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Significance of Phenol−Protein Interactions in Modifying the Antioxidant Capacity of Peas

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The aim of this study is to evaluate the contribution of phenol−protein interaction (PPI) and its binding activity in strengthening the antioxidant capacity of peas after immersion with five phenolics under different heating conditions. The results showed that hydroxycinnamic acids (ferulic acid, coumaric acid, or caffeic acid) are better than hydroxybenzoic acid (gallic acid) in increasing superoxide dismutase (SOD) heat stability. In addition, the higher the temperature, the more evident was the enhancement. DPPH scavenging capacity and reducing power showed the same tendency. Further kinetic analysis proved that SOD with the best heat stability showed the largest activation energy during heating. Moreover, the contribution of phenol−protein binding to the antioxidant capacity was further estimated through complex purification and calculation of binding capacity. Coumaric acid was the most efficient phenolic compound in increasing antioxidant capacity and showed the highest binding capacity with pea protein. These results indicated that phenolic compounds might enhance the antioxidant capacity of peas during heating through phenol−protein interaction.

KEYWORDS: Phenol−protein interaction; binding capacity; antioxidant capacity.

INTRODUCTION

Superoxide dismutase (SOD) activity found in fresh or processed fruits is very weak because of protein deformation induced by heat processing. Numerous studies related to phenolic−protein interaction (PPI) have been carried out to stabilize the protein. Chlorogenic acid and caffeic acid have been reported to affect the heat denaturation of globular proteins (*1*) and enhance the heat stability of milk (*2*). Recent studies indicated that heat stable SOD activity was found in dried peas. The binding of low molecular weight phenolics to the protein matrix may account for the antioxidant potency of the SOD remaining after being dried (*3*). The heat stable antioxidant activity was supposed to be transferred from phenolic compounds to SOD protein. Furthermore, the susceptibility of protein to phenolic complex formation may differ with protein structure (*4*) or polarity of polyphenol (*5*). However, the contribution of the binding activity of PPI in enhancing the SOD activity and how proteins associate with different phenolics are not well-known.

Factors affecting PPI include kind and concentration of phenolic compounds (*6*), protein structure (*4*), and temperature (*7*). Cinnamic acids were reported to show higher antioxidant capacity than benzoic acid through the resonance structure (*8*). The protein binding activity has also proved to be significantly correlated with the antioxidant property, with a correlation coefficient of 0.78 (*9*). In this study, the effect of phenolic compounds with CH═CHCOOH (hydroxycinnamic acid) or COOH (hydroxybenzoic acid) in stabilizing antioxidant activities of pea protein was investigated. Contributions and binding capacity of the PPI complexes to the antioxidant ability were also evaluated through fractionation by Sephadex G-75.

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MATERIALS AND METHODS

Chemicals. Chemicals used were of analytical grade. The phenolic acids were obtained from Sigma-Aldrich, St. Louis, MO.

Samples Preparation. Two procedures were used. (a) Immersion of peas in phenolic solution: The peas were immersed directly in five different kinds of phenolic solutions (gallic acid, catechin, ferulic acid, coumaric acid, and caffeic acid) of 1 g/L for 6 h. After drying the peas at different temperatures (30−70 °C) for different durations (0−8 h), the SOD residual activity was calculated. (b) SOD/phenolic model system: Pea SOD extract (PSODE) was incubated with five kinds of phenolic solutions for binding interaction analysis and confirmation.

Extraction of SOD from Peas. One kilogram of fresh peas was well mixed with five times as much of acetone (w/w) in a blender and then filtered. After filtration, the peas were further extracted by potassium phosphate buffer (50 mM, pH 7.8) in the ratio of 1:5 (w:v) for 12 h at 4 °C. After centrifugation (4 °C, 12000 rpm, 30 min), ammonium sulfate was added to give 50 to 80% of saturation at 4 °C. Precipitated protein was collected by centrifuging and dissolved in the phosphate buffer. The extract was then subjected to dialysis for 48 h. After centrifugation (4 °C, 12000 rpm, 30 min), the supernatant was collected, named PSODE and then stored at −18 °C until use (*4*).

Formation of PPI Complex with PSODE. PSODE was incubated with phenolic compounds in the ratio of 0.68:1 (mg/mL) at 37 °C for 1 h for PPI complex formation (PPI mixture) (*10*), after which the complex was ready for LC purification.

