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Lectin from edible seaweed *Meristotheca papulosa* exhibits a high digestion-resistant property

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Abstract

The physiological impacts of polysaccharides and lipids in seaweed on human health are becoming clearer, but the behavior of the protein components, especially lectins, after ingestion remains poorly understood. In this study, we examined the resistance of edible red algae-derived lectins to digestive enzymes. We found that a lectin extracted from *Meristotheca papulosa* (MPL-1), belonging to the Jacalin-related lectin family, was relatively stable when subjected to both peptic and tryptic digestion and retained its hemagglutination activity after 24 h of digestion. The activity of MPL-1 was also maintained without a large change for 24 h following exposure to enzymes such as papain, Actinase E, and proteinase K, suggesting that MPL-1 possesses a strong resistant property against proteolytic digestion. We examined the anti-proliferation activity of the MPL fraction from the algal body against HT-29, a human colon cancer-derived cell line, and found that it showed a strong inhibitory activity with a half-maximal inhibitory concentration of 4.5 µg/mL. This activity was nullified in the presence of yeast mannan, an inhibitory sugar compound of lectin, demonstrating that MPL expressed its activity through binding to the glycan moieties on HT-29. This study indicates that this proteinase-resistant lectin could play a vital role after ingestion and is expected to have an inhibitory effect against colorectal cancer.

Keywords Lectin · Seaweed · Red algae · Digestion-resistance · Anti-cancer activity

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Introduction

Seaweed is a substantial fishery product that has garnered interest as a nutritious dietary option. The culture of marine algae has been initiated in many countries due the growing interest in their potential to produce essential natural molecules that have unique and algae-inherent properties (El-Beltagi et al. 2022). The brown algae *Saccharina japonica* and *Sargassum fusiforme*, the red alga *Neopyropia yezoensis*, and the green algae *Ulva* sp. are consumed daily as dietary ingredients and food. Extensive research has focused on the physiological effect of seaweed-derived polysaccharides and lipid components, such as fucoidan and fucoxanthin, and elucidation of their role in promoting health (Jin et al. 2021; Tavares et al. 2023). However, the functional attributes of protein elements of algae have remained relatively understudied. With the growing interest in seaweed-based diets, it is essential to understand the functionality of proteinaceous components and determine their potential toxicity.

Lectins, which are sugar-binding proteins, are widely distributed in various organisms, ranging from viruses to

humans. They selectively bind to carbohydrates and usually agglutinate cells or precipitate polysaccharides and glycoconjugates through their carbohydrate-recognition sites (Correia et al. 2008). Lectins function as recognition molecules in cell–cell interactions in diverse biological processes, such as defense against infection, innate immunity, quality control of glycoproteins, and cell cycle regulation (Sharon and Lis 2004). Recently, research has focused on lectins from marine algae due to their unique structures, carbohydrate-binding properties, and functions (Barre et al. 2020; Fernández Romero et al. 2021). Several algal lectins have been isolated from red algae, green algae, and blue-green algae (cyanobacteria), and these exhibit a number of common characteristics, including low molecular weight, thermostability, no requirement for metal ions, and no affinity for monosaccharides but with more specificity for glycoproteins (Hori et al. 1990; Rogers and Hori 1993). Numerous species of edible red algae are currently being utilized, and several species are recognized for their high lectin content, such as *Eucheuma serra* (lectin ESA) (Kawakubo et al. 1997, 1999; Sato et al. 2015), *Hypnea japonica* (lectin HypninA) (Hori et al. 1986a; Okuyama et al. 2009), and *Meristotheca papulosa* (lectin MPL) (Hori and Hirayama 2012). None of these lectins exhibit binding properties to monosaccharides, instead ESA and MPL bind to high mannose-type *N*-glycans (HM-glycans), which are highly expressed on viral surfaces (Watanabe et al. 2020) and cancer cells (Boyaval et al. 2022), while HypninA strictly recognizes only complex-type *N*-glycans with the core α1,6 fucose (Fuc) (Okuyama et al. 2009). In addition, ESA has been shown to induce apoptosis in cancer cells (Hayashi et al. 2012), while HypninA shows biological activities, such as platelet aggregation inhibition, anti-coagulation, and angiogenesis inhibition (Matsubara et al. 1996). Furthermore, α1,6-fucosyltransferase, which mediates core-fucosylation on *N*-linked glycoproteins, has been shown to be involved in biological and tumor characteristics and is upregulated in various cancers (Bastian et al. 2021). The core α1,6 fucosylated α-fetoprotein and haptoglobin are known as cancer markers for hepatocellular carcinoma (Zhu et al. 2014) and pancreatic cancer (Miyoshi and Nakano 2008).

The ubiquitous presence of lectins means that there is a good possibility that most plant-based foods contain substantial amounts of lectins with remarkable biological activities on gut function. In general, lectins are relatively stable against heat denaturation and proteolytic digestion (Pusztai and Bardocz 1996; Muramoto 2017). However, the extent of lectin resistance to degradation by the gut enzymes fluctuates greatly, reaching very high values in some cases. For example, legumes and whole grains contain high levels of various lectins (Shi et al. 2018), and the red kidney bean, which is a cultivar of *Phaseolus vulgaris*, is well-known to have a high level of phytohemagglutinin (toxic lectin) in

their raw form, which causes adverse effects (Banwell et al. 1993; Van Damme et al. 1998). Even though many plant lectins have been found to have a deleterious effect on the gut together with the enzyme inhibitors, in most cases it has not been acknowledged that this effect only arises at a relatively high lectin content in the diet and that lectins can have beneficial effects with low dietary intake, with no measurable negative effect on nutritional activity. Notably, lectins from the tomato *Lycopersicon esculentum* and mushroom *Agaricus bisporus* are known to resist digestion in vivo (Kilpatrick et al. 1985; Ditamo et al. 2016; Ismaya et al. 2020). Most of these foods are eaten raw or slightly cooked, which shows that a significant amount of lectins are consumed in an active form, while still being safe to eat. Hence, the physiological functions of food-derived lectins after consumption have been receiving much attention, especially given the potential for commercial applications.

While for many lectins from terrestrial plants the mechanisms for diet-gut interaction and resistance to digestive enzymes have been elucidated, as described above, these mechanisms have not been reported for edible seaweed-derived lectins. In terms of lectin consumption, the nutritional properties and safety of consuming edible seaweed in the diet have not been clarified as yet. In this context, this study was undertaken to examine the resistance of algal lectins to digestive enzymes, with the aim to estimate their behavior and beneficial properties after oral ingestion.

Materials and methods

Materials

The red alga *H. japonica*, which contains the lectin HypninA (Hori et al. 1986a; Okuyama et al. 2009), was collected at Kagoshima Prefecture, Japan and was kept at – 30°C until use. Dried red alga *E. serra*, which contains the lectin ESA (Kawakubo et al. 1997, 1999) and salted red alga *M. papulosa*, which contains the lectin MPL (Hori and Hirayama 2012) were obtained from a local shop in Miyazaki Prefecture, Japan, and from KANERYO Sea Vegetable Corp. (Kumamoto, Japan), respectively. Hexa-histidine tagged (His)-recombinants of ESA-2 (rESA-2), HypninA-2 (rHypninA-2), and MPL-1 (rMPL-1) were prepared according to the general method using the *Escherichia coli* expression system with the pET system, as described by Hirayama et al. (2016), except for rMPL-1, which was prepared using pCold system (Takara Bio Inc., Shiga, Japan). The edible mushroom *Agaricus bisporus* agglutinin (ABA) and the plant lectins soybean agglutinin (SBA) and kidney bean *Phaseolus vulgaris* agglutinin (PHA-E) were purchased from FUJIFILM Wako Pure Chemical Co. (Osaka, Japan). Pepsin, pancreatin, and trypsin were also obtained from FUJIFILM

Wako Pure Chemical Co. Protease Inhibitor cOmplete™ Mini Tablet, papain, and bovine serum albumin (BSA) were purchased from Merck (Darmstadt, Germany). Actinase E and proteinase K were purchased from KAKEN Pharmaceutical Co. (Tokyo, Japan) and Takara Bio Inc., respectively. Rabbit blood was purchased from the Hiroshima Animal Research Institute (Hiroshima, Japan). Protein size markers for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) were obtained from TEFCO (Tokyo, Japan) and Apro Science (Tokushima, Japan).

