, the digestibility of 5 kinds of major rice allergenic proteins was simultaneously analysed by a multiplex immunodetection method.

2. Materials and methods

2.1. Rice grain

Brown rice grain of the cultivar Koshihikari (*Oryza sativa*, japonica), produced in Ibaraki, Japan, was purchased at the local market. The protein content of the rice grain was 6.7 g per 100 g of grain, determined by the Kjeldahl method (nitrogen-to-protein conversion factor: 5.95). To prepare the rice powder, rice grains were pulverised with a Fritsch Variable Speed Rotor Mill Pulverisette 14 (Idar-Oberstein, Germany) at 20,000 rpm, and sieved to pass a Sieve Ring with a 0.2 mm trapezoidal perforation.

2.2. Pre-processing of rice powder

Rice powder was pre-processed, using the method described by Zhang et al., 2010, with slight modifications. Briefly, unheated rice samples were prepared by soaking 50 mg of rice powder in 1 ml of

MilliQ water overnight at 4 °C. Heat-processed rice samples were prepared by soaking 50 mg of rice powder in 1 ml of MilliQ water for 2 h at 4 °C, followed by incubation in a boiling water bath for 20 min.

2.3. SGF digestion of pre-processed rice powder

For SGF digestion, protocols described by Wong et al. (2010) and Huang, Tsen, Chen, and Hwang (2011) were slightly modified. We prepared 2 × SGF by dissolving pepsin (469 units per mg solid; Sigma, St Louis, MO, USA) in 0.06 M NaCl (pH adjusted to 1.2, 2.0 and 2.5 with HCl) to make a pepsin concentration of 1107 units/ml (2.36 mg per ml). After the pre-processed rice samples and 2 × SGF were pre-warmed separately at 37 °C for 5 min, 1 ml of 2 × SGF was added to each pre-processed rice sample (giving approx. 330 units of pepsin/mg of rice protein). Samples designated as no-pepsin controls were supplemented with 1 ml of 0.06 M NaCl at the respective pH levels. Samples of experimental groups were incubated in a shaking water bath at 37 °C for 10, 30, 60 and 120 min, and no-pepsin control samples were incubated at 37 °C for 120 min. The digestion was stopped by raising the pH to 8.0 by adding 0.5 M ammonium bicarbonate to inactivate the pepsin. Samples were then kept on ice until the following step of protein extraction. The digestion treatment was conducted in duplicate and three independent experiments were performed.

2.4. SIF digestion of pre-processed rice powder

SIF digestion was performed according to the method reported by Fu et al. (2002) with some modifications. 2 × SIF was prepared by dissolving pancreatin (8 × USP specifications; Sigma) in 0.1 M KH₂PO₄ (pH adjusted to 7.5 with 1 N NaOH) to reach a pancreatin concentration of 20 mg/ml. After the pre-processed rice samples and 2 × SIF were separately pre-warmed at 37 °C for 5 min, 1 ml of 2 × SIF was supplemented to and mixed with each pre-processed rice sample. Samples designated as no-pancreatin controls were supplemented with 1 ml of 0.1 M KH₂PO₄ (pH 7.5). Samples of experimental groups were incubated at 37 °C with continuous shaking for 10, 30, 60 and 120 min, and no-pancreatin control samples were incubated for 120 min. At indicated times, the digestion was stopped by adding 0.1 ml of 100 mM phenylmethylsulfonyl fluoride (PMSF) and subjected to protein extraction as soon as possible.

2.5. Protein extraction from both the liquid and the solid phases of digestion mixture

The digestion mixtures were centrifuged at 11,000g at 4 °C for 30 min; the supernatant was designated as liquid phase and the sediment was designated as solid phase. The liquid phase was transferred to a new tube and a 4-fold amount (v/v) of ice-cold acetone was added. After thorough mixing, acetone precipitation of proteins was carried out at −30 °C for 2 h, followed by centrifugation at 11,000g at 4 °C for 30 min. The sediment was dried at room temperature for 15 min, and 0.5 ml of extraction solution, consisting of 125 mM Tris–HCl (pH 6.8), 4% (w/v) SDS, 10% (v/v) glycerol, 4 M Urea and 5% (v/v) 2-mercaptoethanol was added. The sediment was then suspended with a vortex mixer, after which, proteins were solubilised by rotation on a rotary mixer at room temperature (RT) for 2 h. To extract the protein from the solid phase of the digestion mixture, 1 ml of extraction solution was added and mixed with the solid phase sediment, and the mixture was rotated on a rotary mixer at RT for 2 h. Finally, all extraction

mixtures were centrifuged at 15,000g at 4 °C for 30 min, and the supernatants were transferred to new tubes and stored at –30 °C.

