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Full Length Research Paper

Biologically active proteins from *Curcuma comosa* Roxb. rhizomes

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***Curcuma comosa* Roxb., belongs to Zingiberaceae family, is used in folk medicine to relieve postpartum uterine inflammation. The crude protein from *C. comosa* was assayed to find some interesting bioactivities. The hemagglutination activity assay was performed with erythrocytes from seven different species, and revealed a strong specificity towards rabbit erythrocytes only. After isolation by Concanavalin A (Con A) affinity column chromatography and likely identification by tryptic peptide tandem mass spectrometry, two separate proteins with homology to other plant lectins were found. The free radical scavenging capacity and the superoxide dismutase (SOD) enzyme activity were used to determine any potential antioxidant activity. The results suggested that there were three different SODs in the crude rhizome protein extract. These enzymes were stable over a wide range of temperatures (up to 80°C) which may make them useful for further applications. In addition, alpha-glucosidase inhibitory activity also was found in this plant.**

Key words: Hemagglutination, antioxidant, superoxide dismutase, alpha-glucosidase inhibition, *Curcuma comosa.*

INTRODUCTION

Plants in the Zingiberaceae are widely distributed in Southeast Asia, especially in Thailand. There are some two-hundred species of this ginger family which fifty-eight species have been recorded as medicinal plants (Chuakul and Boonpleng, 2003). These plants widely used as a food, flavoring, coloring, spice agent and traditional herbs. Several reports have been published on the biological properties of small organic molecules from these plants, such as anticandidal (Rukayadi et al., 2006), antioxidant (Ali, 2002), antimicrobial (Lai et al., 2004; Wilson et al., 2005), anti-inflammatory (Li, 1985; Makabe et al., 2006; Tohda et al., 2006) and anti-tumor (Fu, 1984; Kim et al., 2000) activities. Furthermore, many of the biologically active proteins from Zingiberaceae plants have been reported such as an antifungal protein from *Curcuma longa* (Wang and Ng, 2005; Petnual et al., 2010), heamagglutinating proteins (lectins) from *C. amarissima* (Kheeree et al., 2010), *C. aromatica* (Tiptara et al., 2008), and *C. zedoaria* (Tipthara et al., 2007) and antioxidant enzymes (SODs) form *C. longa* (Kochhar and Kochhar, 2008), and *C. zedoaria* (Loc et al., 2008).

Curcuma comosa Roxb. (Zingiberales: Zingiberaceae) commonly known as Wan Chak Mod Look which mean uterus tightening. This phytoestrogen herb has been used for century to treatment women suffering from postpartum uterine swelling. Traditional medicine uses it as general tonic after childbirth, believed to make firmly vaginal, promote healthiness and make skin glow naturally. *C. comosa* has been found to exhibit various powerful properties, such as estrogenic (Suksamrarn et

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al., 2008; Winuthayanon et al., 2009), nematocidal (Jurgens et al., 1994), anti-inflammatory (Jantaratnotai et al., 2006; Sodsai et al., 2007; Thampithak et al., 2009), choloretic (Piyachaturawat et al., 1997, 1999) and antioxidant (Niumsakul et al., 2007) activities.

However, these activities have been attributed to non-protein components, whilst the bioactive protein in this plant has not been reported. Therefore, the aim of this research was to investigate the biological activity of the proteins from *C. comosa* rhizomes, which are expected to have at least some similar properties as those already reported in other Zingiberaceous plants and outlined previously.

MATERIALS AND METHODS

Protein extraction and precipitation

Fresh *C. comosa* rhizomes were purchased from the local market in Bangkok, Thailand. The rhizomes (1 kg wet weight) were blended and soaked in 4 L of extraction buffer (20 mM Tris-HCl pH 7.4, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM $MnCl_2$ and 0.15 M NaCl) overnight at 4°C. After filtration through cheesecloth, the crude extract was centrifuged at 15,000× g for 30 min. The supernatant was supplemented with ammonium sulfate to a final level of 90% saturation and then left overnight at 4°C. The precipitate was then collected after centrifugation at 15,000× g for 30 min, resolvated in 50 mL of deionized water and dialyzed (3.5 K MWCO SnakeSkin Dialysis tubing) against 4 L of deionized water to remove the salt. The desalting proteins were dry by using freeze dryer and kept in -20°C until used. The amount of protein was determined using the Bradford assay with bovine serum albumin as the standard (Bradford, 1976).