Preparation of lectin fraction from algal bodies

A 5-g sample of dried *E. serra* was soaked in ultrapure water for 30 min. All of the soaked algal body was utilized in subsequent processing. Salt on the salted *M. papulosa* was washed off with water, and the algal body was soaked in ultrapure water for 5 min. A 50-g sample of the soaked *M. papulosa* was used for the following processing. Fifty grams of *H. japonica* and the two soaked seaweeds, *E. serra* and *M. papulosa*, were frozen in liquid nitrogen and subsequently crushed using a blender, following which the crushed algae were stirred overnight at 4°C with 100 mL of a 20 mM phosphate buffer (pH 7.0) containing 0.85% (w/v) NaCl (phosphate-buffered saline [PBS]). The mixture was then centrifuged at 10,000 g for 30 min (4°C), and the supernatant was recovered. To obtain a protein-rich fraction, solid ammonium sulfate was added to the supernatant to attain a final concentration of 75% saturation. The mixture was kept overnight at 4°C, then centrifuged at 10,000 g for 30 min (4°C). The resulting precipitate was dissolved in a small amount of PBS and then dialyzed thoroughly against PBS. After the non-dialysate was centrifuged at 10,000 g for 30 min (4°C), the supernatant was collected as a salting-out fraction.

Digestion test for lectins from red algae and plants by artificial gastric and intestinal juices

Artificial gastric juice (AGJ), artificial intestinal juice (AIJ), and protease inhibitor solution were prepared immediately prior to the digestion test. AGJ was made by dissolving 0.85% (w/v) NaCl and 0.04% (w/v) pepsin in ultrapure water and the solution adjusted to pH 2.0 with hydrochloric acid. AIJ was composed of 0.04% (w/v) trypsin and 0.04% (w/v) pancreatin in 200 mM phosphate buffer (pH 7.0) containing 0.85% (w/v) NaCl (200 mM PBS). A 2× proteinase inhibitor solution was prepared by dissolving the Protease Inhibitor cOmplete™ Mini Tablet (Merck) in PBS at twofold its working concentration.

For the digestion test of ABA, SBA, PHA-E, rESA-2, rHypninA-2, rMPL-1, and BSA (control), respectively, 25 μg of protein was dissolved with 100 μL of AGJ; the pH

of the resulting solution was checked with pH test paper to ensure its proximity to pH 2. The proteins in the AGJ were incubated for varying durations (1, 2, 3, 8, 12, or 24 h) at 37°C. Following the stipulated time period, 100 μL of 2× protease inhibitor solution was added to each sample to stop the enzymatic digestion and the samples were then frozen until analyzed. The digestion test with AIJ was similar to that with AGJ, with the exception that the incubation times were 2, 4, 8, 12, and 24 h. For the digestion test involving sequential treatment with AGJ and AIJ, 25 μg of the protein in 100 μL of AGJ was incubated for 2 or 3 h, followed by the addition of 100 μL of AIJ and further incubation for 4 or 8 h, respectively. After these treatments, 200 μL of 2× protease inhibitor solution was added to stop digestion. Each digestion test was performed three times to check reproducibility.

Digestion test for digestion-resistant lectins with three proteinases

A sample (25 μg) of PHA-E, rMPL-1, or BSA (control) was dissolved in 100 μL of 200 mM PBS containing 0.04% (w/v) papain, Actinase E, or proteinase K. These samples were incubated for 30 min, 1 h, 3 h, and 24 h. Following the stipulated incubation period, 100 μL of 2× protease inhibitor solution was added to each sample to stop the enzymatic digestion and the samples then frozen until analyzed. Each digestion test was performed three times to check reproducibility.
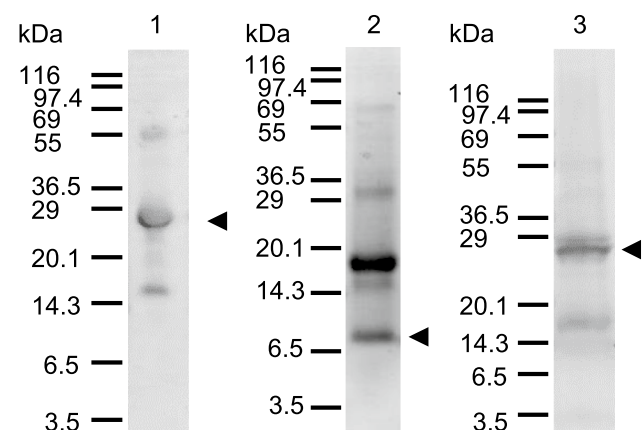