2.6. SDS-PAGE and multiplex immunodetection analyses of digested rice proteins

Proteins, in 5 µl of extraction solution, were resolved by SDS-PAGE, using a 15% (w/v) Acrylamide/Bis (29:1) separating gel with a 4% (w/v) Acrylamide/Bis (29:1) stacking gel. After electrophoresis, the gels were stained with Bio-safe Coomassie Stain (Bio-Rad Laboratories, Hercules, CA, USA) according to manufacturer's protocol. Otherwise, for immunoblotting, resolved proteins were transferred to Hybond-LFP membranes (GE Healthcare, Buckinghamshire, UK) by electroblotting with a Trans-Blot SD Semi-Dry Transfer Cell (Bio-Rad Laboratories). In order to analyse the digestibility of rice allergenic proteins simultaneously, the multiplex immunodetection method described in our previous report (Lang, Kagiya, Ohnishi-Kameyama, & Kitta, 2013) was applied to detect the following rice allergenic proteins: GBSSI, Gly1, α -globulin, α -amylase/trypsin inhibitor family proteins and nsLTP1.

3. Results and discussion

3.1. Rice proteins dissolved in the liquid phase and remaining in the solid phase of SGF digestion mixture

The SDS-PAGE-based method has generally been applied to analyse proteins subjected to pepsin digestion (Nunes et al., 2004; Wong et al., 2010). In the present study, SDS-PAGE was used to compare the solubility and digestibility of rice proteins. In order to evaluate the solubility of rice proteins in SGF, as well as to examine the process of their digestion in the dissolved and undissolved states, digestion mixtures were separated into a liquid phase and a solid phase by centrifugation. Rice proteins dissolved in the liquid phase were recovered by the acetone precipitation method, and proteins that remained in the solid phase were extracted from separated sediment. As judged by the SDS-PAGE bands of proteins in the liquid phase of unheated no-pepsin controls, α -globulin and α -amylase/trypsin inhibitor family proteins were soluble at the three tested pH levels and more kinds of proteins were extracted at pH 2.5 than at the other pH levels (Fig. 1). In heat-processed samples, the intensity of bands corresponding to α -globulin and α -amylase/trypsin inhibitor family proteins decreased compared with that in unheated samples. The results showed that low pH and heat processing decreased the solubility of rice proteins. On the other hand, compared with only a few kinds of protein dissolved in the liquid phase, more rice proteins, including the rice main storage protein, glutelin, remained in the solid phase. On SDS-PAGE gel, glutelin was resolved as three main bands of glutelin precursor, α -glutelin and β -glutelin. In addition, a band corresponding to 13 kDa prolamin was observed from proteins in the solid phase. Moreover, the colour intensity of the 13 kDa prolamin bands in the unheated no-pepsin controls was higher than that in the heat-processed ones at the same pH level, which implies that heat processing decreased its solubility.

3.2. Comparison of rice protein digestibility in SGF by SDS-PAGE analysis

To compare the influence of pH on protein digestibility, three levels of widely used pH—1.2, 2.0 and 2.5—were applied. Judging from the staining intensity of the protein bands resolved by SDS-PAGE (Fig. 1), most rice proteins were more quickly digested at pH 1.2 than at pH 2.0 and 2.5. The highest digestion rate, at pH 1.2, may be due to both the higher enzyme activity of pepsin and the more deeply denatured state of rice proteins.

In the liquid phases of digestion, at the three tested pH levels, α -globulin was diminished or decreased to faint content after as little as 10 min of digestion, while the intensity of bands corresponding to α -amylase/trypsin inhibitor family proteins decreased gradually with the process of digestion. On the other hand, compared with the SDS-PAGE-pattern of proteins in the no-pepsin controls, colour intensity in the range of less than 15 kDa was largely increased; these were protein fragments generated by pepsin digestion of rice proteins, since they were not observed in proteins of no-pepsin controls. Moreover, we found that the colour intensity was correlated with digestion rate. In digestion at pH 1.2, rice proteins were digested most quickly, and thereby the colour intensity of protein fragments of less than 15 kDa was higher than those in digestions at pH 2.0 and 2.5. Since protein digestion rate was obviously different among the three tested pH levels, to obtain comparable digestibility data, it would be necessary to apply the same pH in the pepsinolysis test.

