



Extraction and Partial Characterization of Lectin from Indonesian Brown Algae *Padina australis* and *Padina minor*

Nurrahmi Dewi Fajarningsih^{1*}, Naomi Intaqta², Dinar Praseptiangga², and Choiroel Anam²

¹ Research and Development Center for Marine and Fisheries Product Processing and Biotechnology,
Jalan KS. Tubun Petamburan VI, Slipi, Jakarta Pusat 10260, Indonesia

² Department of Food Science and Technology, Sebelas Maret University (UNS), Indonesia

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Abstract

Extraction and partial characterization of lectin from Indonesian *Padina australis* and *Padina minor* had been carried out. The crude extract of the *P. australis* and *P. minor* were examined for hemagglutination activity (HA) using native and trypsin-treated of rabbit and human A, B, O type erythrocytes. Both extracts agglutinated all of the trypsin-treated erythrocytes tested in the HA assay. Strong HA was detected in the crude extract of *P. minor* with trypsin-treated of human type A and O erythrocytes. However, the sugar-binding specificity study through the quantitative hemagglutination inhibition (HI) assay showed that *P. minor* extract could not specifically recognize the glycans tested. Apparently, the HA of the *P. minor* was more due to its co-extracted polyphenols content than its lectin content. On the other hand, the HI assay showed that asialo transferrin human (aTf) and asialo porcine thyroglobulin (aPTG) were the most powerful in inhibiting the HA of *P. australis*. Those indicated that *P. australis* protein extract was able to specifically recognize aTf and aPTG. The stability of *P. australis* and *P. minor* HA over various temperatures, pH ranges, and divalent cations studies showed that the *P. minor* HA was stable on a wide range of pH and temperature; not affected by the presence of EDTA, but decreased by Ca²⁺ and Mg²⁺ additions showed that *P. minor* protein extract was not a metallic protein. The HA of *P. australis* decreased at 60 °C and was inactivated at 90 °C; increased at strong acidic (pH 3 & 4) and strong basic (pH 9 & 10) and dependent by the presence of either EDTA or Ca²⁺ and Mg²⁺ divalent cation.

Keywords: hemagglutinin, brown seaweed, Phaeophyta, *Padina australis*, *Padina minor*

1. Introduction

Glycans are one of the most vital components of a cell (Ajit, 2017). The glycan, components of glycoconjugates, is formed through the glycosylation process and mediates many physiological and pathological functions (Reily, Stewart, Renfrow, & Novak, 2019). Many studies reported that glycosylation is involved in many crucial biological processes, e.g., receptor activation, molecular trafficking and clearance, signal transduction, cell adhesion, endocytosis, and host-pathogen interactions (Hyono, Mazda, Okazaki, Tadokoro, & Ohshima, 2008). Glycosylation plays a crucial part in many cellular mechanisms of both health and disease, such as tumor cell formation and metastatic diseases (Cadena, Cushman, & Welsh, 2018; Nardy, Freire-de-lima,

Freire-de-lima, & Morrot, 2016), viral immune escape, inflammatory responses, and kidney functions (Reily et al., 2019).

Lectin or hemagglutinin is a protein or glycoprotein that specifically and reversibly binds glycans, in the form of free sugar chains or glycoconjugates, i.e., glycoproteins and glycolipids, without changing its structure (Singh & Walia, 2018). Due to its specific interactions with glycans, lectins have gathered much attention as tools in glycobiology and cell biology research, e.g., lectin microarrays, cell selections, blood typing, bacterial typing, and cell sorting (Cummings, Darvill, Etzler, & Hahn, 2017). Moreover, researches working on lectins exploitation as therapeutic tools are also emerge, e.g., lectins therapy for cancer (Hamid, Masood, Wani, & Rafiq, 2013;

*Corresponding author.

E-mail: nurrahmi.dewi@gmail.com

Mody, Joshi, & Chaney, 1995; Singh & Walia, 2018), anti-bacterial (Marques et al., 2018; Vasconcelos et al., 2014), anti-fungal (Amano, Katayama, Saito, Ando, & Nagata, 2012; Chikalovets et al., 2015), anti-HIV (Alexandre et al., 2011; Ziólkowska & Wlodawer, 2006), and anti-insects (Hamid et al., 2013; Lam & Ng, 2011).