Protein isolation

The desalting crude protein from ammonium sulfate precipitation was dissolved in extraction buffer, and 5 mL of the solution (10 mg/mL) was applied to a Concanavalin A (Con A) Sepharose 4B column (1.6 × 15 cm) installed in an AKTA prime instrument (GE Healthcare, Uppsala, Sweden) equilibrated and eluted at 1 mL/min with buffer A (20 mM Tris-HCl pH 7.4 containing 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 0.15 M NaCl) at a flow rate of 1 mL/ min and collecting 10 mL fractions.

The protein content in each fraction was monitored by measuring the absorbance at 280 nm. After elution of the unbound fractions, the bound proteins were eluted with 100% buffer B (20 mM Tris-HCl pH 7.4 containing 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 0.15 M NaCl and 0.2 M Methyl-α-D-glucopyranoside) at the same flow rate and fraction volume collection. The eluted protein fractions were dialysis against deionized water and concentrated using a speed vacuum for further characterization.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was carried out as described elsewhere (Laemmli, 1970) using 8 × 10 cm sized gels. The recovered Con A-bound protein fractions (that is, those eluted in buffer B) were resolved by reducing SDS-15% (w/v) PAGE and visualized by Coomassie Brilliant Blue R-250 staining.

Hemagglutination activity

Red blood cells (RBC) from seven types of animal, that is mouse, rat, guinea pig, goose, sheep, rabbit and human, and with the human blood divided into the four serotypes or groups (A, B, AB and O), were suspended in 20 mM Tris-HCl pH 7.4 containing 0.15 M NaCl to a final 2% (v/v). Serial twofold dilutions of the protein sample (0.05 mL) in 96-well plates were mixed with 0.05 mL of the RBC. Agglutination was monitored visually after 1 h at room temperature. The activity was defined as the reciprocal highest dilution with positive agglutination (Tipthara et al., 2007). The commercial lectin, phytohemagglutinin, was used as positive control.

Measurement of free radical scavenging capacity by the DPPH assay

The DPPH (2, 2-diphenyl-1-picrylhydrazyl) assay (Udenigwe et al., 2009) was used to determine the antioxidant activity of the crude protein. Several different protein concentrations of each protein sample in the same volume (0.04 mL) were placed in each well of 96-well plates and mixed with 0.16 mL of 100 mM DPPH in methanol and then incubated in dark for 20 min at room temperature. After incubation, the absorbance was read at 517 nm in a micro plate reader. A control containing water instead of the protein sample was run and a sample blank was evaluated by adding methanol without DPPH. Vitamin E and ascorbic acid were used as positive controls. The concentration of the protein sample that resulted in scavenging of 50% of the DPPH was calculated (EC_{50}) graphically.

Riboflavin-nitroblue tetrazolium (NBT) assay for SOD activity

The superoxide dismutase (SOD) activity was determined using the riboflavin-nitroblue tetrazolium (NBT) assay, with slight modifications from the Lai method (Lai et al., 2008). The protein samples (0.1 mL) were first mixed with 2.75 mL of 67 mM phosphate buffer (pH 7.8) containing 0.01 M EDTA and 0.1 mL of 1.5 mM NBT. After incubation at 37°C for 5 min, 0.05 mL of 1.2 mM riboflavin was then added. The reaction mixture was illuminated with a 25 W light tube for 14 min in a foil-lined box. The microtiter plate reader was used to measure the absorbance at 560 nm. Deionized water instead of the protein sample was used as a control along with a sample blank which was evaluated by adding water instead of the riboflavin solution. The concentration of protein that provides 50% inhibition of the riboflavin-mediated reduction of NBT, taken as SOD activity, was calculated.