In the solid phase of both unheated and heat-processed rice samples digested at pH 1.2, most proteins, including glutelin, the major rice SSP, were readily digested by pepsin over time, as revealed by the diminished staining intensity. This result corroborates a previous report that glutelin disappeared by pepsin digestion of separated protein bodies in 1 h (Ogawa et al., 1987). However, in the solid phase of digestion at pH 2.0 and 2.5, the decreasing rate of the colour intensity of most protein bands was slower than that at pH 1.2 over time, and the decreasing rate was greater at pH 2.0 than at pH 2.5. In contrast to the relatively quick digestibility of most rice proteins in the solid phase, a slower digestion rate was observed in a 13 kDa prolamin-like protein. The bands corresponding to the 13 kDa prolamin remained discernible for 120 min, even at pH 1.2. Moreover, judged by the variation of colour intensity, its digestion rate was slower in heat-processed samples than in unheated samples. Ogawa et al. (1987) reported that 13 kDa prolamin remained in pepsin-digested PB-I and its content was not greatly affected by pepsin concentration. Prolamin is widely considered hard to digest, and is accumulated in layer structure protein body-I (PB-I). Furthermore, previous reports have shown that the cooking process decreases the digestibility of PB-I proteins in rats (Resurreccion, Li, Okita, & Juliano, 1993). To the best of our knowledge, the present study is the first to demonstrate the slow digestion process of 13 kDa prolamin in unheated rice samples and, furthermore, that heat processing decreases the digestibility of 13 kDa prolamin *in vitro*.

3.3. Comparison of the digestibility of rice allergenic proteins in SGF by multiplex immunoblotting analysis

3.3.1. The solubility of rice allergenic proteins in SGF

To compare the solubility of rice allergenic proteins, a multiplex immunodetection method, developed in our previous study (Lang et al., 2013), was applied to simultaneously detect five kinds of rice allergenic proteins from the liquid and solid phases of the SGF digestion mixture. As shown in Fig. 2, three allergenic proteins, including α -globulin, α -amylase/trypsin inhibitor family proteins and nsLTP1, were detected from both the liquid and the solid phases of all the no-pepsin controls. The results suggested that these three kinds of allergens were soluble in SFG solutions at the three tested pH levels. Moreover, the signal intensity of soluble allergenic proteins in the liquid phase of no-pepsin controls was lower in heat-processed samples than in unheated samples at the same pH level.

With regard to the other two kinds of analysed allergenic proteins, the solubility of Gly1 was greatly influenced by heat processing and pH level. In heat-processed samples, the Gly1 signal was detected mainly from the solid phase, and only a faint signal was detected from the liquid phase. However, in unheated samples,