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Purification of PPI Complex. The PPI complex was purified by LC method (*11*). Five milliliters of PPI mixture or phenolic standards in buffer was placed in the Sephadex G-75 column and washed with acetate buffer (50 mM, pH 5.0) at a flow rate of 48 mL/h. The elute was collected every 5 mL by a Biorad Econo LC system (BIO-RAD Laboratories, Hercules, CA). Profiles of the absorbance in contrast to elution volume were constructed by determining the absorbance at 280 nm. Each fraction derived from the chromatography was compared with standard phenolic solution and evaluated for its antioxidant capacity.

SOD Activity. The SOD activity of immersed samples was measured by the light induced nitroblue tetrazolium/riboflavin assay (A_{560}) as described by Nice et al. (*4*).

DPPH Radical Scavenging Activity. The DPPH radical scavenging activity of immersed samples was measured according to the method described by Tsai and Huang (*11*).

Reducing Power. The reducing power of immersed samples was determined by FRAP assay, which is a method of measuring the ability of reductants (antioxidants) to reduce Fe^{+3} to Fe^{+2}. This method was described by Tsai and Huang (*11*).

Binding Capacity. The binding capacity was evaluated by HPLC method (*10*). Each fraction from the LC was either extracted by organic solvent (F1) or evaporated directly to dryness and dissolved (F2) in 0.5 mL of methanol/water (50:50, v:v). All the fractions were tested by HPLC for phenolic compounds quantification and binding capacity calculation. There were two types of complex formed: protein-retained phenolics and protein-bound phenolics. The binding of the former was weak and could be broken after being extracted with organic solvent (three times with diethyl ether and ethyl acetate separately). It was calculated as [(Δ phenolics recovery of F1 and F2)/phenolics recovery of standard] × 100. The binding of the latter was calculated as {[(Δ phenolics recovery of standard and PPI mixture)/phenolics recovery of phenolic standard] × 100} − [binding capacity of protein-retained phenolics]. The expression [(Δ phenolics recovery of standard and PPI mixture)/ phenolics recovery of phenolic standard] × 100 was named PPI binding percentage.

Kinetic Analysis of Activation Energy. Samples were heated at 30, 40, 50, 60, or 70 °C, and their SOD activity was calculated every 2 h. By determining the change in SOD activity vs time, the rate of inactivation was calculated. The activation energy was then calculated as $\ln K = -E_a/RT$; K = rate constant, $R = 1.986$, and $T = (°C + 273)$.

RESULTS AND DISCUSSION

Effect of Phenolic Immersion on SOD Activity. Peas were incubated for 6 h with five phenolic compounds, 1 g/L solution. All the samples treated with phenolics demonstrated higher SOD activity than the control, which indicated a possible involvement of phenol−protein interaction. Among the five tested phenolic compounds, hydroxycinnamic acids (ferulic acid, coumaric acid, and caffeic acid) immersed samples showed higher SOD activity (2085.76, 2575.48, and 2605.61U, respectively) than the control (1477.94U) (**Figure 1**). That high SOD activity of hydroxycinnamic acids might result from the contribution of their resonance structure, which favored the stability of the molecule during heating (*8*). However, catechin and gallic acid (hydroxybenzoic acid) samples showed low SOD activity, which might result from the low diffusion of catechin. Indeed, the diffusion content for catechin, ferulic acid, coumaric acid, and caffeic acid was 1.42, 32.99, 80.57, and 47.06 times greater than the control, respectively. Moreover, the masking effect or weak H donor for gallic acid might lead to the low SOD activity of hydroxybenzoic acids (*12*).

Effect of Phenolic Immersion on SOD Thermal Stability. Further investigation related to the thermal stability of antioxidant capacity was carried out by heating the immersed peas at 30−70 °C for 8 h. For samples heated at 70 °C for 8 h, the residual SOD activity (regarding that of peas immersed with each phenolic compounds as 100%) was 74.41% (1552.05 U vs 2085.76 U) in the ferulic samples, while that of the control sample was only 53.55% (791.47 U vs 1477.94U). When samples were heated at 30 °C or 70 °C for a short time (2 h), only a small difference occurred between the ferulic acid and the control samples (**Figure 2**). In order to elucidate the effect of PPI binding on the thermal stability of SOD protein, SOD extract from immersed pea was further heated at 30−70 °C