Fig. 1 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis profile of salting-out fractions in 3 species of edible red algae. Lanes: *1 Eucheuma serra*, *2 Hypnea japonica*, *3 Meristotheca papulosa*. After electrophoresis, the gel was stained with Coomassie Brilliant Blue R-250. Arrowheads indicate the bands of the corresponding lectins from each alga. About 10 μg of protein was applied in each lane
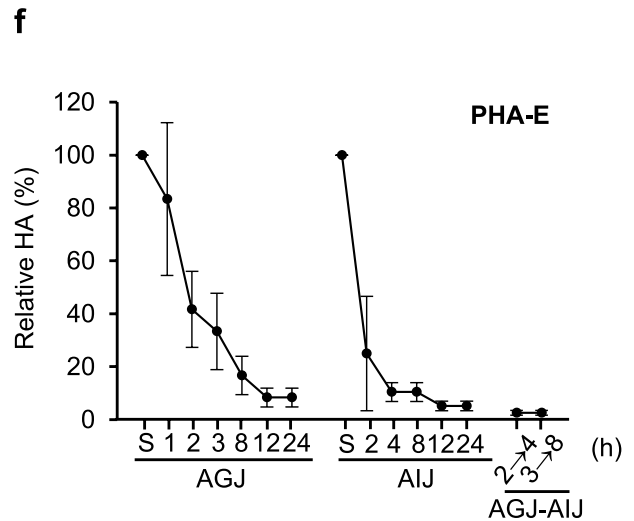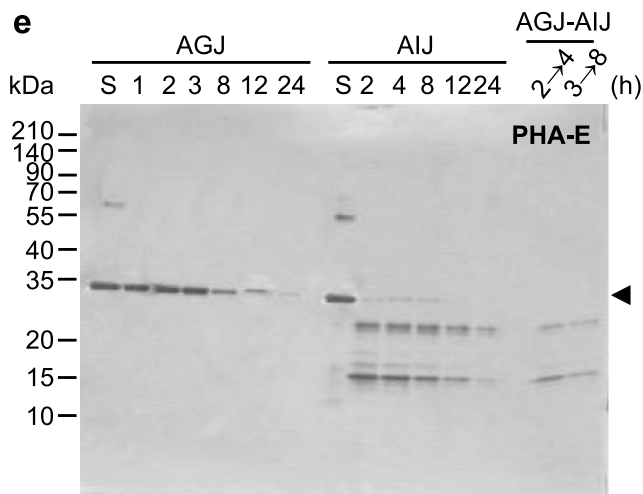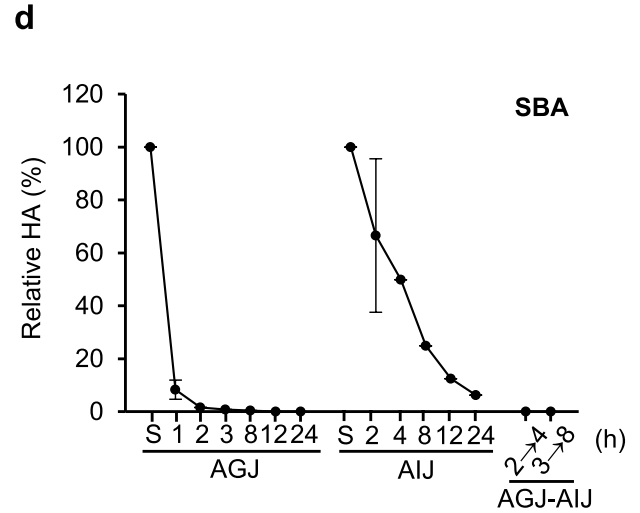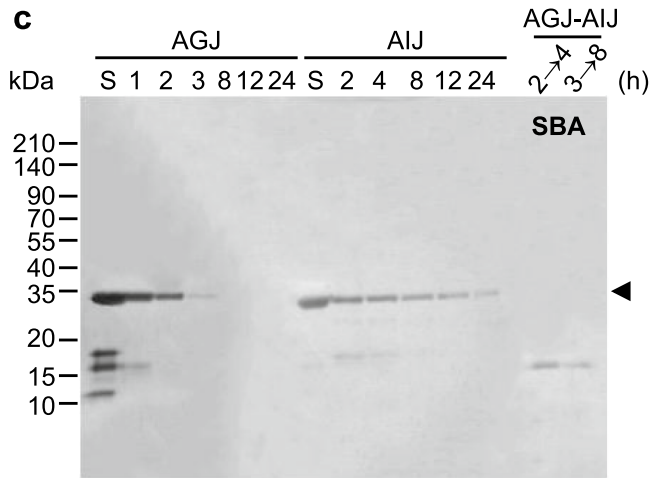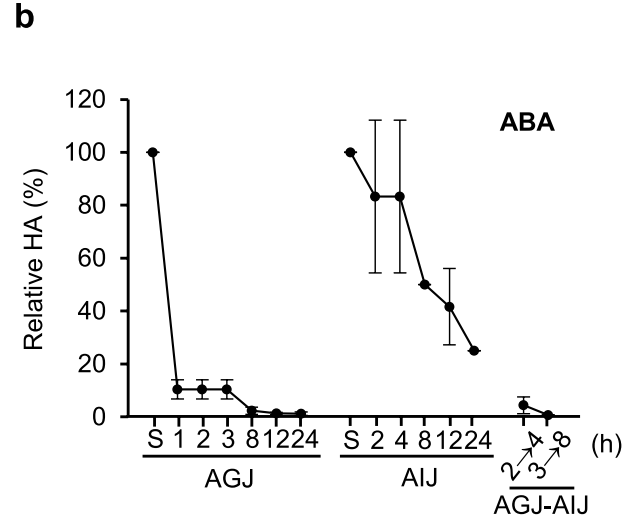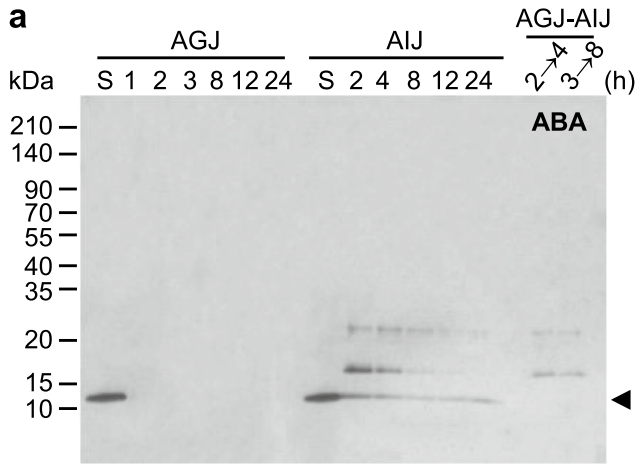
◄**Fig. 2** Representative sodium dodecyl sulfate-polyacrylamide gel electrophoresis profiles (**a, c, e**) and remaining hemagglutination activity (*HA*) (**b, d, f**) of lectins extracted from a mushroom and plants after treatment with simulated gastric and intestinal juices. Digestion tests for the mushroom *Agaricus bisporus* agglutinin (*ABA*) (**a, b**), soybean agglutinin (*SBA*) (**c, d**), and *Phaseolus vulgaris* agglutinin (*PHA-E*) (**e, f**) were performed by incubating the lectins with artificial gastric juice (*AGJ*) for 1, 2, 3, 8, 12, or 24 h and with artificial intestinal juice (*AIJ*) for 2, 4, 8, 12, or 24 h. Sequential digestion with AGJ and AIJ (2 incubation durations: 2 h [AGJ]—4 h [AIJ]; 3 h [AGJ]—8 h [AIJ]) was also examined. A 0.5-µg sample of lectin from each reaction was added to each lane and visualized with silver staining. Arrowheads indicate the bands of the corresponding lectins. Other bands are derived from proteases or degradation products of lectins. HA is shown as a relative value, with the untreated sample having the reference value of 100. Data are presented as means ± standard deviation from triplicate trials. *S* untreated sample

Hemagglutination test

Following the digestive enzyme treatment, a hemagglutination assay was performed using a 2% (v/v) suspension of trypsin-treated rabbit red blood cell (TRBC) as described by Hori et al. (1986b). Briefly, serial twofold dilutions of the sample were prepared in a final volume of 25 µL with 0.85% NaCl in microtiter V-shaped bottom plates, followed by the addition of an equal volume of TRBC to each well. The plate was gently shaken and kept at room temperature for 1 h. Hemagglutination was observed macroscopically, and hemagglutination activity (HA) was expressed as a titer (the reciprocal of the highest twofold dilution exhibiting positive hemagglutination). Furthermore, each activity of lectins in the digestion test was expressed as a relative value, with the HA of the untreated sample set at 100%.

SDS-PAGE and silver staining

Before and afer each treatment, 2 µg of each sample was subjected to SDS-PAGE in a 12% polyacrylamide gel, as described by Schägger and Jagow (1987). Samples were pre-treated by heating at 100°C for 5 min in a loading buffer. Gel electrophoresis was carried out at a constant voltage of 100 V for 2 h. After electrophoresis, the gel was subjected to silver staining using a Silver Stain MS Kit (FUJIFILM Wako Pure Chemical Co.) according to the manufacturer's instructions.

Cell culture and cytotoxicity test

The effect of the digestion-resistant lectin from *M. papulosa* on the HT-29 cell line, which is derived from human colon cancer, was examined. The cells were inoculated at a density of 5×10^3 cells/well in a 96-well culture plate and incubated at 37°C under 5% CO_2. After 24 h of incubation, the culture medium was replaced with 100 µL/well of the medium with

the *M. papulosa* lectin at various concentrations. To obtain medium at various concentrations of *M. papulosa* lectin, we lyophilized and dissolved various amounts of the salting-out fraction of *M. papulosa* in the medium (Fig. 1). The cells were then incubated for an additional 72 h. A control well was prepared in which cells were treated with medium without the lectin, and a blank well without cells was set up. After the incubation, 10 µL of water-soluble tetrazolium salt WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-*2H*-tetrazolium, monosodium salt) from Cell Counting Kit-8 (DOJINDO LABORATORIES, Kumamoto, Japan) was added to each well and the plate incubated for 2 h to determine the amount of viable cells by the colorimetric assay. Absorbance at 450 nm was measured using a multimode plate reader model ARVO X4 (Perki-nElmer, Shelton, CT, USA), and the cell viability in each well (%) was calculated by normalizing to the control well (untreated with the lectin fraction).