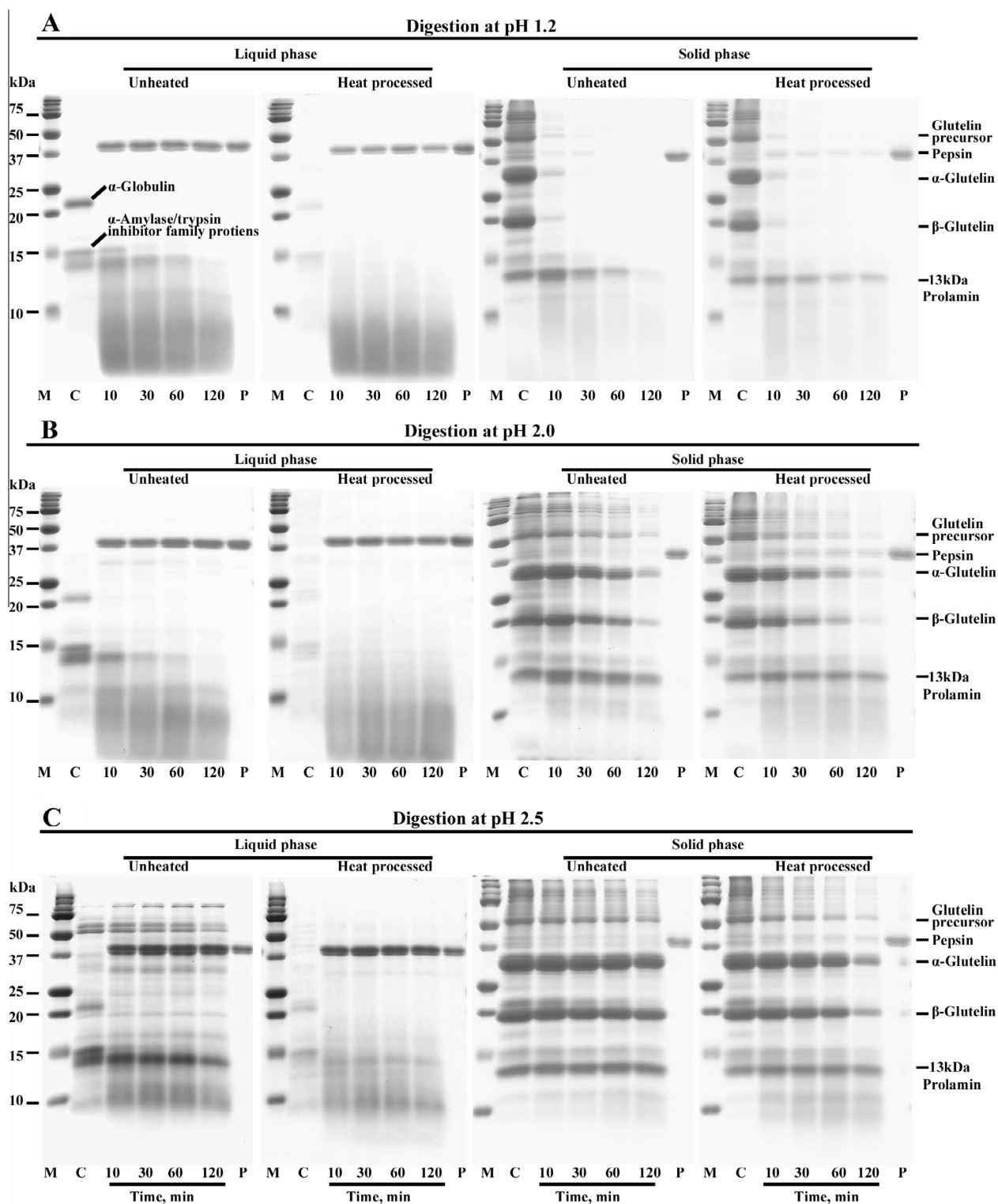


Fig. 1. Comparison of the *in vitro* protein digestion process of rice grain proteins in SGF analysed by SDS–PAGE. Unheated and heat-processed rice grain powder samples were subjected to pepsin digestion in SGF at pH 1.2 (A), 2.0 (B) and 2.5 (C). Proteins in both the liquid and the solid phases were extracted and 5 μ l of protein extracts were resolved by SDS–PAGE analyses. The numbers under the lanes indicate the time (min) of digestion. C represents the no-pepsin control; P is Pepsin, 7.5 μ g; M is Precision Plus Protein Dual Color Standards. Images are shown from a single experiment and are representative of three independent experiments.

solubility was pH-dependent. The largest amount of Gly1 was detected from the liquid phase of the unheated no-pepsin sample at pH 2.5 while, at pH 1.2 and 2.0, Gly1 was mainly detected from the solid phase. This decrease in the solubility of Gly1 could be

explained by the denaturing effect of the heat processing and low pH. It is interesting to find that a pH rise from 2.0 to 2.5 greatly increased the solubility of Gly1, and therefore turned its digestion reaction from the solid phase to the liquid phase.