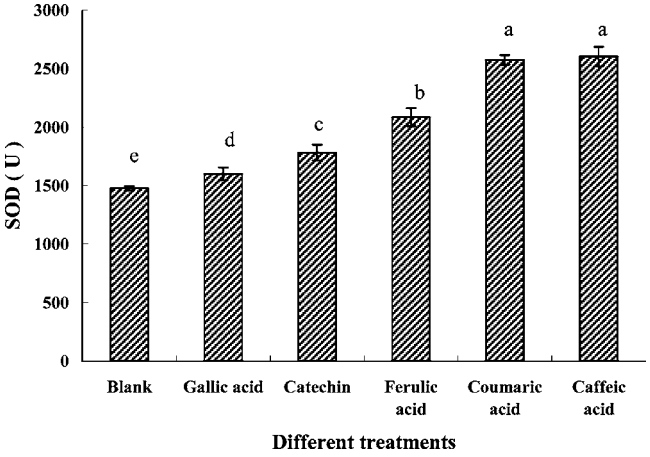

Figure 1. Effect of phenolic compounds on the SOD activity of peas after immersion for 6 h.

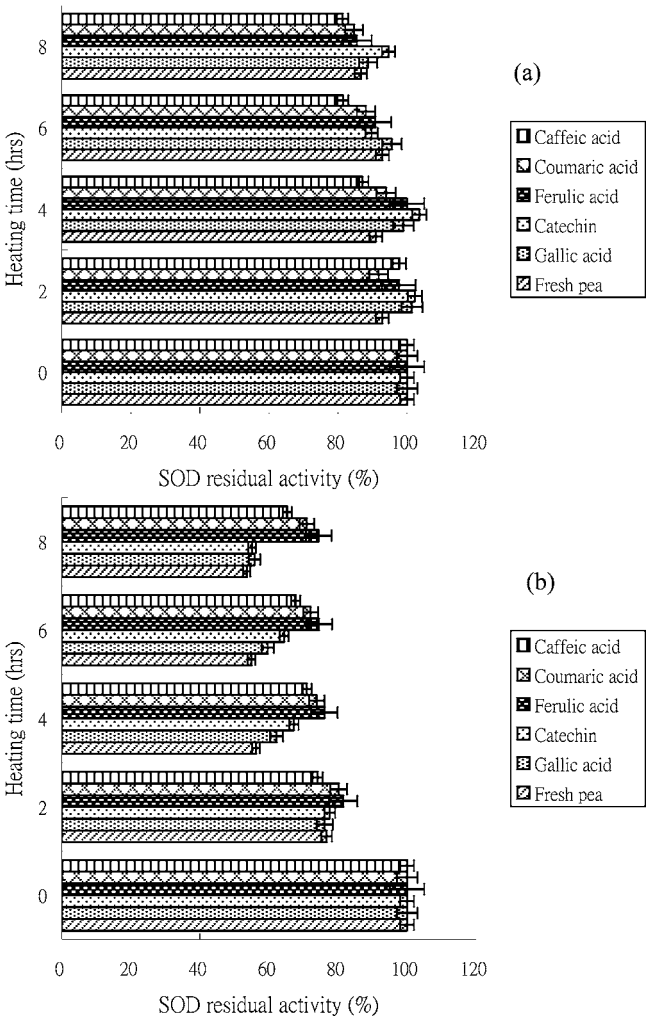

Figure 2. SOD residual activity of peas after immersion in different solutions of phenolic acids and heating at (a) 30 °C and (b) 70 °C for 8 h.

Table 1. Binding Capacity of PSODE with Different Phenolic Compounds

	phenolic recovery of LC (%)		binding capacity evaluation		
phenolic standard	phenolic std. (in buffer)	PPI mixture (PSODE with phenolic std.)	PPI binding percentage (%)	protein-retaine d phenolics (%)	protein-bound phenolics (%)
gallic acid	99	99	-[a]	-	-
catechin	95	83	13	-	13
ferulic acid	99	96	3	-	3
coumaric acid	100	74	26	-	26
caffeic acid	80	62	23	-	23

[a] -: Not detectable

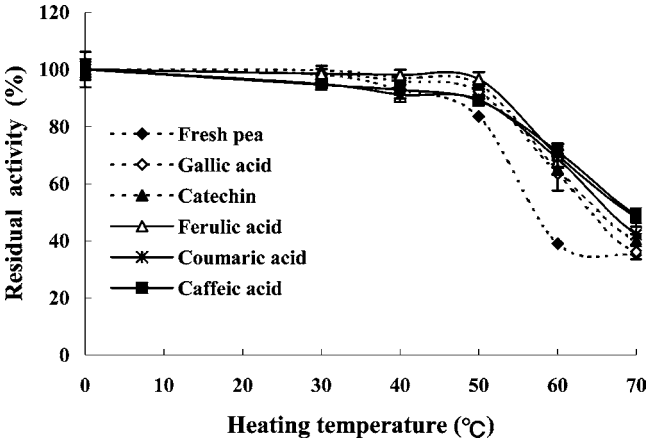

Figure 3. Effect of heating temperature on the residual activity of SOD extract from peas immersed in different solutions of phenolic compounds.