Naturally, lectins are found in almost all living things, e.g. plants, fungi, bacteria, viruses, and animals. However, compared to other sources, lectins from macroalgae have several unique characteristics, i.e. low molecular weight, bind specifically to glycoproteins rather than to monosaccharides, and do not require divalent cations for their activities (Rogers & Hori, 1993; Singh, Thakur, & Bansal, 2015). The first information of macroalgae lectin was reported in 1966 by Boyd, Var, and Boyd (1966). Since then, lectins of macroalgae have been explored and studied quite intensively, even though still in very limited numbers compared to lectin from other sources. As a comparison, a number of results for the keyword search of "lectin" and "lectin" "algae" in Google Scholar in July 2019 were 785,000 and 21,000 hits, respectively.

So far, lectins have been isolated and characterized mostly from red and green macroalgae. Some lectin isolated from red algae, e.g. high-mannose specific lectin KAA-2 that was isolated from *Kappaphycus alvarezii* (Sato, Morimoto, Hirayama, & Hori, 2011), high mannose N-glycan specific lectin EDA-2 was isolated from cultivated *Eucheuma denticulatum* (Hung, Hirayama, Ly, & Hori, 2015), purification dan characterization of *Gracilaria ornata* was also reported by Leite et al. (2005) and lectin from *Tichocarpus crinitus* (TCL) was purified by Molchanova, Chernikov, Chikalovets, and Lukyanov (2010). Lectin from green algae sources was also reported, e.g. lectin of green algae *Caulerpa cupressoides* was purified and showed specificity to porcine mucin glycoprotein (Benevides et al., 2001), a wound-healing lectin bryohelin was purified from *Bryopsis plumosa* (Jung et al., 2010), another lectin (BPL-3) was also purified from *B. plumosa* (Han, Yoon, Klochkova, Hwang, & Kim, 2011), lectin CBA was isolated from *Codium barbatum* green algae (Praseptianga, Hirayama, & Hori, 2012), molecular characterization of green algae *Ulva pertusa* was reported by (Wang et al., 2004) and a high-mannose binding lectin was also isolated from green algae *Boodlea coacta* (Sato et al., 2011). However, up till now, report on brown algae lectin is still limited. Only few publications reported the screening of brown algae lectin (Anam, Praseptianga, Fajarningsih, & Intaqta, 2016; Fajarningsih, Intaqta, Praaseptianga, Anam, & Chasanah, 2018; Fajarningsih et al., 2015; Freitas

et al., 1997; Hung et al., 2012) and to our knowledge only one publication reported the purification of brown algae lectin, an HFL lectin isolated from *Hizikia fusiformis* (Wu, Tong, Wu, Liu, & Li, 2016). According to Harrysson et al. (2018), isolation of lectin or other protein from brown algae is difficult due to the high content of non-protein interfering compounds such as polyphenols and viscous polysaccharide. Thus, in this report we described the extraction and partial characterization of lectin from Indonesian brown algae *P. australis* and *P. minor*.

2. Material and Methods

2.1. Macroalgae Sampling

Sample of *P. australis* alga was collected from Binuangeun Beach, Banten while sample of *P. minor* alga was collected from Pok Tunggal Beach, Jogjakarta, Indonesia. Upon collection, the algae were washed using freshwater, cleaned from impurities and stored in cold storage (-20 °C) until utilization. The specimen samples were sent to the Research Center for Oceanography, Indonesian Institute for Sciences for species identification.