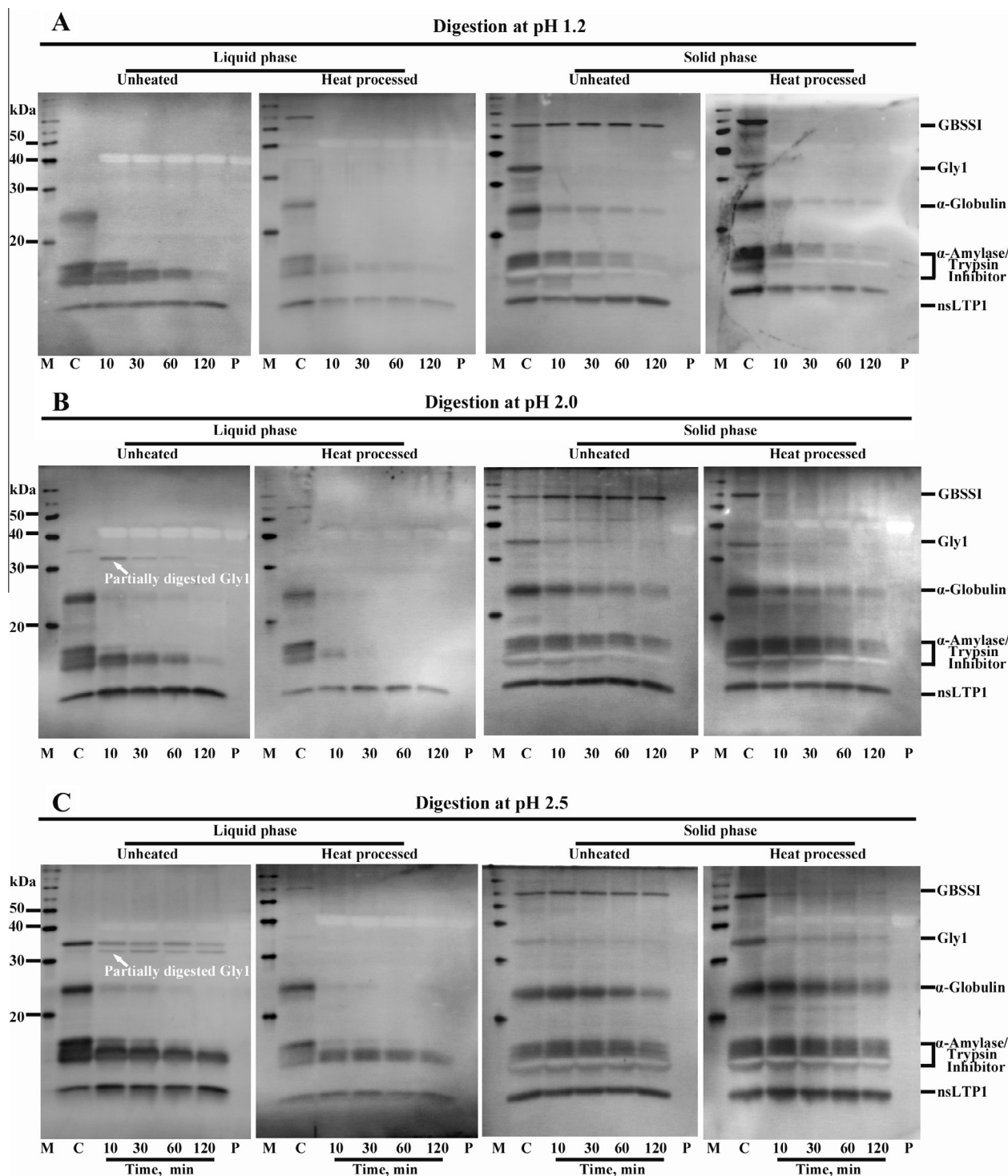


Fig. 2. Comparison of the digestion process of rice allergenic proteins in SGF detected by multiplex immunodetection. Unheated and heat-processed rice grain powder samples were subjected to pepsin digestion in SGF at pH 1.2 (A), 2.0 (B) and 2.5 (C). Rice allergenic proteins, including GBSSI, Gly1, α -globulin, α -amylase/trypsin inhibitor family proteins and nsLTP1, were detected by multiplex immunodetection. Five μ l of protein extracts from the liquid and the solid phases of digestion mixtures were loaded in each lane. The numbers under the lanes indicate the time (min) of digestion. C represents the no-pancreatin control; P is Pepsin, 7.5 μ g; M is MagicMark XP Western protein standard. Images are shown from a single experiment and are representative of three independent experiments.

On the other hand, as expected, GBSSI, the protein that has been suggested to be bound to the starch matrix, was mainly detected from the solid phases of all samples. However, weak signals of GBSSI were also detected from the liquid phase of all heated no-pancreatin control samples, which implies that a small portion of GBSSI is released from starch granules swelled by the heat processing.

3.3.2. The digestibility of GBSSI in SGF

During the process of SGF digestion, GBSSI was constantly detected from the solid phase of all unheated samples, in which no obvious change in signal intensity was observed. This result is in accord with a report that a 60 kDa waxy gene product (GBSSI) remained in pepsin-digested uncooked indica rice (Zhang et al.,

2010). However, in the present study, we found that GBSSI disappeared from heat-processed samples in as little as 10 min of digestion. As GBSSI could not be extracted by conventional methods, it was suggested to be bound to starch granules (Taira et al., 1995). When starch is heated in water, the breaking of hydrogen bonds allows water to enter into the starch granules and swells them, which is the gelatinisation process of starch. We speculated that, owing to the increased permeability of the heat-swelled starch granules to pepsin, GBSSI was quickly digested in heat-processed samples; on the contrary, GBSSI was shielded from pepsin digestion by starch granules and remained intact over the digestion process in unheated samples. In addition, the small portion of GBSSI released from swelled starch granules in heat-processed samples was degraded within 10 min. The above finding suggests that the starch matrix can restrict the digestion of GBSSI, and GBSSI was susceptible to pepsin digestion in heat-processed rice samples.

3.3.3. The digestibility of Gly1 in SGF

Because the solubility of Gly1 was influenced by both heat processing and pH level, the digestion of Gly1 occurred in the solid and/or liquid phases. After being digested for 10 min, Gly1 was diminished at pH 1.2 and decreased to a weak signal at pH 2.0 and 2.5 in the solid phase. Interestingly, however, partial digestion of Gly1 was found in the unheated samples at pH 2.0 and 2.5. At pH 2.5, two bands, one with a molecular mass similar to Gly1 and another with molecular mass a little smaller than that of Gly1, were detected from the liquid phase and continued to be detectable for as long as 120 min. At pH 2.0, a weak signal of Gly1 was detected from the liquid phase of the unheated no-pepsin control sample and was diminished after being digested for 10 min. Moreover, partially digested Gly1 fragments, showing higher colour intensity than that of Gly1 in the no-pepsin control, turned up after digestion for 10 min, and were then progressively digested over time. The partially digested Gly1 fragments were subsequently proved to specifically immunoreact with anti-Gly1 antibody (data not shown). These results proved that the digestibility of Gly1 was significantly affected by pH level, and that partially digested Gly1 had higher solubility than Gly1 at pH 2.0. Since Gly1 has a theoretical isoelectric point of 5.4 (Usui et al., 2001), the solubility variation among the tested pH levels should not be caused by isoelectric precipitation. Acid-induced unfolding of proteins has been reported to be critical in proteolysis (Herman, Gao, & Storer, 2006; Noda, Fujiwara, Yamamoto, Fukuno, & Segawa, 1994). Besides variation of pepsin activity at different pH levels, the denaturing effect of various acid levels might also greatly influence the solubility and digestibility of Gly1. Further study is needed to clarify the partial digestion mechanism of Gly1 at pH 2.0 and 2.5.