(**Figure 3**). Apparently, hydroxycinnamic acids including ferulic acid, caffeic acid, and coumaric acid were better than gallic acid or catechin in strengthening the SOD heat stability. Furthermore, the higher the temperature, the more the binding effect. On the other hand, this demonstrated that heat might damage the PPI complex (*7*). Kinetic analysis showed that, the activation energy (E_a) of SOD denaturation in the samples treated with gallic acid, catechin, ferulic acid, coumaric acid, and caffeic acid was 8.12, 8.80, 10.19, 10.57, and 8.74 kcal/mol, respectively, which was higher than that of control samples (7.83 kcal/mol). Apparently, phenolic binding with protein increased the activation energy of SOD and led to better thermal stability (*1*).

Effect of Phenolic Immersion on DPPH Scavenging Ability and Reducing Power. The changes in DPPH scavenging ability and reducing power were also evaluated after pea immersion. As shown in **Figure 4**, both DPPH scavenging ability and reducing power in all the samples treated with phenolic compounds were higher than those of the control. However, the DPPH scavenging ability decreased while FRAP increased as heating time or temperature increased. For example, DPPH scavenging ability was around 40% for fresh peas or 30 °C samples, whereas only 19.63, 23.16, 24.94, and 11.84% was found in the ferulic acid, courmaric acid, caffeic acid, and control samples, respectively, after heating at 70 °C for 8 h. FRAP in the 70 °C samples is significantly higher than that in 30 °C samples after being heated for 8 h (for instance, 1327.57 μmol/L vs 408.67 μmol/L for caffeic acid treatment). It seems that binding with hydroxycinnamic acids results in higher antioxidant properties (*13*). This, also suggested that phenolic compounds might change after heating and contributed to the decrease of DPPH scavenging ability or increase of FRAP (*14*, *15*).

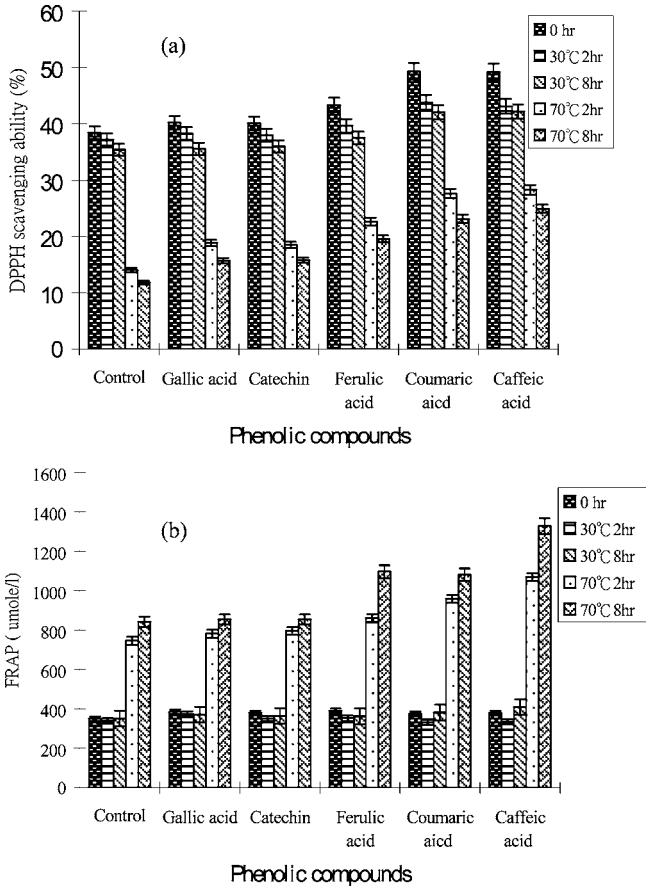

Figure 4. DPPH radical scavenging ability (a) and reducing power (b) of peas after immersion in different solutions of phenolic acids and heating at 30 °C or 70 °C for 0, 2 or 8 h.

