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ORIGINAL PAPER

Antioxidant and Antithrombotic Activities of Rapeseed Peptides

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Abstract The antioxidant and antithrombotic activities of crude rapeseed peptides (CRPs) and peptide fractions (RP25 and RP55) prepared from aqueous enzymatic extraction (AEE) of rapeseed were determined. The reducing power of RP55 and CRPs was higher than that of RP25 at the same concentrations. Rapeseed peptides exhibited marked antioxidant activities. The median effective dose (ED_{50}) values of CRPs, RP25 and RP55 for α,α-diphenyl-β-picrylhydrazyl (DPPH) radical scavenging were 72, 499 and 41 μg/mL, respectively. The ED_{50} values for RP25 and RP55 for hydroxyl radicals scavenging were 2.53 and 6.79 mg/mL, respectively while the ED_{50} values of RP55 and CRPs for inhibition of lipid peroxidation in a liposome model system were 4.06 and 4.69 mg/mL, respectively. The inhibitory effect on lipid oxidation of RP55 was similar to that of ascorbic acid at a concentration of 5.0 mg/mL. A good positive correlation existed between the peptide concentration and antioxidant activity. RP55 generally showed more potent antioxidant activities except for hydroxyl radicals scavenging ability than RP25 and CRPs at the same concentrations, which was thought to relate to the significantly higher contents of hydrophobic amino acid, tannin, and the brown color substances in RP55. Rapeseed peptides possessed marked inhibitory activities on the thrombin-catalyzed coagulation of fibrinogen, however, their inhibitory effects were not comparable to that of heparin.

Keywords Rapeseed peptides · Antioxidant · Antithrombotic · Reducing power · α,α-Diphenyl-β-picrylhydrazyl (DPPH) · Hydroxyl radical · Lipid peroxidation

Introduction

Rapeseed (*Brassica napus*) is one of the major sources for vegetable oil production. Conventional rapeseed processing for oil production generates low-valued meal as a co-product which is mainly used in animal feeds and fertilizers. Aqueous enzymatic extraction (AEE) has emerged as a novel oil extraction technique and provides a new approach for the effective utilization of rapeseed protein when applied to rapeseed processing. The optimal conditions for AEE of rapeseed and subsequent downstream processes including refinement of rapeseed protein hydrolysates have been reported [1, 2]. The rapeseed slurry was treated by the combination of pectinase, cellulase, and β-glucanase, which was followed by sequential treatments consisting of alkaline extraction and an alkaline protease (Alcalase 2.4L) hydrolysis to simultaneously produce free oil and protein hydrolysates. Crude rapeseed peptides (CRPs), containing no glucosinolates and phytic acid, were obtained by treatment of the protein hydrolysates with macroporous adsorption resin (MAR). Three peptide fractions (RP25, RP55, and RP85) with different levels of bitterness and protein content were also achieved by stepwise desorption from the resin column with different ethanol concentrations.

Recently, there has been an increasing interest in bioactivities of peptides derived from many animal and plant origins. Antimicrobial, blood pressure-lowering (ACE inhibitory), cholesterol-lowering, antioxidant,

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immunomodulatory and opioid activities have been reported [3, 4]. However, the studies investigating bioactivities of rapeseed peptides are limited due to the high concentration of antinutrients in the protein hydrolysates. Marczak et al. [5] used subtilisin to produce antihypertensive peptides from rapeseed protein. Yust et al. [6] reported HIV protease inhibitory activity of rapeseed protein hydrolysates which was produced using Alcalase 2.4L. Aerobic organisms must deal with free radicals that are generated from sequential reduction of oxygen during the normal course of aerobic metabolism. These radicals may cause cellular damage leading to a number of pathological conditions including atherosclerosis, arthritis, diabetes and carcinoma, if produced in an uncontrolled manner [7]. It is well known that thrombi (blood clots) may cause heart attacks if they lodge in the coronary artery. A key phase of the blood clotting process is the interaction between thrombin and fibrinogen to form the fibrin clot [8]. Laudano and Doolittle [9] reported two synthetic short peptides which effectively prevented the polymerization of fibrin monomers. Some peptides from κ-casein which could prevent blood clotting have been reviewed by Silva and Malcata [10]. More recently, Yang et al. [11] reported that egg white protein hydrolysate possesses antithrombotic activity.