2.2. Macroalgae Lectin Extraction

The extraction of *P. australis* and *P. minor* lectin was carried out according to Fajarningsih et al. (2015) and Praseptianga et al. (2012). The extraction was carried out in triplicates. As many as 100 grams of each alga thallus was cut into small sizes and grounded in liquid nitrogen into powder. The powdered alga were extracted using a 20 mM phosphate buffer (pH 7.0) containing 0.85 % NaCl (PBS) with a ratio of 1: 2 then stirred at 4 °C for 8 h. The alga homogenate was then centrifuged at 15.302 xg (Beckman Coulter, rotor JA-14) for 30 min at 4 °C. The supernatant was precipitated with ammonium sulfate (75 % saturation) overnight at 4 °C and then centrifuged at 15.302 xg for 30 min at 4 °C. The precipitate was dissolved by adding as little volume as possible of PBS then thoroughly dialyzed (SnakeSkin dialysis tubing 10K MWCO) against PBS for 10 h, with PBS replacement every 2 h. The internal fraction was centrifuged at 15.302 xg for 30 min at 4 °C and the supernatant obtained, referred to as protein extract, was then stored at -20 °C.

2.3. Protein Content Determination

The protein content was measured using the BCA protein assay kit (Pierce-Thermo Scientific) following the product manual instructions.

2.4. Hemagglutination Activity (HA) Assays

Hemagglutination activity assay, a basic assay to define the lectin presence in the sample, was carried

out according to Praseptiangga et al. (2012). The assay was carried out in 96-well V-bottom microplates using a 2 % solution of native and trypsin-treated rabbit, and human A, B, and O blood cells (RBC). The human A, B, and O erythrocytes were obtained from the Indonesian Red Cross Society. As much as 25 μ L of protein extracts were two-fold diluted using salt solution (0.85 % NaCl). Into each well, 25 μ L of RBC was added. The microplate was then gently shaken and the mixture was allowed to stand for 1 h at room temperature. The hemagglutination was macroscopically observed and marked as positive if more than 50 % of the erythrocytes were agglutinated. The negative results were indicated by the formation of erythrocyte dot at the bottom of the microplate well. The HA assay was carried out in triplicates and the activity was recorded as the minimum protein concentration (μ g/mL) that caused agglutination (Goldstein, Winter, & Poretz, 1997).

2.5. Hemagglutination Inhibition (HI) Assay

The HI assay was carried out to determine the lectin binding specificity to various types of sugars and glycoproteins. In this assay, the interaction of lectin and the RBC-carbohydrate is inhibited by certain sugar or glycoprotein that was added in the assay which then resulted in agglutination inhibition. This assay was conducted in 2 steps, namely qualitative and quantitative tests. The qualitative test was carried out to determine the types of sugar and/or glycoprotein that positively bound to lectins. The positive types of sugar and glycoprotein bound to the *P. australis* and *P. minor* protein extracts will be continued to the quantitative HI assay to acquire the minimum inhibitory concentration (MIC) data.

The qualitative HI assay was carried out by putting in 25 μ L of sugar (100 mM) or a glycoprotein (2 mg/mL), prepared in saline, into a V-bottom microplate well. Twenty five microlitres of extract that was already diluted to titer of 4 was added to each well. The microplate was then gently shaken and allowed to stand at room temperature for 1 h. Then, 25 μ L of 2 % of the trypsin-treated rabbit red blood cell (TRBC) was added to each well. The microplate was then gently shaken and allowed to stand for 1 h at room temperature, and the HI was macroscopically observed.

The quantitative HI assay was done by serially two-fold diluting (25 μ L) of a sugar or a glycoprotein that gave positive results in the qualitative assay, in V-bottom microplate well. The highest sugar concentration was 100 mM, while the highest glycoprotein concentration was 2 mg/mL in PBS.

Twenty five microlitres of protein extract that was already diluted to titer of 4 was added to each well. The microplate was then gently shaken and allowed to stand for 1 h at room temperature. Lastly, 25 μ L of 2 % of TRBC was added to each well. The microplate was then gently shaken and allowed to stand for 1 h at room temperature, and the HI was macroscopically observed. The inhibitory activity is expressed as a minimum inhibitory concentration (MIC, mM or μ g/mL), which is the smallest concentration of sugar or glycoprotein which inhibited the hemagglutination.