To elucidate whether the anti-proliferative effect of the lectin fraction from *M. papulosa* on HT-29 cells was caused by the action of lectin, yeast mannan, which has HM-glycans and is an inhibitory sugar compound against MPL (Hori and Hirayama 2012), was applied for an inhibition assay. Briefly, the cells were inoculated at 5×10^3 cells/well in a 96-well plate. After 24 h of incubation, the culture medium in each well was replaced with 100 µL of the medium containing 20 µg/mL salting-out fraction of *M. papulosa* with yeast mannan at various concentrations (ranging up to 10 mg/mL), and the cells were incubated for another 72 h. The WST-8 treatment, absorbance measurement at 450 nm, and calculation of the cell viability were performed as described above.

Statistical analysis

Data are expressed as the means ± standard deviation (SD). Statistical significance was evaluated by Student's or Welch's *t*-test. Significance was set at $P < 0.05$.

Results

Salting-out fractions of edible red algae

Three species of edible algae, *E. serra*, *H. japonica*, and *M. papulosa*, were examined with the aim to extract lectin from the algal body of each species. SDS-PAGE of the salting-out fractions from each of these algal species revealed a few major bands. Western blotting using antibodies specific to each lectin was performed on the salting-out fractions from *E. serra* and *H. japonica* to clarify which bands in the fractions were derived from lectins (Electronic Supplementary Material [ESM] Fig. S1). The results showed that the 28
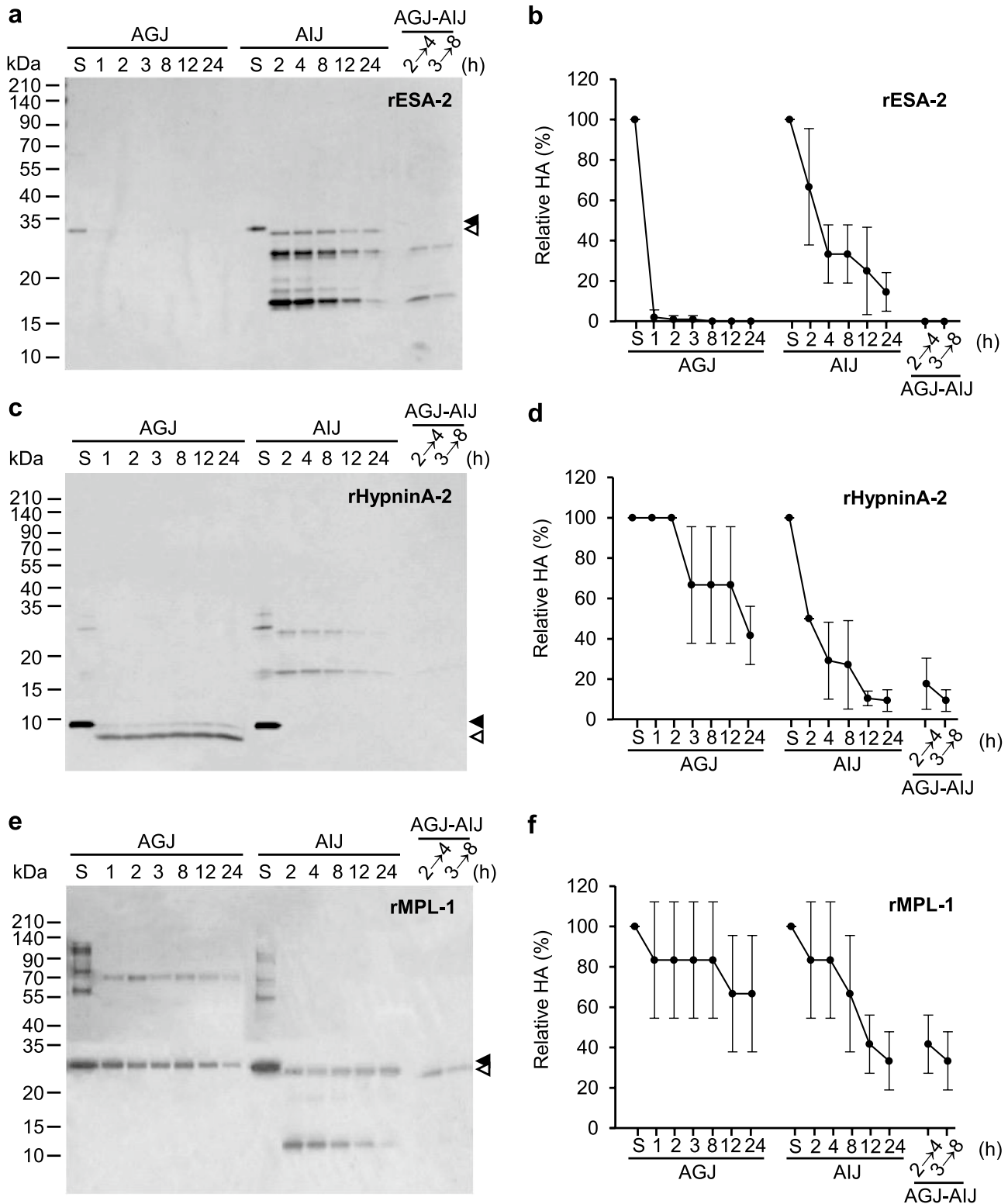
kDa and 8 kDa components in the fractions from *E. serra* and *H. japonica* (Fig. 1) were ESA and HypninA, respectively, as previously reported (Kawakubo et al. 1997; Hori et al. 2000). For *M. papulosa*, Hori and Hirayama (2012) previously demonstrated that the 26 kDa protein band in the salting-out fraction corresponded to the MPL lectin. Thus, it was determined that these three edible red algae contained relatively large amounts of lectins.

◄**Fig. 3** Representative sodium dodecyl sulfate-polyacrylamide gel electrophoresis profiles (**a**, **c**, **e**) and remaining hemagglutination activity (*HA*) (**b**, **d**, **f**) of lectins extracted from edible red algae after treatment with simulated gastric and intestinal juices. Digestion tests for recombinant *Eucheuma serra* lectin (*rESA-2*) (**a**, **b**), recombinant *Hypnea japonica* lectin (*rHypninA-2*) (**c**, **d**), and recombinant *Meristotheca papulosa* lectin (*rMPL-1*) (**e**, **f**) were performed by incubating lectins with artificial gastric juice (*AGJ*) for 1, 2, 3, 8, 12, or 24 h) and with artificial intestinal juice (*AIJ*) for 2, 4, 8, 12, and 24 h. Sequential digestion with AGJ and AIJ (2 incubation durations: 2 h [AGJ]—4 h [AIJ] and 3 h [AGJ]—8 h [AIJ]) was also examined. Arrowheads indicate the bands of the corresponding lectins. A 0.5-µg sample of lectin from each reaction was added to each lane and visualized with silver staining. Filled arrowheads indicate the corresponding lectins; blank arrowheads indicate the lectins from which the His-tag portion was presumably removed. Other bands are possibly derived from proteases or degradation products of lectins. HA is shown as a relative value, with the untreated sample having the reference value of 100. Data are presented as means ± standard deviation from triplicate trials. *S* Untreated sample