3.3.4. The digestibility of α -globulin in SGF

After being digested for 10 min, α -globulin was diminished in the liquid phase of digestion at pH 1.2, and showed a faint band in the solid phase of digestion at pH 1.2, as well as in the liquid phase of digestion at pH 2.0 and 2.5. In the solid phase, the digestion rate of α -globulin decreased with rise of pH level, which was reflected by the higher colour intensity at pH 2.0 and 2.5 than at pH 1.2 at the same digestion stage. Compared with digestion in the liquid phase, α -globulin in the solid phase was more slowly digested at all tested pH levels. This was probably because of the already identified accessibility problem in solid-phase reaction where the enzyme might have difficulty in accessing the substrate, which resulted in a lower reaction rate in the solid phase than in the liquid (Laurent, Haddoub, & Flitsch, 2008). In the present study, α -globulin and glutelin, the two large amounts of SSP stored in PB-II, were both found to be quickly degraded by digestion at a pH of 1.2.

3.3.5. The digestibility of α -amylase/trypsin inhibitor family proteins in SGF

Some homologous rice seed proteins of around 14–16 kDa (RAG2, RA5, RA5B, RA14, RA14B, RA14C, RA16, RA17) have been characterised as major rice allergens (RAs) (Nakase et al., 1996). These proteins have been identified as belonging to the α -amylase/trypsin inhibitor family of wheat and barley (Adachi et al., 1993). Such α -amylase/trypsin inhibitor family proteins were detected simultaneously with antibodies raised against recombinant RAG2 in our previous report (Lang et al., 2010). From the immunoblotting images (Fig. 2), we unexpectedly found that members of the rice α -amylase/trypsin inhibitor family proteins were digested at different rates. In the liquid phase, the high and low molecular mass fractions were preferentially digested, while the fraction showing medium molecular mass was progressively digested at a low rate, and remained detectable from unheated samples for as long as 120 min. Moreover, the digestion rate of the medium molecular mass fraction was greater at pH 1.2 and 2.0 than at pH 2.5. In the solid phase, α -amylase/trypsin inhibitor family proteins were more quickly digested at pH 1.2 than at pH 2.0 or 2.5, and were digested to a faint signal after 120 min at pH 1.2. Among members of the rice α -amylase/trypsin inhibitor family proteins remaining in the solid phase, low molecular mass fractions were found to be more preferably digested at pH 1.2 and 2.0. It would be interesting to study the distribution of rice α -amylase/trypsin inhibitor family protein members in rice matrix, which might be related to their variable digestibility.

3.3.6. The digestibility of nsLTP1 in SGF

Another unexpected finding revealed by the multiplex immunoblotting was that nsLTP1 remained detectable in both liquid and solid phases after being digested for as long as 120 min. Decrease in signal intensity was only observed in heat-processed samples by digestion at pH 1.2. This result showed the relatively low digestibility of nsLTP1. Plant nsLTP1s are basic polypeptides (pI 8–10) with 90–95 amino acid residues. Members of this family, isolated from different plant species, share sequence identities of 30–95%. Moreover, all nsLTP1s contain four disulphide-bonds, formed by eight conserved cysteine residues, which are essential for maintaining their molecular folding and are related to their high thermostability, protease resistance, and allergenic features. So far, nsLTP1s have been proven to be widely cross-reacting plant pan-allergens (Salcedo, Sanchez-Monge, Barber, & Diaz-Perales, 2007). Unlike α -globulin and α -amylase/trypsin family proteins, nsLTP1 is a trace protein in rice grains that cannot be detected on SDS-PAGE gel stained with Bio-safe Coomassie stain under the conditions applied in the present study. It could be concluded that the digestion rate of rice proteins is not always correlated with protein content.