SOD activity staining on non-denaturing PAGE

Protein samples were resolved by non-denaturing -12.5% (w/v) PAGE (8 × 10 cm gel size) without boiling. Following electrophoresis, the gel was stained for SOD activity by the method of Chopra (Chopra and Sabarinath, 2004), except with some modifications as outlined below. Gels were first soaked in 50 mL riboflavin-NBT solution (5 mg riboflavin, 12.5 mg NBT) for 15 min at room temperature in the dark. After incubation, the riboflavin-NBT solution was removed and 50 mL of 0.1% (v/v) TEMED was added. Gels were incubated again in the dark for 15 min. The solution was then removed and superoxide synthesis was induced by exposure to a 25 W light tube for 15 min in foil-lined box. The gel color turns into blue-purple and the SOD bands are white. To identify the prosthetic group of SOD, two inhibitors (KCN and H_2O_2) were used. For hydrogen peroxide treatment, the gel was first soaked in 8 mM H_2O_2 for 30 min, followed by the SOD activity staining as detailed

earlier. For KCN treatment, 8 mM KCN was included in the riboflavin-NBT solution. To determine the effect of temperature upon the SOD activity, the crude protein (1.5 mg/mL) was incubated at one of eight temperatures (30, 40, 50, 60, 70, 80, 90 and boil) for 5 min. After incubation, the protein was cooled immediately in an ice bath and 0.02 mL aliquots were used for determination of the SOD enzyme activity by *in situ* staining after resolution by non-denaturing PAGE, as detailed previously.

Alpha-glucosidase inhibition

Alpha-glucosidase inhibition was assayed in 50 mM sodium acetate buffer at pH 5.5 with 1 mM *p*-nitrophenyl-α-D-glucopyranoside (PNPG) as the substrate (Boonmee et al., 2007). A 0.02 mL aliquot of α-glucosidase enzyme (1 Unit/mL) was mixed with 0.01 mL of the protein sample, 0.04 mL of 50 mM sodium acetate buffer (pH 5.5) and 0.02 mL of deionized water. After incubation at 37°C for 10 min, 0.01 mL of PNPG, was then added and incubated again at 37°C for 30 min. To stop the enzyme reaction, 0.1 mL of 0.5 M Na_2CO_3 was added. Alpha-glucosidase activity was determined by measuring the release of the yellow *p*-nitrophenol at 400 nm using a microtiter plate reader, and the percentage of inhibition was calculated. Acarbose (Sigma) was used as the positive control.

Protein characterization

To characterize the proteins, they were first resolved in denaturing SDS-PAGE and the resolved protein bands were excised, in-gel digested with trypsin and then recovered, (Chokchaichamnankit et al., 2009) as reported.

The tryptic peptides were then analyzed by electrospray ionization quadrupole-time of flight mass spectrometry (ESI-Q-TOF) experiments, as reported (Chokchaichamnankit et al., 2009), performed on a Q-TOF (Micromass, Manchester, UK) mass spectrometer. For identification of the proteins, the tandem mass spectra were interpreted by De Novo sequencing software on Masslynx and the resulting peptides were searched against the non-redundant database with the modified mass spectrometry-driven BLAST searching protocol (MS-BLAST), using the European Molecular Biology Laboratory (EMBL) web interface and default settings.

RESULTS AND DISCUSSION

Hemagglutination activity

Hemagglutination activity is one property of lectins (Rüdiger and Gabius, 2001), a family of sugar-specific binding proteins that are potentially biologically active proteins and are used as tools in the fields of biochemistry, cell biology and immunology, as well as for diagnostic and therapeutic purposes in cancer research (De Mejía and Prisecaru, 2005).