Digestion of algal and plant lectins with artificial gastric and intestinal juices

The digestion resistance of the identified lectins was determined in experiments using AGJ and AIJ and subsequently evaluated by examining the remaining HA and band signals by SDS-PAGE (Figs. 2, 3). The mushroom lectin ABA (16 kDa; Figs. 2a, b) and plant lectin SBA (31 kDa; Figs. 2c, d) immediately lost their HA upon incubation with AGJ, with the disappearance of their corresponding bands in SDS-PAGE. The HA and the signal intensities of the lectin protein bands in the SDS-PAGE gel exhibited a gradual decrease over time following their treatment with AIJ. Sequential treatment with AGJ and AIJ resulted in complete digestion of ABA and SBA. BSA, which was used as a control, also showed similar digestion behavior to these two lectins in the SDS-PAGE gel (ESM Fig. S2). On the other hand, PHA-E (30 kDa), which is known to be resistant to proteolytic degradation (Pusztai et al. 1979), retained 40% of its HA following a 2-h treatment with AGJ, although it was susceptible to digestion over an extended period. Notably, PHA-E displayed a tendency to be more readily digested by AIJ compared to AGJ (Figs. 2e, f). After sequential digestion with both AGJ and AIJ, PHA-E exhibited minimal HA (Fig. 2f) with no band (Fig. 2e). The results of SDS-PAGE and HA generally corresponded well.

For the algal lectins, the behavior of rESA-2 to digestion with AGJ and AIJ resembled that of ABA and SBA (Figs. 2a, d). Interestingly, rHypninA-2 retained 40% of its HA even after 24 h of treatment with AGJ, and digested products were detected, which were considered to have remained biologically active (Figs. 3c, d). Also, rHypninA-2 appeared to be more susceptible to digestion by AIJ than by AGJ, and the bands corresponding to rHypninA-2 disappeared after AIJ treatment even though HA to some extent

was detected. Due to this result, no bands of rHypninA-2 were observed, and little HA appeared after sequential treatment with AGJ and AIJ. In contrast, rMPL-1 retained its activity at > 60% even up to 24 h of treatment with AGJ and AIJ (Figs. 3e, f). In the SDS-PAGE analysis, the bands corresponding to rMPL-1 remained detectable even after sequential treatment with AGJ and AIJ; it should be noted that rMPL-1 was the only lectin from among all of the lectins tested that showed bands after the sequential treatment. Figure 4 shows a comparison of the relative activities of all the lectins tested after sequential treatment with AGJ and AIJ for 3 and 8 h, respectively. The residual activity of rMPL-1 was significantly higher than that of the other lectins. Based on these results, rMPL-1 exhibited the highest resistant properties to digestion enzymes, surpassing even the resistance demonstrated by PHA-E, which is known as a digestion-resistant plant lectin. The SDS-PAGE profiles of the lectin alone incubated at 37 °C for 24 h are shown in ESM Fig. S3 as a reference of non-enzymatic degradation of lectins; ESM Fig. S4 shows enzyme-derived bands in the AIJ. In addition to the pH 2.0 condition applied for AGJ in the above study, the digestion of MPL-1 in AGJ at pH 1.5 and 1.0 was also investigated to evaluate the further stability of MPL-1 after ingestion (ESM Fig. S5). These results indicated that 40% of HA was retained at pH 1.5 even after 24 h of incubation. On the other hand, MPL subjected to AGJ at pH 1.0 exhibited a substantial decrease in HA over time although 30% of HA was retained after 3 h incubation. This lectin displays remarkable resistance to digestion by gastric juice at acidic conditions, particularly those up to pH 1.5.

rMPL-1 was not digested with AGJ, but it was digested with AIJ, with the SDS-PAGE showing a slightly reduced molecular weight (Fig. 3e). To check the digestion-resistant property of the rMPL-1 molecule, western blotting analysis was performed using antibodies exhibiting reactivity with MPL-1 or the 6-His-tag for AGJ- or AIJ-treated rMPL-1 (ESM Fig. S6). The analysis using anti-MPL-1 antibody showed that the bands corresponded to rMPL-1 in both treatments, even though rMPL-1 treated with AIJ was slightly smaller in size than intact rMPL-1 (Fig. S6b). In contrast, using the anti-His-tag antibody, we detected a band for rMPL-1 treated with AGJ while there was no band for the rMPL-1 treated with AIJ (Fig. S6c). These results indicated that the His-tag region in rMPL-1 is to be digested with AIJ and that the structure of the MPL-1 molecule could be intact even after proteolytic treatment. The slightly smaller bands observed in the AIJ-treated rESA-2 and AGJ-treated rHypninA-2 (Fig. 3a, c) were presumed to be those of recombinant lectins without His-tag, removed by digestion.
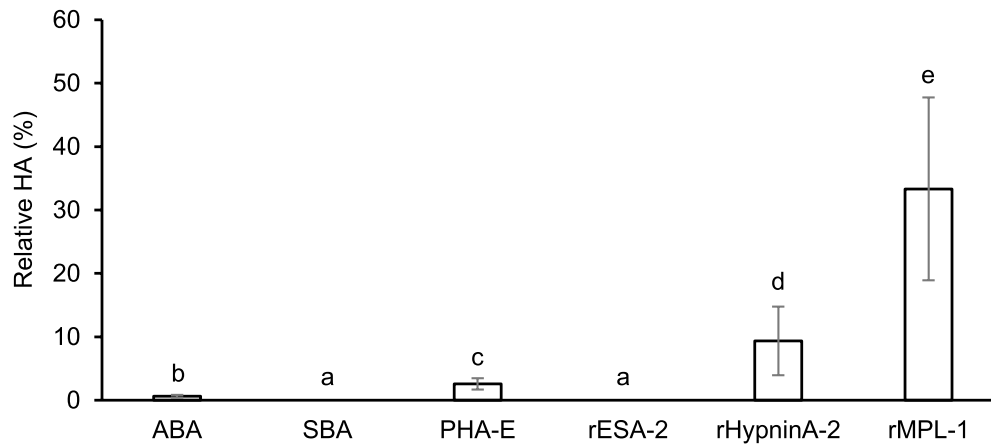
Fig. 4 Statistical comparison of the hemagglutination activity (*HA*) of the lectins after sequential treatment with simulated gastric and intestinal juices. ABA, SBA, PHA-E, rESA-2, rHypninA-2, and rMPL-1 were sequentially treated with AGJ and AIJ for 3 and 8 h, respectively. HA is shown as a relative value, with the untreated sample having the reference value of 100. Data are presented as means ± SD from triplicate trials. Means without a lowercase letter in common are significantly different at *P* < 0.05, as determined by Student's or Welch's *t*-test. *ABA* Mushroom *Agaricus bisporus* agglutinin, *SBA* soybean agglutinin, *PHA-E Phaseolus vulgaris* agglutinin, *rESA-2* recombinant *Eucheuma serra* agglutinin, *rHypninA-2* recombinant *Hypnea japonica* lectin, *rMPL-1* recombinant *Meristotheca papulosa* lectin, *SD* standard deviation

Resistance of rMPL-1 and PHA-E to digestion with other enzymes

rMPL-1, characterized by its notable resistance to digestion by both AGJ and AIJ, and PHA-E, which is known as a proteinase-resistant lectin (Pusztai et al. 1979), were separately subjected to further digestion tests utilizing three distinct proteinases, namely, papain, Actinase E, and proteinase K, with HA as the indicator (Fig. 5). BSA was also used as a control, and SDS-PAGE showed that BSA was degraded over time by each of the three above-mentioned proteinases, with the band disappearing after 24 h (ESM Fig. S2b-d). The HA of PHA-E exhibited a significant reduction within just 30 min of exposure to papain (Fig. 5a) and Actinase E (Fig. 5c); also, proteinase K treatment gradually rendered PHA-E inactive over time (Fig. 5e). In contrast, rMPL-1 retained at least approximately 15%, 30%, and 80% of its initial activity even after 24 h of treatment with papain, Actinase E, and proteinase K, respectively (Fig. 5b, d, f). A comparison of the relative activities of PHA-E and rMPL-1 after treatment with the three proteinases for 24 h is shown in Fig 6. The residual activity of rMPL-1 with each of these proteinases was significantly higher (Actinase E, proteinase K) or tended to be higher (papain) than that of PHA-E with the same proteinase. These results suggested that rMPL-1 possesses a significantly broader enzymatic digestion-resistance capacity which greatly exceeds that of PHA-E.