Radical mediated oxidation of fats and oils is one of the most important reasons for the deterioration of oil-containing foods during processing and storage. Butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) as synthetic antioxidants are widely used to retard lipid peroxidation, which, however, have increasingly caused public concerns due to their potential health hazards. Therefore, there has been a growing interest in the identification and characterization of natural antioxidants during the last few decades. Antioxidant activity has been identified in some protein hydrolysates such as those from fish protein [12], egg-yolk protein [13], wheat germ protein [14], milk proteins [15] and porcine haemoglobin [16]. We speculated that rapeseed protein hydrolysates may also contain some peptides which can inhibit oxidation and thrombin-catalyzed coagulation of fibrinogen. Little information about the antioxidant and antithrombotic activities of rapeseed protein hydrolysates is available. Thus, the objectives of this study were to evaluate the antioxidant and antithrombotic activities of rapeseed protein hydrolysates.

The present study reports on the antioxidant and antithrombic activities of rapeseed peptides obtained from the AEE process. Different measurements, including reducing power, the scavenging effects on the α,α-diphenyl-β-picrylhydrazyl (DPPH) and hydroxyl free radicals, the ability to inhibit lipid peroxidation in a liposome model system, as well as the ability to inhibit the thrombin-catalyzed coagulation of fibrinogen were used to evaluate the antioxidant and antithrombic activities.

Materials and Methods

Materials

CRPs and peptide fractions (RP25 and RP55) were prepared according to our previous studies [1, 2]. DPPH, ascorbic acid, 2-deoxy-D-ribose, phosphatidylcholine (PC), 2-thiobarbituric acid (TBA), heparin, thrombin and fibrinogen were purchased from Sigma Chemical Co. (St Louis, MO, USA). All other chemicals used in the experiments were of analytical grade.

Preparation of Rapeseed Peptides

The rapeseed slurry (containing 800 g original dry rapeseeds) from a wet-milling was diluted to seeds-to-water ratio of 1:5 (w/v). The suspension was heated to 48 °C and adjusted to pH 5, followed by addition of the combination of pectinase (1.2×10^5 IU), cellulase (2.2×10^3 IU), and beta-glucanase (4.2×10^3 IU) in the ratio 4:1:1 (v/v/v) at 2.5% (v/w) and further incubation for 4 h. Then, the slurry was heated to 60 °C and the pH was adjusted to 10, incubation for 30 min followed by readjusting the pH to 9. Alcalase 2.4L (2.9×10^5 IU) was added at 1.4% (v/w) of the dry rapeseed and the slurry was incubated for 3 h followed by heating at 90 °C for 10 min. After that, the slurry was centrifuged at 1,819*g* for 15 min to obtain the free oil, the aqueous phase, the emulsion and the solid phase.

The aqueous phase (50 mL) was adjusted to pH 4 with acetic acid and then was pumped through a glass column (180 mL) with MAR at a flow rate of 1 mL/min. Next, the column was washed with 1 L deionized water previously adjusted to pH 4 at a flow rate of 1.5 mL/min. Then, 1 L of 85% (v/v) ethanol was used to desorb the peptides without changing the flow rate and the ethanol-eluent was concentrated and freeze-dried to obtain the CRPs. For preparation of RP25 and RP55, stepwise desorption was carried out with 25 and 55% ethanol. The procedure is as follows: after the column was loaded with 50 mL sample, it was washed with deionized water as described above, 600 mL of 25% (v/v) ethanol was used to desorb the peptides and the ethanol-eluent was concentrated and freeze-dried to obtain the RP25. Then the same volume of 55% (v/v) ethanol was used to obtain the RP55.