Lectins are found in almost all organisms, including vertebrates, invertebrates, bacteria, viruses and plants. In this study, proteins from *C. comosa* rhizomes were extracted using an aqueous extraction buffer followed by ammonium sulfate precipitation and dialysis, which are general methods for the isolation of biologically active hydrophilic proteins. To investigate hemagglutination activity, red blood cells from six animals; rabbit, rat, sheep, mouse, guinea pig and goose were used. The crude rhizome protein extract from *C. comosa* exhibited a strong agglutination activity against only rabbit erythrocytes, with no detectable activity against the erythrocytes from the other five species. The protein was then tested with human erythrocytes of the four serotypes, but no hemagglutinating activity was detected. In agreement with this data, the crude proteins of various *Curcuma* species, obtained by Mg/NP-40 extraction, have been reported to exhibit heamagglutinating activity against rabbit erythrocytes (Sangvanich et al., 2007). However, comparison of the hemagglutinating activity between *C. comosa* (this study) and that for other *Curcuma* plants (Sangvanich et al., 2007) suggests that the hemagglutination activity of the *C. comosa* protein(s) are stronger than those found in other species of *Curcuma*. This difference may arise from the method of protein extraction and precipitation.

To isolate the hemagglutination protein(s) from *C. comosa*, Con A affinity column chromatography was used. When the protein extract of *C. comosa* was passed through a Con A Sepharose 4B column, only the adsorbed fraction (Figure 1) exhibited hemagglutinating activity towards rabbit erythrocytes. The proteins from this peak were separated by SDS-PAGE (Figure 2) and the two dominant bands, marked D1 and D2, were excised and subjected to in-gel trypsin digestion. The resultant mixture of tryptic peptides was evaluated with tandem mass spectrometry and the tandem mass spectra so obtained were used to deduce the amino acid sequences of each principal peptide using the Masslyxn software. The sequences of peptides were compared against existing known proteins using the MS-Blast algorithm to search the NCBI database.

This revealed that the peptides from D1 and D2 significantly matched the two sets of (one each, no overlap) lectins from different plants, as shown in Table 1. For the D1 protein, the MS-Blast result revealed that seven lectin proteins (Table 1) from various plants with a total HSP score of more than the threshold score (HSP = 170).

These lectins were sub grouped as ~25.3 kDa (five lectins) and ~31 kDa (two lectins), which is in broad agreement with the apparent ~27 kDa size for D1 estimated by reducing SDS-PAGE. The amino acid sequences of the three tryptic peptides from the D1 protein were compared with the six homologous lectins from various plants species (Table 1), which revealed that the tryptic peptide amino acid sequences were, however, mostly identical to the larger 31.4 kDa lectin from *Cratylia mollis*. For the D2 protein, the MS-Blast result revealed that the tryptic peptide amino acid sequences were identical to the lectins from *Allium sativum* and *Gastrodia elata*. These two lectins are quiet different in size, being 33.9 and 18.1 kDa, respectively, with the smaller being in broad agreement with that estimated for D2 of ~15 kDa.