Anti-proliferative activity of *M. papulosa* lectin against human colon cancer-derived cell

The proliferation-inhibitory effect of *M. papulosa* lectin on human colon cancer-derived HT-29 cells was assessed in vitro. The results showed that *M. papulosa* lectin has anti-proliferative effects against HT-29 cells. The extent of inhibition exhibited a direct sigmoidal correlation with increasing concentrations of *M. papulosa* lectin. The half maximal inhibitory concentration (IC_{50}) of MPL-1 against HT-29 proliferation was 4.5 µg/mL (Fig. 7a) and the anti-proliferative effect was nullified in the presence of yeast mannan (Fig. 7b), a glycoprotein known to inhibit the HA of MPL-1.

Discussion

Dietary lectins, especially those of plant origin, have been studied for their biological activities in view of their high resistance to digestion (Pusztai and Grant 1998; Kelsall et al. 2002). SBA, PHA, Concanavalin A (ConA), and wheat germ agglutinin (WGA) are among the plant proteins that have been found to show resistance to degradation by the gut enzymes (Pusztai et al. 1979; Nakata and Kimura 1985; Carbonaro et al. 1997; Pan et al. 2018). ABA and tomato lectin have been reported to be resistant to digestive processes in the gastrointestinal tracts of both rats and humans, and when consumed in their raw form they appear not to be harmful (Kilpatrick et al. 1985; Rhodes 1999; Ismaya et al.
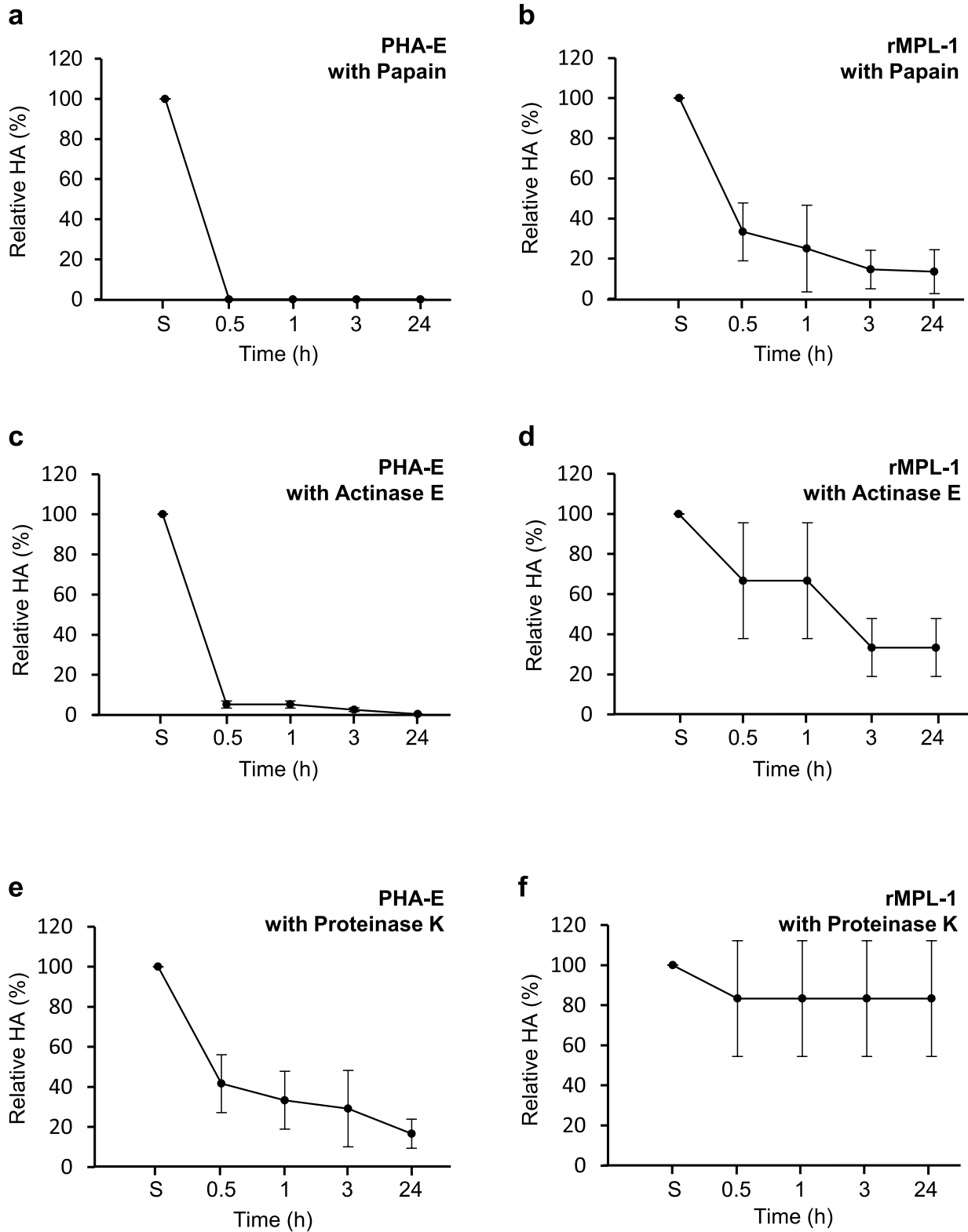
Fig. 5 Hemagglutination activity (*HA*) of PHA-E (**a**, **c**, **e**) and rMPL-1 (**b**, **d**, **f**) after treatment with each of three proteinases: papain (**a**, **b**), Actinase E (**c**, **d**), and proteinase K (**e**, **f**). The digestion was performed by dissolving each lectin in the reaction solution containing each proteinase for different durations of incubation time (0.5, 1, 3, and 24 h). HA is shown as a relative value, with the untreated sample having the reference value of 100. Data are presented as means ± standard deviation from triplicate trials. *PHA-E Phaseolus vulgaris* agglutinin, *rMPL-1* recombinant *Meristotheca papulosa* lectin, *S* untreated sample
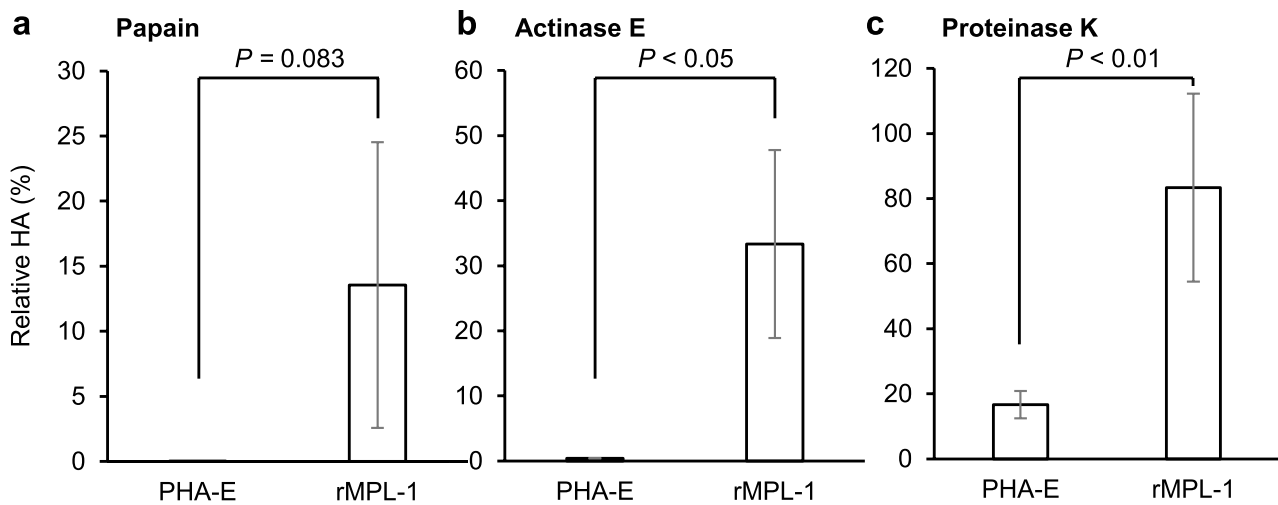
Fig. 6 Statistical comparison of the hemagglutination activity of PHA-E and rMPL-1 after 24 h of treatment with papain (**a**), Actinase E (**b**), and proteinase K (**c**). HA is shown as a relative value, with the untreated sample having the reference value of 100. Data are presented as means ± standard deviation from triplicate trials. *PHA-E Phaseolus vulgaris* agglutinin, *rMPL-1* recombinant *Meristotheca papulosa* lectin

