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A Novel α-Glucosidase Inhibitor Protein from the Rhizomes of *Zingiber ottensii* Valeton

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Abstract The objective of this study was to investigate a new protein with α-glucosidase inhibitory activity from the rhizomes of *Zingiber ottensii*. With a simple salting-out technique followed by single-step anion-exchange purification, the protein was successfully purified from the rhizomes. This protein was found to have three likely sub-unit types, 32.5, 15.2, and 13.8 kDa, as revealed by native and reducing SDS-PAGE analysis. Determination of the kinetics of the inhibition of α-glucosidase from *Saccharomyces cerevisiae* by standard enzymatic methods indicated the maximum percent inhibition; IC_{50} and K_i of this protein were 77.5%, 30.15 μg/ml, and 140 μmol, while the K_m and V_{max} were 2.35 μmol and 0.11 mM/min, respectively. The inhibitory action was pH-independent within the pH range 2–10, but was potentially affected by buffer salts, and was relatively temperature-stable between 4–35 °C, with a maximum activity at 65 °C. The amino acid sequence of an internal fragment of this purified *Z. ottensii* rhizomal protein had a similarity to the sequence from the plant cysteine proteinase family. Although this α-glucosidase inhibitory protein was purified from *Z. ottensii* rhizomes and preliminarily characterized, further studies are needed prior to firm applications being envisaged.

Keyword α-Glucosidase inhibitor · *Zingiber ottensii*

Introduction

The α-glucosidases (EC 3.2.1.20) [1, 2] (EC 3.2.1.10, EC 3.2.1.48, and EC 3.2.1.106) [3] or maltases [4] are a group of enzymes that catalyze the cleavage of the α-glycosidic

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linkage of carbohydrate molecules from the non-reducing end. They function particularly well with disaccharides and oligosaccharides as substrates [4, 5]. In the case of humans, the enzyme is located around the brush-border cells of the intestine and plays a fundamental role in releasing glucose from dietary carbohydrates, thereby allowing efficient absorption. However, this can lead to an undesired increase in the blood sugar levels for diabetes type II patients and people who are in obesity treatment. Thus, α-glucosidase inhibitors, a relatively recent therapeutic topic, are gaining interest because they could not only decrease the postprandial blood glucose levels, but also prevent or cure some chronic diseases such as, cancer, viral infections, and hepatitis [1]. For instance, castanospermine, an alkaloid from the black bean or Moreton Bay chestnut tree, has already been characterized as an α-glucosidase inhibitor that can effectively prevent mice from succumbing to dengue virus infection and also to allow infected mice to recover more quickly [6]. The effect was shown to be due to the ability of castanospermine to block the viral α-glucosidase enzyme activity. Apart from medical or healthcare benefits, α-glucosidase inhibitors are also involved in modern biotechnological studies to improve beer [4], sake [7], and other brewing industries, as certain glucose/total sugar ratios of the ferment are necessary for good product qualities. One more advantage of this inhibitor group is that some of them contain a trans-glycosylation activity which may be used in specific oligosaccharide synthesis [8].

To overcome these challenges, various α-glucosidase inhibitors have been discovered and studied. The three most familiar inhibitors currently used in medicine are acarbose (pseudotetrasaccharide), miglitol (1-deoxynojirimycin) [9], and *N*-butyl-1-deoxynojirimycin [5]. However, to date, almost all reported inhibitors are sugar or oligosaccharide derivatives [10], and other small molecules, such as flavonoid analogs [2], β-lactam, which already contains antibiotic activity at the same time [11], the isoflavone genistein from soy bean [12], and alkaloid [6]. Moreover, uncharacterized inhibitors include the unknown substances from plant extracts, such as the methanol extract from the Devil tree, *Alstonia scholaris* [13], the water extract of the *Morus alba* leaf [14], *n*-butanol extract of *Chaenomeles sinensis* fruit [15], and the ethanol extracts of *Entada rheedii* and *Archidendron jiringa* seed coats, *Albizia lebbeck* and *Albizia lebbeckoides* bark, and *Parkia speciosa* pericarp [16]. Most sources were found to have from a moderate to a very high inhibition activity. Although some, but not all, of the extraction buffers above are suitable for protein extraction, it still remains that there are still very few reports of protein inhibitors of α-glucosidase activity so far, especially in herbs.