3.4. The digestibility of rice proteins in SIF analysed by SDS-PAGE analysis

Pancreatin consists of several digestive enzymes, including amylase, lipase, and protease. As a result, several bands corresponding to these enzymes were observed in pancreatin control resolved by SDS-PAGE gels (Fig. 3A). The corresponding bands of rice proteins dissolved in the liquid phase were rapidly diminished within 10 min, and only the bands corresponding to enzymes of pancreatin were observed. Moreover, the rice proteins remaining in the solid phase also rapidly disappeared within 10 min. In addition, it was unexpected to find that protein fragments with molecular mass of less than 15 kDa were recovered from the solid phase. These low molecular mass protein fragments were not observed in the no-pancreatin controls, which were assumed to be peptides generated from the digestion of rice proteins by pancreatin. These

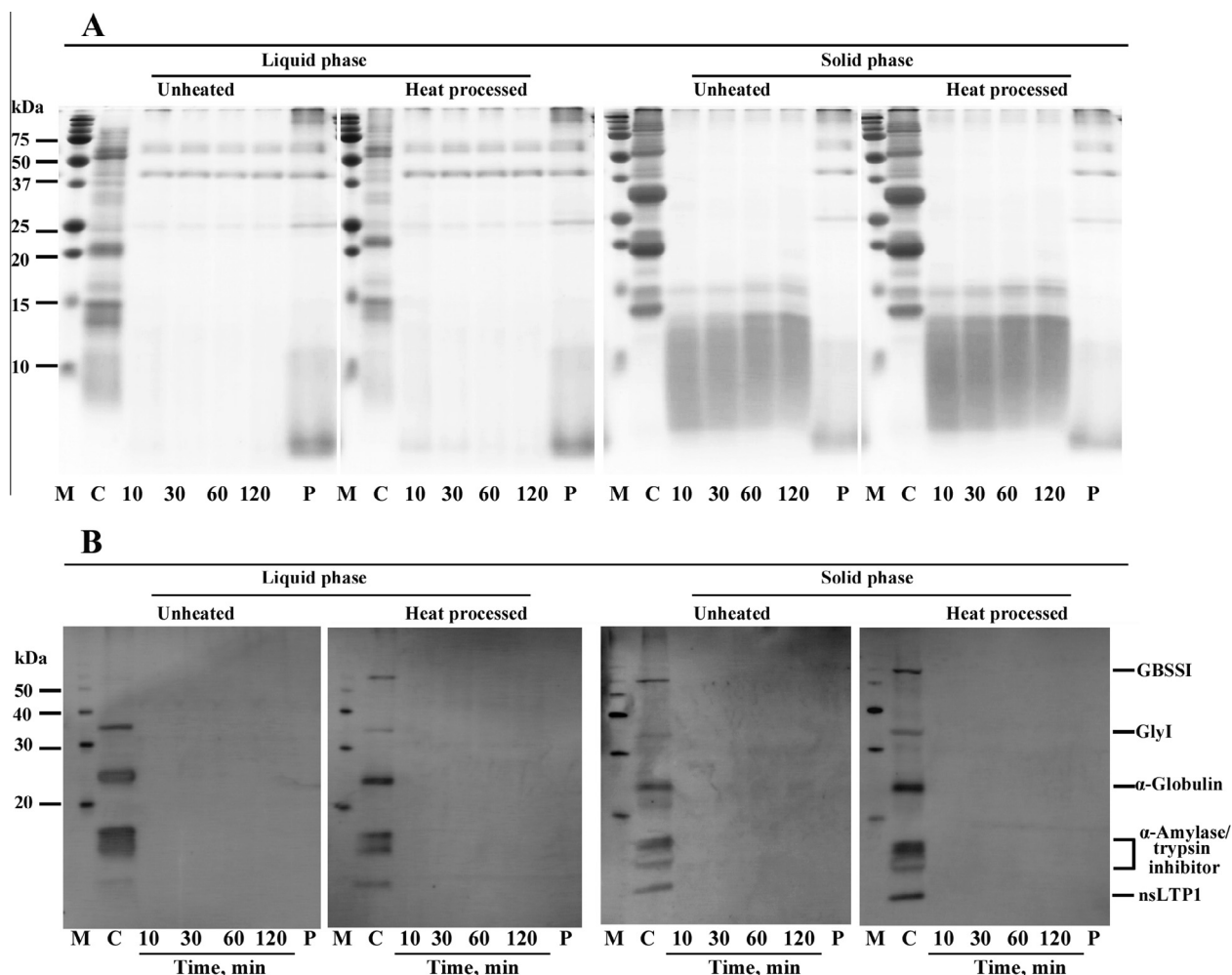


Fig. 3. Comparison of the digestion process of rice allergenic and non-allergenic proteins in SIF detected by SDS-PAGE and multiplex immunodetection. Unheated and heat-processed rice grain powders were subjected to pancreatin digestion in SIF. Proteins in both the liquid and the solid phases were extracted and 5 μ l of protein extracts were resolved by SDS-PAGE analyses (A), and rice allergenic proteins were detected by multiplex immunodetection (B). The numbers under the lanes indicate the time (min) of digestion. C represents the no-pancreatin control; P is Pancreatin, 10 μ g; M is Precision Plus Protein Dual Color Standards. Images are shown from a single experiment and are representative of three independent experiments.

peptides appeared within 10 min and remained throughout the digestion process.