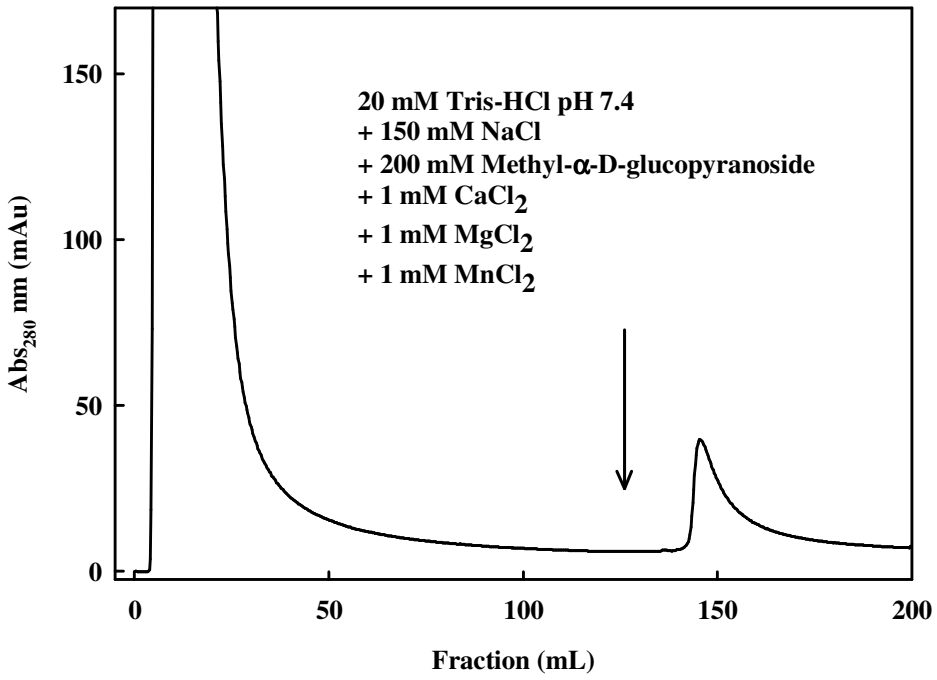

Figure 1. Affinity chromatogram of *C. comosa* rhizome proteins on a Con A Sepharose column equilibrated and then washed with elutant buffer (start of elution at the arrow)

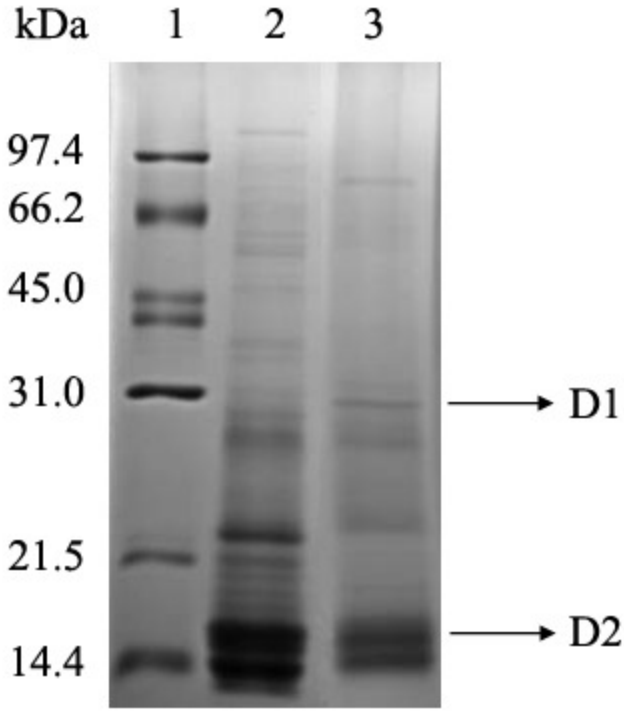

Figure 2. Reducing SDS-PAGE resolution of the crude protein extract and the ConA Sepharose-bound fraction, from the rhizomes of *C. comosa*. Lane 1 molecular weight standards; lane 2, crude lane was loaded with 30 µg of protein in total; and lane 3, bound Con A was loaded with 50 µg of protein in total.