Reducing Power Assay

The reducing power of the peptide fractions was measured according to the method of Oyaizu [17] with minor

modifications. Various concentrations of the hydrolysates in solution (2.5 mL) were added to 2.5 mL of 1% potassium ferricyanide (2.5 mL distilled water in place of the sample solution as the blank). The mixture was incubated at 50 °C for 20 min. Then 2.5 mL of 10% trichloroacetic acid (TCA) was added to the mixture followed by vortex mixing for 2 s. Next, 2.5 mL from each of the mixture was further mixed with 2.5 mL of distilled water and 0.5 mL of 0.1% ferric chloride in a 10-mL test tube. After 2 min, the absorbance of the resulting solution was measured at 700 nm. Ascorbic acid was used as a positive control. At each concentration of the hydrolysates from one batch and ascorbic acid triplicate determinations were carried out. A high absorbance was indicative of strong reducing power.

DPPH Radical Scavenging Assay

The scavenging effects of peptide fractions on DPPH free radicals were measured according to the method of Shimada et al. [18] with some modifications. The sample solution (2 mL) with various hydrolysates concentrations was added to 2 mL of 0.1 mM DPPH in 95% ethanol (2 mL of 95% ethanol in place of the DPPH solution as sample blank). The mixture was shaken and left for 30 min at room temperature, and the absorbance of the resulting solution was measured at 517 nm. Ascorbic acid was used as positive control. At each concentration of the hydrolysates from one batch and ascorbic acid, determinations were carried out in triplicate. The scavenging effects were calculated according to Eq. 1.

$$\text{Scavenging effect, } \% = \frac{[\mathrm{C}-\mathrm{CB}]-[\mathrm{S}-\mathrm{SB}]}{[\mathrm{C}-\mathrm{CB}]} \times 100\% \tag{1}$$

where S, SB, C, and CB represent the absorbance of the sample, the sample blank, the control, and the control blank, respectively.

Hydroxyl Radical Scavenging Assay

The hydroxyl radical scavenging effects of the rapeseed peptide fractions were assayed using the method of Halliwell et al. [19]. The reagents were added to a test tube in the following order: 0.4 mL KH_2PO_4–KOH buffer (pH 7.5), 0.1 mL sample solution with various concentrations, and 0.1 mL of 1 mM EDTA, 10 mM H_2O_2, 60 mM 2-deoxy-D-ribose, 2 mM ascorbic acid, and 1 mM $FeCl_3$ (0.1 mL distilled water in place of the $FeCl_3$ solution as sample blank). The reaction solution was incubated at 37 °C for 1 h. Next, 1 mL of 20% TCA was added to stop the reaction. The color was developed by addition of 1 mL of 1% TBA into the reaction tubes, which were placed in boiling water for 15 min. The tubes were cooled to room temperature and then the absorbance was read at 532 nm. At each concentration of the hydrolysates from one batch, determinations were carried out in triplicate. The scavenging effects were calculated according to Eq. 1.

Assay of the Ability to Inhibit Lipid Oxidation

PC liposomes were prepared from 300 mg PC by homogenization and sonication in 30 mL of phosphate buffer solution (10 mM, pH 7.4). Lipid oxidation and assay of thiobarbituric acid-reactive substances (TBARS) were performed according to the methods of Peña-Ramos et al. [20] and Sakanaka et al. [21] with some modifications. The reagents were added to a test tube in the following order: 1 mL PC liposomes, 1 mL of 400 μM $FeCl_3$, 1 mL of 400 μM ascorbic acid, and 1 mL sample solution with various hydrolysate concentrations distilled water (3 mL) was used as sample blank instead of the $FeCl_3$, ascorbic acid and sample solution. The reactants were mixed by vortex mixing for 2 s, incubated in the dark at 37 °C for 30 min, and then 2 mL TCA/TBA solution (consisting of 15% TCA and 0.375% TBA in 0.25 M HCl) was added to the reaction tubes. The tubes were placed into boiling water for 10 min, cooled to room temperature, and then centrifuged at 1,710*g* for 10 min. The absorbance of the supernatant was determined at 532 nm. Ascorbic acid was used as a positive control. At each concentration of the hydrolysates from one batch and ascorbic acid, determinations were carried out in triplicate. The inhibitory effects were calculated according to Eq. 1.