Nine types of sugars, i.e. monosaccharide D-galactose, D-glucose, D-mannose, D-xylose, L-fucose, L-rhamnose, N-acetyl-D-galactosamine, N-acetyl-D-glucosamine, and disaccharide lactose and 11 types of glycoprotein, i.e. fetuin from fetal bovine (Fe), mucin from bovine submaxillary gland (BSM), thyroglobulin from bovine thyroid gland (BTG), thyroglobulin from porcine thyroid gland (PTG), human transferin (Tf), yeast mannan, asialo BSM (aBSM), asialo BTG (aBTG), asialo Fe (aFe), asialo PTG (aPTG), and asialo Tf (aTf) were used in the HI assay.

2.6. Effect of pH, Temperature and Divalent Cations on the Hemagglutination Activity (HA)

To determine the effects of pH on the HA of the *P. australis* and *P. minor* protein extract, 500 μ L of each extract was dialyzed (SnakeSkin dialysis tubing 10K MWCO) with 100 mL of 50 mM buffer solutions (various pH value of 3 to 10) overnight at 4 °C. The pH treated sample was then dialyzed with PBS (pH 7.0) for 10 h and subjected to the HA assay. The buffers used were: Glycine-HCl 2 M pH 3.0, Acetate 2 M pH 4.0 and 5.0, Phosphate 2 M pH 6.0 and 7.0, Tris-HCl 2 M pH 8.0, and Carbonate 2 M pH 9.0 and 10.0. To determine the effect of temperature on the HA of the *P. australis* and *P. minor* protein extract, a 500 μ L sample of each extract was incubated at various temperature ranges of 30 °C to 100 °C for 30 min in a thermo block. Right after the incubation completed, the sample was directly cooled with ice and was then subjected to hemagglutination assay. To find out the effect of divalent cations on the HA of the *P. australis* and *P. minor* lectin-protein extract, 500 μ L of each extract was dialyzed (SnakeSkin dialysis tubing 10K MWCO) with 50 mM EDTA in PBS for 10 h at 4 °C. The dialyzed extracts were then subjected for HA assay with three treatments, i.e. without the addition of divalent cation solution, with the addition of 25 μ L of 20 mM CaCl₂ and MgCl₂. The mixtures were then kept for 1 h at room temperature followed with the HA assay. Each of the treatments was carried out in triplicates.

3. Result and Discussion

The HA of *P. australis* and *P. minor* protein extracts are presented in Table 1. Both extracts gave positive hemagglutination for all erythrocytes tested indicating the presence of lectin in the extracts. Treatment of trypsin enzyme reduces the negative charge and decreases the distance between erythrocytes which then improve the hemagglutination (Fernandes, Cesar, & Barjas-Castro, 2011). Thus, both of the algae protein extracts agglutinated the enzyme-treated erythrocytes better than the native cells. Furthermore, the *P. australis* extract only agglutinated the trypsin treated human blood-group A, B and O erythrocytes whilst showed a negative result for the non-treated. The minimum concentration of protein (0.08 µg/mL) required to produce hemagglutination was showed by *P. minor* extract to human blood group O type.

The ability of *P. australis* and *P. minor* protein extracts to specifically bind carbohydrates was studied using the HI assay approach. In this assay, protein extracts of both algae were reacted to a certain type of sugar or glycoprotein. If the lectin has a binding site for a certain type of sugar or glycoprotein, a bond will occur between the two, so that when TRBC was added into the assay reaction, hemagglutination will not occur. We have tested the ability of the *P. australis* and *P. minor* extracts to bind 9 types of simple sugar and 11 types of glycoproteins qualitatively (Figure 1.) and quantitatively (Table 2.).

Based on the qualitative HI assay, both of the *P. australis* and *P. minor* extracts did not have an affinity to the various simple sugars tested. This result is in line with previous reports that macroalgae lectins generally do not have an affinity to simple sugars but have more affinity to glycoconjugates (Hung et al., 2015; Mu, Hirayama, Sato, Morimoto, & Hori, 2017; Nagano et al., 2002; Singh & Walia, 2018). Lectin-containing extract of *P. australis* bond 7 glycoproteins, i.e., thyroglobulin from bovine thyroid gland (BTG), thyroglobulin from porcine thyroid gland (PTG), Asialo-Fetuin (aFe), Asialo-BSM (aBSM), Asialo-PTG (aPTG), Asialo-Tf (aTf), and Asialo-BTG (aBTG), while *P. minor* extract only bound 3 asialo glycoproteins, i.e. aPTG,

aTf, and aBTG. Both of the *Padina* lectin-containing extracts recognized asialo glycoproteins, glycoprotein that its sialic acid terminal has been removed, better.