Table 1. Likely identification of the D1 and D2 protein bands by MS-BLAST

Accession number	Organism	Sequence		Mass (Da)
	***Curcuma comosa* (D1)**	**RVSSDGSPKGSSVGRT--**		
P02866	*Canavalia ensiformis* (Concanavalin-A)	DGNLELTRVSSNGSPQGSSVGRALFYAPVHIWESSAVVASFEATFTF	108	31,480
P55915	*Canavalia brasiliensis* (Concanavalin-Br)	T--EGNLRLTRVSSNGSPQGSSVGRALFYAPVHIWESSAVVASFEATFTF	197	25,568
P81364	*Canavalia maritime* (Concanavalin-Ma)	T--DGNLXXTRVSSNGPSQGSTVGRALFYAPVHIWESSATVAGFDATFXX	196	25,364
P81637	*Dioclea guianensis* (Lectin alpha chain)	S--DGNLELTKVSSSGDPQGSSVGRALFYAPVHIWEKSAVVAGFDATFTF	197	25,399
P81517	*Cratylia floribunda*(Lectin alpha chain)	S--DGNLQLTRVSN-GSPQSNSVGRALYYAPVHVWDKSAVVASFDATFTF	196	25,398
P08902	*Dioclea grandiflora* (Lectin alpha chain)	S—DGNLELTKVSSSGDPQGNSVGRALFYAPVHIWESSAVVASFDATFTF	197	25,567
	***Curcuma comosa* (D1)**	**VGLSASTG-LLGLFPDANLL**		
P02866	*Canavalia ensiformis* (Concanavalin-A)	LIKSPDSHPADGIAFFISNIDSSIPSGSTGRLLGLFPDANVIRNSTTIDF	158	31,480
P55915	*Canavalia brasiliensis* (Concanavalin-Br)	LIKSPDSHPADGIAFFISNIDSSIPSGSTGRLLGLFPDAN----------	237	25,568
P81364	*Canavalia maritime* (Concanavalin-Ma)	LIKSPDSHPADGIAFFISNIDSSIPSGSTGRLLGLFPDAN----------	236	25,364
P81637	*Dioclea guianensis* (Lectin alpha chain)	LIKSPDRDPADGITFFIANTDTSIPSGSGGRLLGLFPDAN----------	237	25,399
P81517	*Cratylia floribunda*(Lectin alpha chain)	LIKSTDSDIADGIAWFIANTDSSIPHGSGGRLLGLFPDAN----------	236	25,398
P08902	*Dioclea grandiflora* (Lectin alpha chain)	LIKSPDREPADGITFFIANTDTSIPSGSGGRLLGLFPDAN----------	237	25,567
	***Curcuma comosa* (D2)**	**DGHVVLYG DELWSSGTNTAAA**		
Q38787	*Allium sativum* (I lectin precursor)	VYDADGRPLWASHSVRGNGNYVLVLQEDGNVVIYG--SDIWSTGTYVKAA	148	33,984
Q1M0Y9	*Gastrodia elata* (Gastrodianin-4A)	----------TNGKASNCILKMQRDGNLVIYSGSRAMWASNTNRQDG	109	18,192
	***Curcuma comosa* (D2)**	**FQTDGN**		
Q38787	*Allium sativum* (I lectin precursor)	GQSLDVEPYHFIMHEDCNLVLYDHSTAVWASNTDIPGKKGCKAVLQSDGN	248	33,984
Q1M0Y9	*Gastrodia elata* (Gastrodianin-4A)	----------YYLILQRDRN	120	18,192
	***Curcuma comosa* (D2)**	**FLLFKKESRALWSSDTRKGS RHATVVLYGNLPFK**		
Q38787	*Allium sativum* (I lectin precursor)	FVVYDAEGRSLWASHSVRGNGNYVLVLQEDGNVVIYGSDIWSTDTYRKSA	298	33,984
Q1M0Y9	*Gastrodia elata* (Gastrodianin-4A)	VVIYDNSNNAIWASGTNVGNAEITVIAHSNGTAAASGAAQNKVNELYISM	170	18,192

Antioxidant activity

The antioxidant activity of the crude rhizome protein extract, as measured by the DPPH assay, revealed that the crude protein presented antioxidant activity with an EC_{50} value of 0.0619 mg/mL, which is lower than that for ascorbic acid (EC_{50} = 0.0098 mg/mL) or Vitamin E (EC_{50} = 0.0179 mg/mL). The antioxidant proteins turmeric (Smitha et al., 2009) and super stable superoxide dismutase have been reported before from the related *C. longa* (Kochhar and Kochhar, 2008) Thus, we determined the SOD activity in the crude rhizome protein extract. Firstly, the crude extract was evaluated using the riboflavin-NBT assay to measure the SOD activity, and this revealed a positive result. To then confirm SOD activity in the *C. comosa* rhizome protein extract, the *in situ* SOD enzyme activity was performed after Native-PAGE resolution, and revealed at least three SOD bands (Figure 3). The largest of the three bands in