2017). *Phaseolus vulgaris* serves as a substantial source of lectins in the human diet, and the ability of *Phaseolus vulgaris* lectins to bind to glycoconjugates is considered to be the primary factor for their poor nutritional value (Bardocz et al. 1995; Woodley 2000). The apparent in vivo indigestibility of plant lectins is a consequence of their ability to bind to intestinal epithelial cell receptors, as well as their molecular structure (Pusztai and Bardocz 1996). In the present study, PHA-E presented moderate resistance to simulated gastric juice and simulated intestinal juice (Fig. 2e–f), a result which is consistent with previous reports indicating that this lectin reaches the intestine in an active form (Pusztai et al. 1979; Hara et al. 1984). When consumed, remnants of partially undigested lectins in the gut may account for reported deleterious effects, reducing enzyme secretion and inhibiting membrane digestion absorption (Jaffé 1960; Oliveira et al. 1988). Although all of these ideas are based on studies with PHA, some plant lectins have been found to show similar effects (Hajos et al. 1995). In addition, it is noteworthy that the effect of lectins depends critically on the degree of resistance to proteolytic degradation. For example, red algal lectins are regarded as part of the human diet because they are often consumed raw or sometimes lightly cooked. Although these algal lectins might not be digested and may induce a reaction in the gut, there have been no reported cases of any toxic effects due to the consumption of these algal lectins so far. In our study, the red algal lectin ESA-2 (Fig. 3a, b) was rapidly degraded by simulated gastric fluid (AGJ), but the red algal lectin HypninA-2 (Fig. 3c, d), which is considered to have a highly stable structure due to its small molecular size and the presence of disulfide bonds

(Hori et al. 1986a, 2000) and which also possesses significant biological activity (Okuyama et al. 2009), was found to be partially digested with AGJ and completely digested with simulated intestinal fluid (AIJ). Moreover, among the algal lectins tested in the present study, MPL-1 showed marked resistance against enzymatic degradation by gastric and intestinal fluids (Figs. 3e, f, 4). SDS-PAGE analysis of this lectin showed a small change in the band intensity and in its HA even after 24 h of exposure to the digestive fluids. This observation stands in contrast to the findings with other tested lectins, including the digestion-resistant PHA-E (Fig. 2e, f). The gastric pH normally fluctuates roughly between 1 and 4 in the human body (Merki et al. 1988). The resistance to AGJ at lower pH (1.0 and 1.5) was also observed, as shown in ESM Fig. S5. Since the stomach retains digestible (< 2–3 mm) and indigestible (> 5–7) solid foods for roughly 3 and 4 h, respectively (Feldman et al. 1984; Pal et al. 2007; Goyal et al. 2019), these results infer that MPL-1 can maintain some activity and migrate to the intestine even in the presence of fairly strong gastric acid.

The tertiary structure of proteins can impact both the levels of detergent loading and the polypeptide migration rates in SDS-PAGE (Rath et al. 2009). For example, disulfide bonds in a protein can decrease SDS binding to the molecule (Pitt-Rivers and Impiombato 1968; Dornmair et al. 1990; Ohnishi et al. 1998) and have been associated to the unusually rapid migration of unreduced proteins compared to their reduced counterparts, likely due to the intact disulfide bonds causing the proteins to adopt a more compact shape (Dunker and Kenyon 1976; Kleinschmidt et al. 1999). In the present study, rHypninA-2 (calculated molecular weight [MW] 11.7
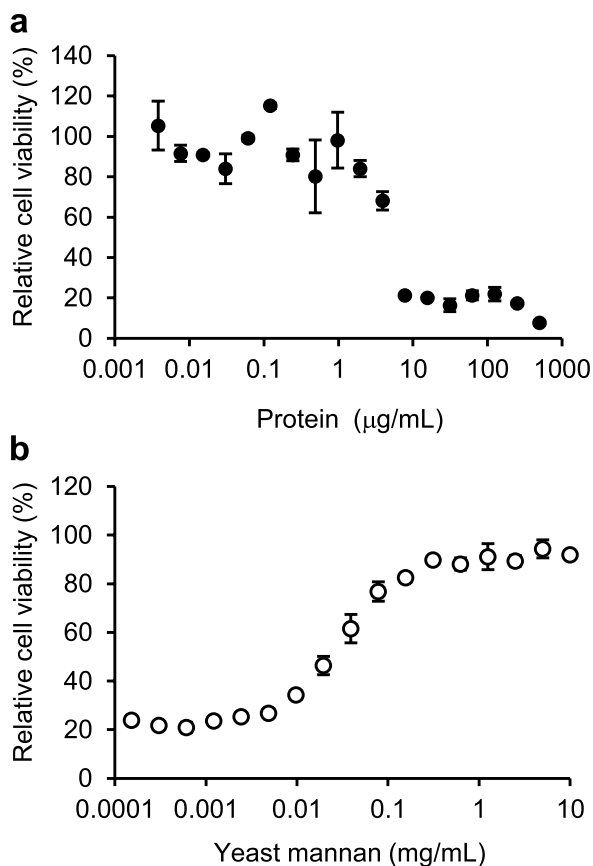
Fig. 7 The proliferation-inhibitory effect of the *Meristotheca papulosa* lectin (MPL-1) fraction on cells of the human colon cancer-derived cell line HT-29. **a** Cell viability of HT-29 cells after treatment with MPL-1 fraction. **b** Nullification of the anti-proliferative effect of the lectin fraction from *M. papulosa* with yeast mannan, which is the inhibitory glycoprotein against MPL-1. Cell viability is shown as a relative value, with that of the cell treated without lectin having the reference value of 100. Data are presented as means ± standard deviation from triplicate trials

kDa) and rMPL-1 (MW 35.2 kDa), but not ESA-2 (MW 30.3 kDa), have disulfide bond(s) in their molecules (Hori et al. 2000, 2007; Hori and Hirayama 2012). As shown in Fig. 3, the apparent MWs of rHypninA-2 and rMPL-1 during SDS-PAGE were observed to be smaller than the calculated MWs. In a previous study using natural lectins of HypninA-2 (calculated MW 9.1 kDa) and MPL-1 (calculated MW 31.1 kDa) obtained from algal bodies, it was also observed that the apparent MWs on SDS-PAGE were smaller than the calculated MWs or the MWs determined by mass spectrometry (Fig. 1), with the differences attributed to be the result of intramolecular disulfide bonds (Hori et al. 2000; Hori and Hirayama 2012). In the present study, intramolecular disulfide bonds could also have had a significant effect on the unexpected behavior of rHypninA-2 and rMPL-1 during SDS-PAGE (Fig. 3).