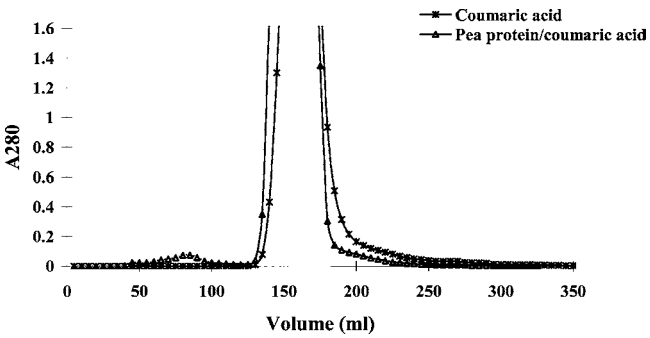

Figure 5. Sephadex G-75 elution profiles of coumaric acid alone and coumaric acid–pea protein mixture.

Binding Capacity of PPI in Peas. PSODE was incubated at 37 °C for 1 h with phenolic compounds for complex formation, purification, and analysis. **Figure 5** presents the sephadex G-75 elution profiles of coumaric acid/buffer and pea

Table 2. Contribution of the PPI to the Superoxide Anion Scavenging Ability, DPPH Radical Scavenging Ability, and Reducing Power of Peas

treatments	gallic acid	catechin	ferulic acid	coumaric acid	caffeic acid
binding percentage (%)	-[d]	13	3	26	23
relative SOD activity (%)[a]	100	122	76	148	146
contribution to SOD activity[b]	-	1586	228	3848	3358
relative contribution (%)[c]	-	41	6	100	88
relative DPPH scavenge %[a]	100	185	140	107	69
contribution to DPPH scavenge	-	2405	420	2782	1587
relative contribution (%)[c]	-	86	15	100	57
relative FRAP(%)[a]	100	38	33	47	48
contribution to FRAP[b]	-	494	99	1222	1104
relative contribution (%)[c]	-	40	8	100	90

[a] All these three relative activities were represented regarding gallic acid as basis (100%). [b] Calculated by multiplying binding percentage and a. [c] All these three relative contributions were represented regarding coumaric acid as 100%. [d] -: Not detectable.

protein/coumaric acid. The fraction at 130−240 mL showed a peak corresponding to the elution of free phenolic/buffer. In the fractionation of the pea protein/coumaric acid, the peak eluted earlier resulted from the formation of protein−phenolic complex with larger molecular weight. The same tendency appeared in all the other phenolic samples. **Table 1** shows the recovery of phenolic compounds and binding capacity evaluation between protein and phenolics. The behavior of the different phenolic−protein structure was determined by calculating protein-bound phenolics and protein-retained phenolics. The former corresponded to the percentage of phenolics that interact with protein and cannot be broken or extracted by the diethyl matrix. Coumaric acid and caffeic acid exhibited the highest value for protein-bound phenolics (26 and 23%), whereas gallic acid showed the lowest (not detectable). The *o*-dihydroxy group of caffeic acid was supposed to favor the formation of a stronger binding than others (*5*, *6*, *10*). However, its low recovery (80% for caffeic acid and 100% for coumaric acid) during the LC process might affect the results. On the other hand, not detectable values (<0.1%) were obtained in all samples for protein-retained phenolics. It suggested that there was a very weak interaction between nonpolar regions of phenolics and protein.

Contribution of Phenolic−Protein Binding to the Antioxidant Capacity. As mentioned above, coumaric acid showed the strongest binding affinity to pea protein and the highest antioxidant capacity (*4*, *5*). The relative contribution of the binding was calculated by multiplying the original antioxidant ability and the binding percentage, regarding coumaric acid as 100%. As listed in **Table 2**, coumaric acid showed the highest contribution to scavenging superoxide anion, DPPH radical, and reducing power, while gallic acid showed the lowest (not detectable). Then, we can conclude that the binding between phenolics and protein matrix might account for the enhancement of antioxidant capacity in peas, since phenolic−protein interaction is able to stabilize the protein and its antioxidant capacity is increased during heating. The application of this interaction in food processing such as manufacture of textured vegetable protein with extruder or pasteurization of milk could be practical.

ABBREVIATIONS USED

PPI, phenol−protein interaction; SOD, superoxide dismutase; PSODE, SOD extract from pea; DPPH radical scavenging, 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging; FRAP assay, ferric reducing ability of plasma assay.

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