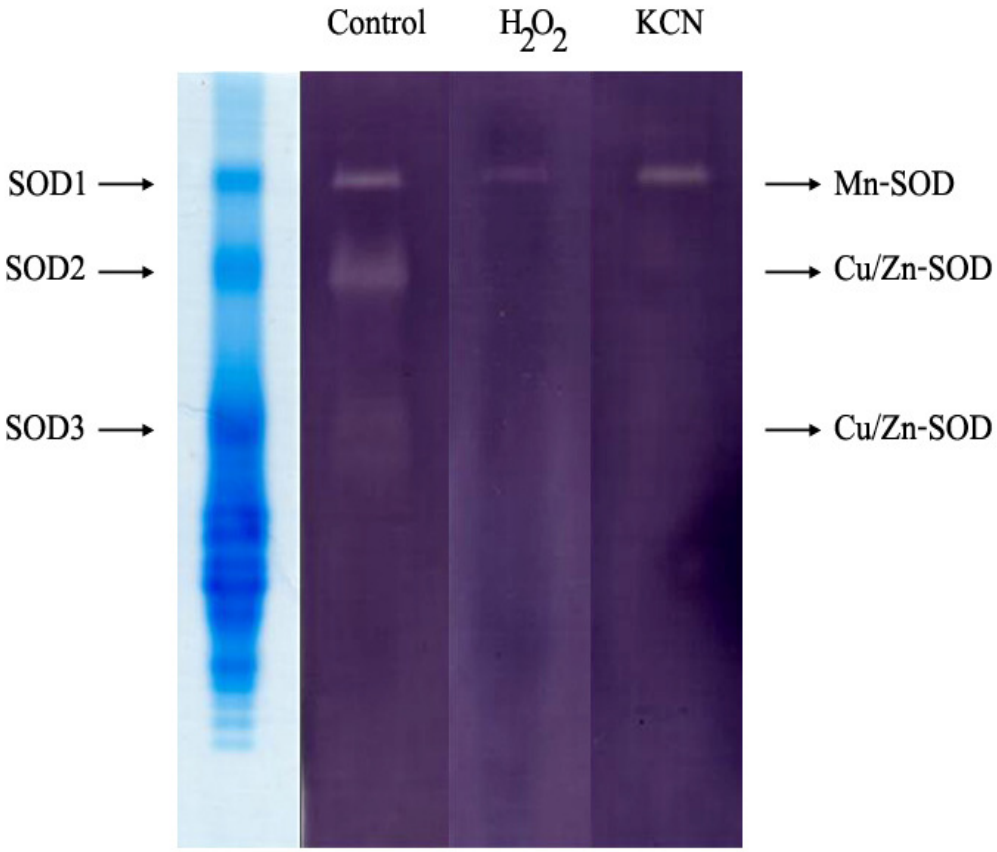

Figure 3. Determination of the type of SOD. Left is coommasie staining, right are SOD activity staining, of the Native-PAGE resolved crude protein extracts of *C. comosa* rhizomes. Each lane was loaded with 30 g of total protein.

the gel (SOD1) was classified as a Mn-SOD due to its insensitivity to KCN and H_2O_2 inhibition. The two smaller proteins (bands) SOD2 and SOD3, respectively, are likely to be Cu/Zn-SODs since they are sensitive to both KCN and H_2O_2. This *in situ* enzyme activity gel staining technique was also used to determine the temperature stability of the three apparent SODs from *C. comosa* rhizomes, with the results shown in Figure 4. The higher molecular weight Mn-SOD was stable up to 80°C and then completely lost all detectable activity (by this assay) at 90°C, while the two lower molecular weight Cu/Zn-SODs were both slightly less thermostable, being stable at up to 70°C. SODs are widely used in medicine and the pharmaceutical and food industries. They have been isolated in various plants, including from members of the Zingiberaceae plant family, such as to the highly thermostable Cu/Zn-SOD found in turmeric (*C. longa)* leaves (Kochhar and Kochhar, 2008), which is very thermotolerant being stable at up to 100°C. The thermostability of SOD enzymes is important in biotechnology, since it allows their use under the harsh conditions in the pharmaceutical and cosmetically industries. *C. zedoaria* have been reported to display antioxidant activity in various antioxidant assays, including SOD activity (Loc et al., 2008). Thus, the finding of SODs in these two plants are consistent with the finding here of SOD activity in *C. comosa*, a plant in same family, although potentially three different SODs from at least two classes (Cu/Zn- SOD and Mn-SOD) are present in *C. comosa*.