Digestion-resistant lectins also resist breakdown by proteinases from gut bacteria and survive the digestive tract conditions in an immunologically and functionally intact form by employing their vigorous biological activities (Bardocz et al. 1995). To elucidate the resistant property of MPL-1 against proteinases other than digestive enzymes in the gut, we performed digestion tests using three proteinases: papain, Actinase E, and proteinase K. These three proteinases were examined for their effect on MPL-1 and PHA-E. The tests showed that MPL-1 exhibited a higher digestion-resistant profile than PHA-E against all three proteinases (Figs. 5, 6). Papain is a cysteine protease that is present in the latex of the *Carica papaya* (papaya) plant. Papain has broad specificity for peptide bonds and prefers amino acids with large hydrophobic side chains at the P2 position on the cleavage region but does not accept valine in the P1 position (Kamphuis et al. 1985). Actinase E is a mixture of proteolytic enzymes derived from the culture filtrate of the actinomycete *Streptomyces griseus*, and it has an extremely broad substrate specificity which is capable of hydrolyzing almost all peptide bonds in proteins (Nomoto et al. 1960). In contrast, the cleavage site of proteinase K, which is a serine protease, is the peptic bond adjacent to the carboxyl group of aliphatic and aromatic amino acids with blocked alpha amino acids (Ebeling et al. 1974). Since all of the proteinases tested in the present study, including pepsin (UniProt ID: P00791), trypsin (UniProt ID: P00760), chymotrypsin (UniProt ID: P00772), papain (UniProt ID: P00784), and proteinase K (UniProt ID: P06873), are not glycosylated—with the exception of the proteinase mixture Actinase E, for which no information is available in UniProt—the strong resistance of MPL-1 to proteinase digestion is not due to its ability to stabilize its molecular structure by binding to the sugar moiety or to inhibit its catalytic activity but, rather, to its indigestible molecular structure. To obtain some understanding about the digestion-resistance property of MPL-1, we performed conformational prediction on MPL-1 using ColabFold (Mirdita et al. 2022), which is a derivative of AlphaFold2, an artificial intelligence (AI) system used to predict a protein's three-dimensional structure from its amino acid sequence (Jumper et al. 2021), as well as on the other two algal lectins, ESA-2, and HypninA-2 (ESM Fig. S7). The known lectin structures obtained from the Protein Data Bank (PDB) were also represented. The prediction was that ESA-2, which belongs to the OAAH family (Koharudin et al. 2012), has large loops in the molecule (Fig. S7d). The structure of HypninA-2, a proteinase which does not belong to any known lectin family, was predicted with moderate confidence by ColabFold (Fig. S7e), and MPL-1, which belongs to the Jacalin-related lectin (JRL) family (Hori and Hirayama 2012), was predicted to have a β-prism I fold similar to that of other mannose-specific JRL families (Fig. S7f) (Meagher et al. 2005), with a compact

and tight structure with smaller loops compared to the other lectins tested. It is noteworthy that banana lectin BanLec, belonging to the mannose-specific JRL family, has also been found to have digestion-resistant properties when exposed to simulated gastric and intestinal fluid, maintaining its structural integrity, carbohydrate-binding capability, and immunological reactivity (Dimitrijevic et al. 2010). This observation entails that MPL-1 and other lectins belonging to the mannose-binding JRL family are resistant to digestion. Considering these features, MPL-1 can promisingly reach the intestines, including the colon and rectum, after ingestion while retaining its sugar-binding activity and biological activity.

Colorectal cancer (CRC) is the third most commonly diagnosed cancer and the second deadliest cancer in the world (World Health Organization, 2023); as such, it is a major health threat. A number of risk factors have been proposed for the development of CRC, including age, family history, personal history, and lifestyle factors (World Health Organization 2023). If CRC can be prevented by choosing beneficial food or food-derived components, this will be a major contribution to human health. Although there are no definite factors, all CRC develops when epithelial cells acquire a series of genetic or epigenetic changes that allow for hyperproliferation (Testa et al. 2018; Hussain et al. 2022). As is the case with various cancers, alteration in glycosylation also occurs in CRC. Some reports show that HM-glycans are increased in CRC tissues (Kaprio et al. 2015; Sethi et al. 2016), although there is no clear information on whether they are in the outer or inner regions of the cells. As shown in Fig. 7, the proliferation of HT-29, a human CRC cell lines, was potently inhibited with a salting-out fraction from *M. papulosa* containing HM-glycan binding lectin MPL-1 in a dose-dependent manner. The inhibitory effect of MPL-1 against the cancer cells was nullified by a glycoprotein yeast mannan possessing HM-glycans, suggesting that MPL-1 inhibited the proliferation of HT-29 via binding to the glycans on the cell. HM-glycan-specific bacterial lectin PFL (*Pseudomonas fluorescens* lectin) has been reported to mediate the downregulation of integrins, which are cell surface receptors ensuring the mechanical connection between cells and the extracellular matrix, and of epidermal growth factor receptor (EGFR), a transmembrane receptor regulating cell proliferation and differentiation, on gastric cancer cells, and also to inhibit tumor growth in vitro and in vivo (Sato et al. 2012, 2016). HM-glycan-bearing glycoproteins on cancer cell surfaces could be the targets of HM-glycan-specific lectins, thereby contributing to the inhibition of cancer growth. These results suggest that MPL-1, which is highly resistant to proteinases, may reach the colorectal tissue in an intact form without being degraded by digestive enzymes and proteases from gut bacteria, and may have an inhibitory effect on the growth of CRC cells generated on

the epithelial tissue. Recently, Boyaval et al (2022) reported that HM-glycan levels are increased in adenoma, pre-cancerous tissue, with high cell proliferation in the colorectal tissue. MPL-1 might be a food ingredient that could prevent the development of CRC. Further analysis focused on the mechanism of anti-proliferative activity of MPL-1 and also using an oncogenic mouse model on CRC would be required to clarify the usefulness of the lectin as a functional food component.

MPL-1 exhibited extremely low digestibility, and so it is expected to pass through the digestive tract and retain its carbohydrate-binding property and biological activities. The algal body of *M. papulosa* contains a large amount of lectin, as shown in Fig. 1. Food allergens exhibit common characteristics, including resistance to digestion in the gastrointestinal tract (Astwood et al. 1996; Metcalfe et al. 1996; Taylor and Lehrer 1996) and proteins with high digestion resistance could be allergens (Pali-Schöll et al. 2018), such as BanLec (Koshte et al. 1992). Although there are no reports to date on any definite negative effects of *M. papulosa* on nutritional activity, including allergy, further analysis needs to be directed towards the possibility that the algal lectin could be an allergen.

Declarations

Conflict of interest The authors declare no conflict of interest.

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