Assay of the Ability to Inhibit the Thrombin-Catalyzed Coagulation of Fibrinogen

The ability to inhibit the coagulation of fibrinogen was measured according to Yang et al. [11] with minor modifications. A microplate reader was set at a wavelength of 405 nm, at 37 °C. The fibrinogen, thrombin, and the peptide sample were all dissolved in 0.05 M Tris–HCl buffer (pH 7.2) containing 0.12 mM NaCl. A 0.1% fibrinogen solution (140 μL) and 40 μL sample solution with various hydrolysate concentrations were added into the plate wells, mixed, and then the absorbance of the sample blank was measured. Next, 10 μL thrombin solution (12 IU/mL) was added to the wells to start the reaction of thrombin-catalyzed coagulation of fibrinogen. After incubation for 10 min, the absorbance of the sample was read again. 40 μL Tris–HCl buffer (pH 7.2, 0.05 M) was in place of the sample solution for measurement of the absorbance of the control blank and the control. Heparin was used as a positive control. At each concentration of the hydrolysates from one batch and heparin, determinations were carried out in triplicate. The inhibitory effects were calculated according to Eq. 1.

Results and Discussion

Reducing Power

The properties of RP85 were not investigated in this study due to its objectionable bitterness, low protein content and smallest proportion (about 5%) among three peptide fractions. The reducing power of a compound can be used to measure its potential antioxidant activity and has been applied to different products, including peptides [14, 16]. The reducing power of CRPs and peptide fractions (RP25 and RP55) was determined by the Fe^{3+}–Fe^{2+} transformation, respectively and compared with that of ascorbic acid. As shown in Fig. 1, all samples exhibited marked reducing power and their effects were concentration-dependent. The reducing power of CRPs and RP55 was higher than that of RP25 at the same concentrations. Ascorbic acid exhibited potent reducing power even at low concentrations (<0.2 mg/mL). CRPs and RP55 at the respective concentration of 1.2 mg/mL showed close reducing power to 0.1 mg/mL ascorbic acid. These results indicate that rapeseed peptides have potential antioxidant activities.

Radical Scavenging Activities

DPPH is a stable free radical that shows maximum absorbance at 517 nm in ethanol. When DPPH encounters a proton-donating substance such as an antioxidant, the radical would be scavenged and the absorbance is reduced [18]. This radical-scavenging assay is quick, convenient and reproducible and thus widely used in predicting the antioxidant activities of compounds. Figure 2 shows the results of DPPH radical scavenging activity of rapeseed peptides at various concentrations. With increasing peptide concentrations, the scavenging effect on DPPH radical increased until about 90%, thereafter reaching a plateau. At a concentration of 0.2 mg/mL, the scavenging effects of RP55 and CRPs (89.9 and 89.4%, respectively) are similar and their effects were much higher than that of RP25 (25.0%). As is shown in Fig. 2, ascorbic acid exhibited higher activity (91.0%) than RP55 (38.7%) and CRPs (20.5%) at a concentration of 0.02 mg/mL. The median effective dose (ED_{50}, meaning the concentration that scavenges 50% of the initial DPPH radical) values for CRPs, RP25, RP55, and ascorbic acid were 72, 499, 41, and 7 µg/mL, respectively. These results revealed that rapeseed peptides probably contained substances that were proton donors and could react with free radicals to convert them to stable diamagnetic molecules.