Alpha-glucosidase inhibition activity

Alpha-glucosidase is an enzyme that hydrolyzes carbohydrate to glucose in the small intestine. Inhibition of this enzyme could suppress postprandial hyperglycemia and help to control type II diabetes. Therefore, the development and search for new inhibitors of this enzyme is potentially advantageous. Natural products are still a good source for α-glucosidase inhibitors, which have included acarbose and voglibose, two natural inhibitors in clinical use. Alpha-glucosidase inhibition activity is also found in Zingiberaceous plants, such as *Zingiber officinale* Roscoe. Thus, the presence of active inhibitors of α-glucosidase were screened for in the

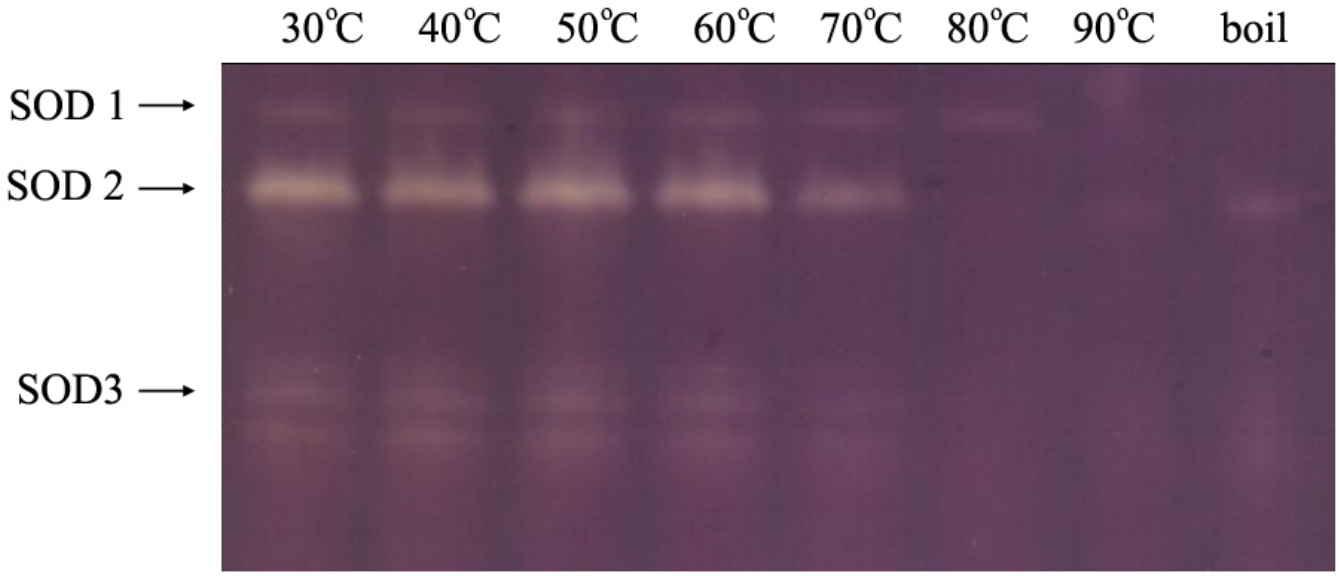

Figure 4. Effect of temperature (30-100°C) on the activity of the three SODs from *C. comosa* rhizomes. Each lane was loaded with 30 g of total protein.

C. comosa rhizome protein extract using the colorimetric α-glucosidase inhibition assay, which revealed an inhibitory activity with an EC_{50} value of 0.0572 mg/mL. Even though this inhibition activity is lower than the commercially used acarbose, it is stronger than some microalgae (Xiancui et al., 2005).

However, it should be borne in mind that the *C. comosa* rhizome extract was screened as a crude rhizome protein extract only, which would be rich in other non-α-glucosidase inhibitory proteins, and so the actual protein inhibitor(s) specific activity is likely to be significantly higher and may even exceed that of acarbose, but this awaits its purification and characterization. It thus remains that the protein(s) responsible for the glucosidase activity may be a new choice for disease control.

Conclusion

It was observed in *C. comosa* that the crude protein showed several biological activities; agglutination of rabbit erythrocytes, inhibition of glucosidase enzyme activity and both Mn- and Cu/Zn-SOD activities, including an antioxidant activity in the DPPH assay.

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