The quantitative hemagglutination assay result (Table 2) showed that asialo transferrin human (aTf), a complex N-glycan type glycoprotein, was most powerful in inhibited the HA of *P. australis* extract. Asialo PTG, a complex high-mannose type glycoprotein, is also a good inhibitor for *P. australis* HA. Those indicating that *P. australis* lectin-containing extract was able to specifically recognize aTf and aPTG. Asialo-transferrin human or Tau protein is a glycoprotein present in cerebrospinal fluid (CSF) which commonly assessed for Alzheimer’s disease diagnosis and also a relevant marker for CSF leakage after an injury (Oudart et al., 2017). Recently, Ito, Hoshi, Honda, and Hashimoto (2018) reported the possible use of lectin ability to specifically recognize glycoprotein as a simple yet rapid histopathological diagnosis for α2,6-sialylated transferrin. The ability of lectins to specifically recognize biomarker-glycans can be used as the basis for its potential utilization, e.g., the use of lectins in cancer diagnostic tools (Bertolini, Shaked, Mancuso, & Kerbel, 2006; Drake et al., 2006; Mody et al., 1995), lectin in the ELISA technique to detect sialylated glycoform in immunoglobulin samples (Srinivasan et al., 2015), and lectin as affinity chromatography resin in the purification of glycoproteins (Freeze, 1995; Kaji et al., 2003; Mao, Qin, & Lin, 2007).

In the preliminary hemagglutination study (Table 1), the protein extract of *P. minor* showed better HA than the *P. australis*, however, based on the HI assay (Table 2), extract of *P. australis* showed better specific binding to the glycoproteins. Protein extract of *P. minor* that showed a very high HA to the trypsin treated human type A and O erythrocytes could not specifically recognize the glycans tested (high MIC value). Apparently, HA was more due to the polyphenols content of the *P. minor* that was co-extracted with the lectins than the *P. minor* lectin. According to Rogers and Loveless (1985), polyphenols were reported to have strong HA. However, lectin HA is based on the specific interaction of lectin with the erythrocytes’

Table 1. Minimum concentration (µg/ml) of *P. australis* and *P. minor* protein extracts to produce HA on different types of erythrocytes

Species	Rabbit		Human type A		Human type B		Human type O	
	N	T	N	T	N	T	N	T
<i>Padina australis</i>	124.5 ± 0	2.92 ± 0.97	-	23.34 ± 7.78	-	31.12 ± 0	-	23.34 ± 7.78
<i>Padina minor</i>	78.81 ± 0	3.71 ± 1.24	39.4 ± 0	1.64 ± 0	630.49 ± 0	39.4 ± 0	19.7 ± 0	0.08 ± 0

Note: N: Non treated erythrocyte; T: Trypsin treated erythrocyte; - : No detectable activity

glycans while polyphenols agglutinating activity is non-specific. It was reported that the HA of the polyphenols was two orders of magnitude lower than the lectin's (Cortés-giraldo, Girón-calle, Alaiz, Vioque, & Megías, 2012).

The false hemagglutinating capacity of polyphenols might become a concern for research on lectin of brown algae (Cortés-giraldo et al., 2012) which resulted in the small number of the Phaeophyta lectin researches. Yet, only 17 lectins of green algae, 31 lectins of red algae and only one brown alga that were isolated and characterized (Praseptianga, 2015; Teixeira et al., 2012; Wu et al., 2016).