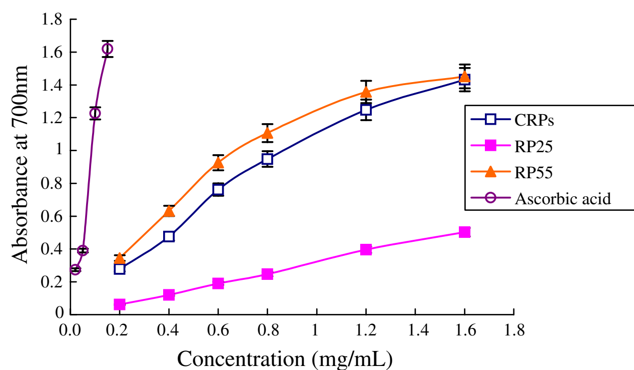

Fig. 1 Reducing power of rapeseed peptides using spectrophotometric detection of the Fe^{3+}–Fe^{2+} transformation. Ascorbic acid was used as positive control. Data were expressed as means with standard deviations ($n = 3$)

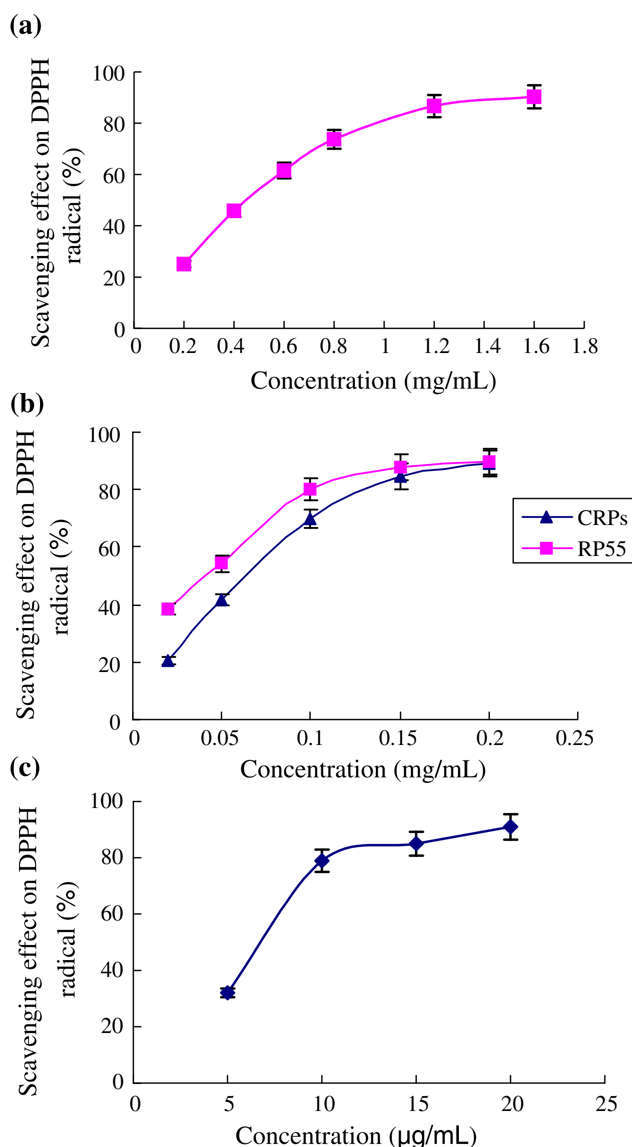

Fig. 2 Scavenging effects on DPPH radical: **a** RP25; **b** CRPs and RP55; **c** ascorbic acid. Data were expressed as means with standard deviations ($n = 3$)