3.5. The solubility and digestibility of rice allergenic proteins in SIF analysed by multiplex immunodetection analysis

As shown in Fig. 3B, strong signals of GlyI, α -globulin and α -amylase/trypsin inhibitor family proteins and a weak signal of nsLTP1 were detected from proteins extracted from the liquid phase of unheated no-pancreatin control samples. From proteins of heat processed no-pancreatin control samples, relatively strong signals of α -globulin and α -amylase/trypsin inhibitor family proteins and weak signals of GBSSI, GlyI and nsLTP1 were detected. Five kinds of allergenic proteins were detected from proteins extracted from solid phases of both unheated and heat-processed no-pancreatin controls. These results proved that GlyI, α -globulin and α -amylase/trypsin inhibitor had relatively high solubility in SIF solution, while the solubility of nsLTP1 was low. GBSSI in unheated rice samples was not soluble in SIF solution, but it was partially released from heat-processed samples.

In contrast to the variation of digestibility among five kinds of rice allergenic proteins in SGF, all five kinds of rice allergenic proteins were rapidly diminished within 10 min, and no differences

were observed in their digestibilities. Moreover, the multifunction of pancreatin was found to apparently contribute to starch digestion. So, matrix influence on protein digestion might have been quickly decreased by SIF digestion, which was found to greatly influence the digestion of GBSSI in SGF, as mentioned above.

Food allergens have been reported to show different stabilities between SGF digestion and SIF digestion. Fuchs and Astwood (1996) indicated that allergens with a low stability in SGF tended to have some stability in SIF, while Fu et al. (2002) reported that food allergens that were stable in SGF were not necessarily stable in SIF, and food allergens that were rapidly degraded in SGF were not necessarily resistant to SIF digestion. In the present study, five kinds of major rice allergenic proteins showed different digestibilities in SGF digestion; however, they were all rapidly digested in SIF.

4. Conclusions

Similar results were obtained from three independent experiments. The kinds of rice proteins dissolved in SGF decreased with the drop in pH level and the amount of soluble proteins was greatly decreased by heat processing. Among five kinds of analysed allergenic proteins, α -globulin, α -amylase/trypsin inhibitor family

proteins and nsLTP1 were soluble in SGF at the three tested pH levels, and were detected in both the liquid and solid phases of digestion mixtures. The solubility of Gly1 was greatly influenced by pH levels; it was soluble at pH 2.5, but became insoluble at pH 1.2. GBSSI in unheated samples could not be dissolved in SGF, while a little GBSSI was released into the liquid phase from heat-processed samples. The digestion of insoluble proteins only occurred in the solid phase, while the digestion of soluble proteins was carried out in both the liquid and solid phases. Therefore, in order to evaluate the digestibility of rice grain proteins, proteins, in both the liquid and solid phases, all need to be subjected to analysis.

pH level greatly influenced the digestibility of rice proteins in SGF. Most rice proteins were most rapidly digested at pH 1.2, rather than at the other two tested pH levels. At pH 2.0 and 2.5, the digestion rate of rice proteins decreased and partial digestion of Gly1 occurred. Heat processing not only decreased the solubility of rice proteins but also changed the structure of the starch matrix. After heat processing, the digestion of larger amounts of proteins was carried out in the solid phase; and GBSSI was quickly digested by pepsin that may have penetrated into the heat-swelled starch granules; otherwise, it remained intact in unheated samples.

Besides the pH of SGF, the solubility of rice proteins and the starch matrix protein properties all greatly influenced the digestibility of rice proteins. Unlike the digestion pattern of other rice allergens, partial digestion of Gly1 occurred at pH 2.0 and 2.5. Notably, members of the rice α -amylase/trypsin inhibitor family proteins in the liquid phase were digested at different rates and the medium molecular mass type needed a relatively longer time to be degraded even at pH 1.2. In addition, the digestion rate of rice proteins was not always correlated with the relative content of the proteins. 13-kDa prolamin and nsLTP1 were relatively difficult to digest compared with the high digestion rate of the major rice storage proteins, glutelin and globulin. Since pH, heat processing, solubility and the matrix connected with the proteins are all important factors affecting protein digestibility, we conclude that applying a constant pH, introducing the matrix influence into pepsinolysis analysis, and analysing proteins in both the liquid phase and the solid phase will provide a more comprehensive understanding of the digestibility of grain proteins in SGF.

In addition, different from the variation in digestibility of rice proteins in SGF, major rice allergenic proteins and non-allergenic proteins were all rapidly digested in SIF.

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