The stability of *P. australis* and *P. minor* HA over various temperature and pH ranges (Figure 2.), as well as the effects of divalent cations, were studied. Generally, algae lectins were reported as thermosensitive (Oliveira, Nascimento, Lima, Leite,

& Benevides, 2002; Rogers & Hori, 1993). However, we found that the HA of *P. minor* was stable on a wide range of pH and temperature. Lectin of *Dictyosphaeria versluysii* green algae was also reported stable in a wide range of temperature and pH (Hung et al., 2012). The HA of *P. minor* protein extract was not affected by the presence of chelating agent EDTA, indicating that it did not require divalent cation to maintain its biological activity (Oliveira et al., 2002). Furthermore, the *P. minor* HA was decreased by Ca^{2+} and Mg^{2+} additions showing that *P. minor* lectin-containing extract was not a metallic protein. The HA of *P. australis* decreased at 60 °C and was inactivated at 90 °C. Interestingly, the *P. australis* HA increased at strong acidic (pH 3 & 4) as well as at strong basic (pH 9 & 10). The HA of the *P. australis* protein extract was dependent on the presence of either EDTA or Ca^{2+} and Mg^{2+} divalent cation.

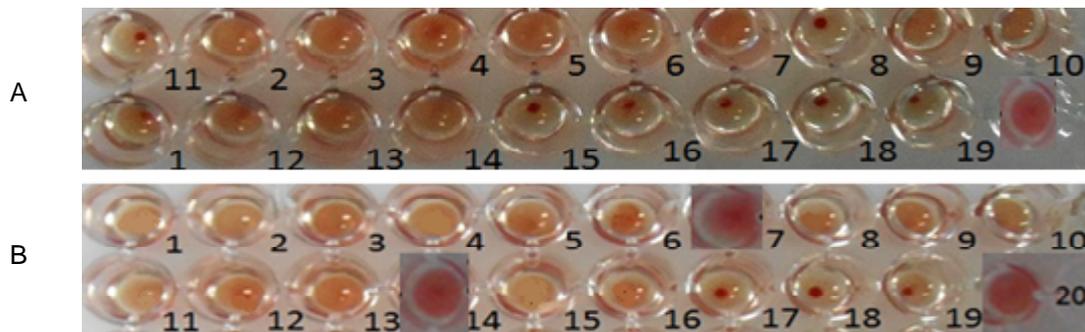


Figure 1. Qualitative inhibitory effects of sugars and glycoproteins on the *P. australis* (A) and *P. minor* (B) HA

Annotation:----

- 1 : Fetuin from fetal bovine serum (Fe)
- 2 : Lactose
- 3 : D-galactose
- 4 : Transferin human (Tf)
- 5 : L-rhamnose
- 6 : Mucin from bovine submaxillary gland (BSM)
- 7 : N-acetyl-D-glucosamine
- 8 : Thyroglobulin from bovine thyroid gland (BTG)
- 9 : D-glucose
- 10 : L-fucose
- 11 : Thyroglobulin from porcine thyroid gland (PTG)
- 12 : Yeast mannan
- 13 : N-acetyl-D-galactosamine
- 14 : D-xylose
- 15 : Asialo-Fetuin (aFe)
- 16 : Asialo-BSM (aBSM)
- 17 : Asialo-PTG (aPTG)
- 18 : Asialo-Tf (aTf)
- 19 : Asialo-BTG (aBTG)
- 20 : D-mannose

Table 2. Quantitative inhibitory effects of sugars and glycoproteins on the *P. australis* (A) and *P. minor* (B) HA

Sugars or Glycoprotein	Minimum Inhibitory Concentration ^a	
	<i>P. australis</i>	<i>P. minor</i>
1. Monosaccharide and disaccharide (mM)		
D-galactose	-	-
D-glucose	-	-
D-mannose	-	-
D-xylose	-	-
L-fucose	-	-
L-rhamnose	-	-
N-acetyl-D-galactosamine	-	-
N-acetyl-D-glucosamine	-	-
Lactose	-	-
2. Glycoprotein (µg/mL)		
N-Glycan		
Complex type		
Transferin human	-	-
Asialo-Transferin human	15.625 ± 0	333.33 ± 144.33
High-mannose type		
Yeast mannan	-	-
Complex type and high-mannose type		
Bovine thyroglobulin (BTG)	125 ± 0	-
Asialo-BTG	125 ± 0	500 ± 0
Porcine thyroglobulin (PTG)	250 ± 0	-
Asialo-PTG	20.83 ± 9.02	666.66 ± 288.67
O-Glycan		
Bovine submaxillary mucin (BSM)	-	-
Asialo-BSM	250 ± 0	-
N/O-Glycan		
Fetuin	-	-
Asialo-fetuin	125 ± 0	-