Figure 3 shows that rapeseed peptides possess the ability to quench the hydroxyl radicals. The scavenging effects with regard to hydroxyl radicals of rapeseed peptides were concentration dependent. The scavenging effect of RP25 was more potent than those of RP55 and CRPs at the same concentration which was different from the experimental results of reducing power and DPPH radical scavenging activity. The ED_{50} values for RP25 and RP55 were 2.53 and 6.79 mg/mL, respectively. These findings indicate that rapeseed peptides are good hydroxyl radical scavengers and might be used as functional ingredients in food products. Hydroxyl radicals were generated by inducement of the ferrous ion in a Fenton reaction in this assay. Rajapakse et al. [22] presumed that the peptide with higher hydroxyl radical scavenging ability was probably because of the combined effects of radical scavenging and ion chelating activity. However, rapeseed peptides showed very poor ferrous ion chelating abilities in this study (data not shown).

Lipid Oxidation Inhibition in a Liposome Model System

The ability of the rapeseed peptides to inhibit lipid oxidation was tested in the PC liposome model system and compared with that of ascorbic acid. As shown in Fig. 4, all samples inhibited lipid peroxidation and their antioxidant activities increased with increasing concentration. At a concentration of 1.0 mg/mL, the inhibitory effects of rapeseed peptides were lower than that of ascorbic acid. However, the inhibitory effect of RP55 was very close to that of ascorbic acid at a concentration of 5.0 mg/mL. The ED_{50} values of ascorbic acid, RP55 and CRPs were 3.14, 4.06, and 4.69 mg/mL, respectively. The lipid oxidation in this liposome model system was induced by the ferrous ion. Erickson and Hultin [23] reported that the capability of histidine-related compounds capable of inhibiting lipid oxidation might stem from their abilities to coordinate with

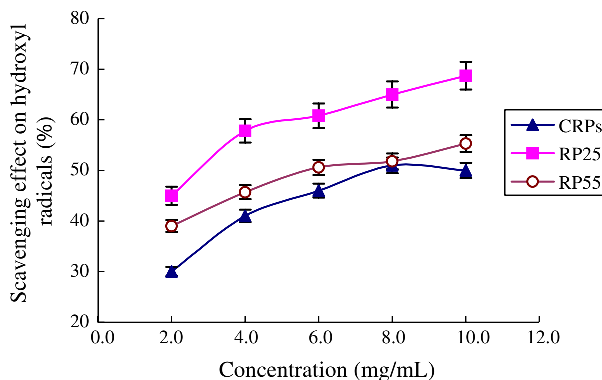

Fig. 3 Scavenging effects on hydroxyl radicals of rapeseed peptides. Data were expressed as means with standard deviations ($n = 3$)

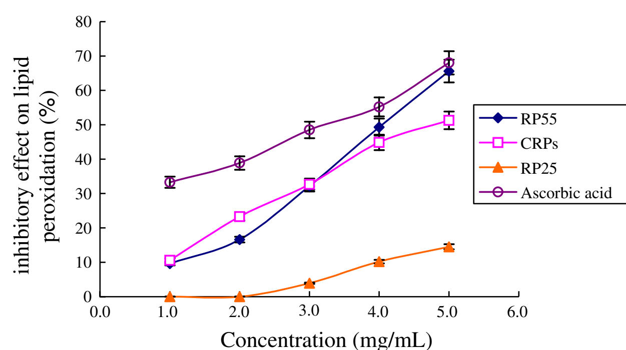

Fig. 4 Inhibitory effects on lipid oxidation of rapeseed peptides. Ascorbic acid was used as a positive control. Data were expressed as means with standard deviations ($n = 3$)

iron. It has been mentioned above that rapeseed peptides had poor chelating ferrous ion abilities. Thus, it was speculated that rapeseed peptides inhibited lipid oxidation by directly donating protons to lipid-derived radicals to stop chain reactions rather than by chelating ions to prevent the initiation of chain reactions. Several studies demonstrated that hydrophobic amino acids act as antioxidants by increasing the solubility of peptides in lipids and thereby facilitating better interaction with free radicals [22, 24, 25]. We reported previously [2] that RP55 had significantly higher mole percentage of hydrophobic amino acid (47.2%) than either CRPs (38.6%) or RP25 (32.5%). This may partially explain why RP55 possessed higher inhibitory activity than CRPs and RP25. As was discussed above, there existed obvious differences between ascorbic acid and RP55 with respect to DPPH scavenging effect at the same concentration (0.02 mg/mL). But for the lipid oxidation inhibitory activities, ascorbic acid and RP55 were almost the same at a concentration of 5.0 mg/mL (Fig. 4). A possible explanation is that RP55 had better distribution than ascorbic acid at the interface in the o/w liposome system due to its high hydrophobic amino acid content, hence, more easily interacting with free radicals.

Generally, RP55 showed higher antioxidant activities (except for hydroxyl radical scavenging ability) than RP25 and CRPs at the same concentrations. Chen et al. [26] proposed that the antioxidant activity of peptides depended on amino acid compositions and their sequences. They reported that histidine and proline played an important role in the antioxidant activity of designed peptides. Dávalos et al. [27] further reported that among the amino acids, Trp, Tyr, and Met showed the highest antioxidant activity, followed by Cys, His, and Phe. The rest of the amino acids did not exhibit any antioxidant activity. Aromatic amino acids are generally considered as effective radical scavengers, because they can donate protons easily to electron deficient radicals at the same time maintaining their stability via resonance structures [22]. In one of our previous

studies [2], for RP55, CRPs and RP25, the total contents of His and Pro were 12.97, 10.07, and 9.23%, respectively. The total contents of six amino acids (Trp, Tyr, Met, Cys, His, and Phe) in RP55, CRPs, and RP25 were 20.71, 17.11, and 15.3%, respectively. Additionally, the total contents of the aromatic amino acid (Phe and Tyr) in RP55, CRPs, and RP25 were 13.26, 10.09, and 7.62%, respectively. Therefore, the unique amino acid composition of RP55 could contribute to its strong antioxidant activity.

In addition to the antioxidant amino acids, the role of some non-protein compounds such as phenols and pigments in rapeseed should not be ignored when evaluating the antioxidant activities of rapeseed peptides. Cheng et al. [28] reported that phenolic compounds afforded their protective actions in lipid oxidation by scavenging the lipid-derived radicals. More recently, Yoshie-Stark et al. [29] demonstrated that the ED_{50} values for DPPH radical of the de-oiled rapeseeds were much smaller than those of the protein concentrates. They presumed that it was because a high proportion of the phenols were removed during the processing of the protein concentrates, relative to the de-oiled rapeseeds. Likewise, RP55 with higher antioxidant activities had markedly higher tannin content (3.08%) than either CRPs (1.05%) or RP25 (0.91%) [2]. Furthermore, most brown color substances in RP55 may also be important for its superior antioxidant activities. The brown color substances were probably derived from the Maillard reaction and phenolic oxidation during the oil extraction, especially at the last step (heating to inactivate the enzymes). It has been reported that Maillard reaction products possess notable antioxidant properties [30]. As discussed above, rapeseed peptides contain not only antioxidant peptides but also some other antioxidant compounds. Therefore, further fractionation studies are necessary to identify and elucidate the compounds in rapeseed peptides that play a decisive role in antioxidant activities.

Inhibition of the Thrombin-Catalyzed Coagulation of Fibrinogen

The thrombin-catalyzed conversion of fibrinogen (F) to fibrin consists of three reversible steps, with thrombin (T) being involved in only the first step which is a limited proteolysis to release fibrinopeptides A (FpA) and B (FpB) from fibrinogen to produce fibrin monomer. In the second step, fibrin monomers form intermediate polymers through non-covalent interactions. In the third step, the intermediate polymers aggregate to form the fibrin clot [8]. As shown in Fig. 5a, rapeseed peptides possessed marked inhibitory activities on the thrombin-catalyzed coagulation of fibrinogen at certain concentrations although their inhibitory effects were not Dose-Dependent. For CRPs, its inhibitory effect