Note: - : No detectable inhibitory activity; ^a: Values (mean of triplicates) represent the lowest concentration of sugar (mM) and glycoprotein (µg/mL) that inhibited the HA of titer 4 TRBC

Table 3. The effects of divalent cations on the *P. australis* and *P. minor* extract HA

Macroalgae	Hemagglutination Titer ^a			
	Control (Untreated)	EDTA	MgCl ₂	CaCl ₂
<i>P. australis</i>	512 ± 0	128 ± 0	26.67 ± 9.23	16 ± 0
<i>P. minor</i>	64 ± 0	64 ± 0	8 ± 0	8 ± 0

Note: ^aData represents mean of triplicates

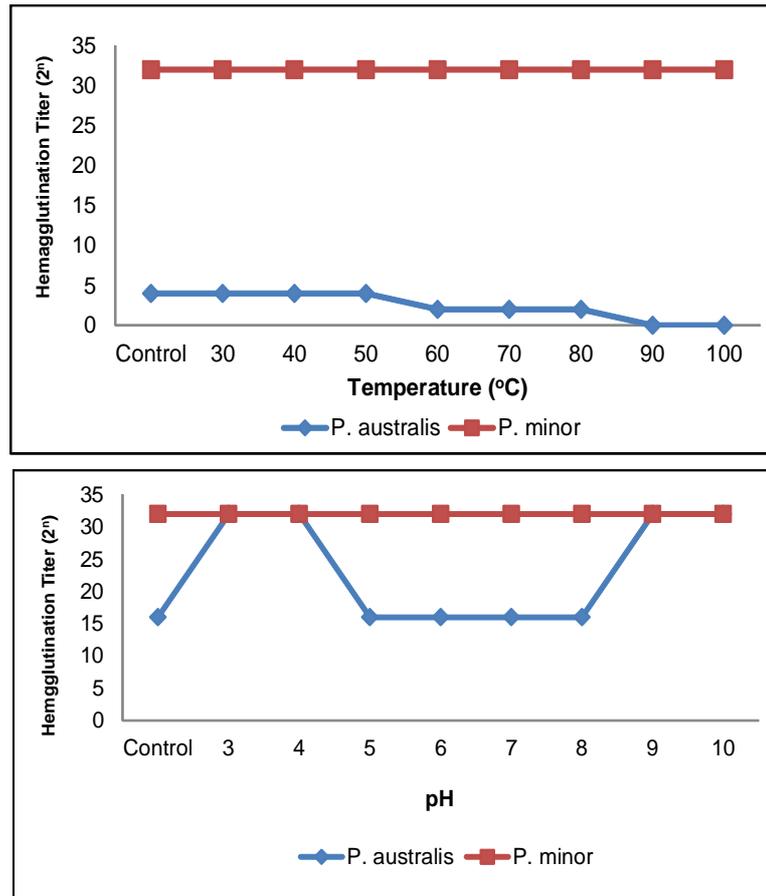


Figure 2. Temperature (A) and pH (B) effects on the HA of the *P. australis* and *P. minor* extract

4. Conclusion

Our study on the *P. australis* and *P. minor* protein extract revealed that both agglutinated all of the trypsin-treated erythrocytes tested. Strong HA was detected in lectin-containing extract of *P. minor* with trypsin-treated of human type A and O erythrocytes. However, the sugar-binding specificity study showed that *P. minor* lectin extract could not specifically recognize the glycans tested. Apparently, the HA of the *P. minor* was more due to its co-extracted polyphenols content than its lectin content. The ability of *P. australis* extract to specifically recognize aTf and aPTG can be used as the basis for its potential utilization. Isolation and characterization studies on the pure lectins of *P. australis* and *P. minor* should be conducted.

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