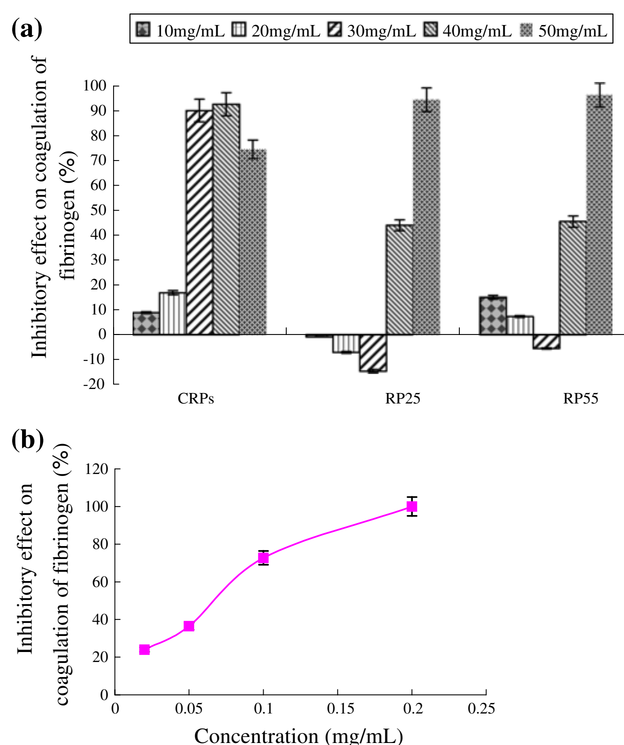

Fig. 5 Inhibitory effects on coagulation of fibrinogen: **a** rapeseed peptides; **b** heparin. Data were expressed as means with standard deviations ($n = 3$)

reached around 90% at concentrations of 30 and 40 mg/mL but decreased at higher concentration of 50 mg/mL. On the other hand, potent inhibitory effects (about 90%) at a high concentration of 50 mg/mL were observed for RP25 and RP55 which were similar to those of CRPs at concentrations of 30 and 40 mg/mL. When the respective concentration was decreased to 40 mg/mL, the inhibitory effects of both RP25 and RP55 declined sharply to about 45%. At lower concentrations, RP25 and RP55 exhibited weaker antithrombotic activities (even the inhibitory effects had low values). Heparin, commonly used as an antithrombotic drug, had the potent ability of retarding the formation of fibrin in a dose-dependent manner (Fig. 5b) with an ED_{50} value of 0.07 mg/mL under the assay conditions. Rapeseed peptides did not show antithrombotic activity comparable to heparin, but they were superior to the reported value for egg white hydrolysate at the same concentration [11]. Though protein hydrolysates are much less potent than heparin, they have the advantage of having no known side effects. Little information has been available about the antithrombotic mechanisms of peptides until now. Laudano and Doolittle [9] first demonstrated that the synthetic short peptides beginning with the sequence Gly-L-Pro-L-Arg, which corresponded to the amino(N-)-terminal segment of the fibrin α chain after the release of the fibrinopeptide A (FpA) by thrombin, could bind to fibrinogen and prevent the polymerization of fibrin monomers. The

peptide beginning with the corresponding mammalian β-chain sequence, Gly-L-His-L-Arg-L-Pro, bound to fibrinogen but did not prevent polymerization. Competition studies indicated that this peptide bound to a different set of sites. The binding and/or inhibitory activities were very sensitive to simple structural alterations [31]. Whether rapeseed peptides contain the Gly-Pro-Arg sequence and their specific antithrombotic mechanisms still requires further studies.

It is well known that rapeseed protein and rapeseed hydrolysates have high nutrition values [32, 33], which can be used for the nutritional enhancement in some kinds of foods. The antioxidant and antithrombotic activities are new functional characteristics of rapeseed peptides and these activities will expand their applications as functional food ingredients.

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