Thailand has a high diversity of plants and possesses a rich biodiversity of herbs, many of which have widely accepted therapeutic properties. Since enzyme inhibition is one of the natural life mechanisms, it is therefore possible, if not likely, that this favorable activity will be present in many kinds of plants. On the other hand, although the existing inhibitors give good glycemic control, there are still many serious side effects reported. For example, the acabose application was found to contribute a high risk from adverse gastrointestinal effects [17], while pneumatosis cystoides of intestine was linked to steroid-induced diabetic patient treated with vogibose [18]. For these reasons, the discovery of new inhibitors of α-glucosidase may be beneficial, if not necessary, as they will be expected to become alternative weapons in the struggle against various healthcare problems, as well as being potentially highly valuable research and industrial tools in future.

Zingiber ottensii Valeton [called (phonetically) “Plai-Dam” in Thai] is a medicinal plant that belongs to the Zingiberaceae family. Asides from its medicinal properties, this Southeast Asia native ginger can also be used for ornamental purposes and is even used as a spice and fresh food [19]. Most medical applications are reported to be achieved from the use of its rhizome, but the reported properties of the plant appear to depend on local

knowledge. This may suggest potential cultivar (genetic) or environmental influences in the chemical composition, but this remains unknown. Nevertheless, these medicinal uses have included as a traditional sedative [20] and remedy for convulsion and lumbago treatment in Malaysia [21]. In addition, it is linked to poultice in postnatal treatment and as an appetizer [19]. In Thailand, *Z. ottensii* has been traditionally used to cure external bruises and gastrointestinal ulcers. Although some reports have addressed the chemical constituents of *Z. ottensii*, and in particular the essential oils, there still are no reports about bioactive proteins from this plant. Thus, from the above rationale, the objective of this study was to purify and investigate proteins with α-glucosidase inhibitory activity from *Z. ottensii* rhizomes.

Materials and Methods

Materials

The fresh rhizomes of *Z. ottensii* were periodically (October 2008–June 2009) purchased from Chatuchak park market in Bangkok, Thailand. A voucher specimen (BKF No. 60689) is deposited at The Forest Herbarium (BKF), Royal Forest Department, Bangkok, Thailand. Ammonium sulfate, acrylamide, bis-acrylamide, hydrochloric acid, mercaptoethanol, TEMED (tetramethylethylenediamine), and tris(hydroxymethyl) aminomethane (MERCK) were purchased from the Merck group, Germany. Ammonium persulfate, Coomassie Blue G-250, glacial acetic acid, methanol, sodium chloride, sodium hydroxide, and sodium dodecylsulfate (BDH) were purchased from VWR International, USA. The α-glucosidase from *Saccharomyces cerevisiae* was purchased from Sigma-Aldrich Co. Ltd, USA. All chemicals were analytical grade.

Extraction of Protein from the Rhizomes of *Z. ottensii*

Protein was isolated from the rhizomes using the procedure described by Tiptara [22] and Samarkina [23] with some modification. Briefly, the rhizomes were peeled, minced into small cubes (about 5×5×5 mm), and soaked in 20 mM Tris–HCl buffer pH 7.2, containing 0.15 M NaCl, with care to cause minimal oxidation by air exposure. With a prepared rhizome to buffer (20 mM Tris–HCl, pH 7.2) ratio of about 1:4 (*w*/*v*), the small cubes were homogenized using a blender (Philips, HR 2061, Indonesia) until no remaining small pieces could be seen. The homogenate was then stirred overnight (IKa, RW 20 N, Labotechnik Inc., Japan) at 4 °C. After centrifugation at 15,000×*g* at 4 °C for 15 min, the supernatant was collected and transferred into a new container (5 L plastic tub) and further agitated at 4 °C overnight. Anhydrous ammonium sulfate was gradually added to make an 80% saturated solution and left for 6 h to precipitate. The suspension was then harvested by centrifugation at 15,000×*g* at 4 °C for 15 min, and the pellet, after resolvation in 20 ml of deionized water, was then dialyzed against 5 L of deionized water, at under 4 °C temperature, using dialysis tubing with 3500 Dalton cut-off (SnakeSkin, Thermo scientific Co., Ltd., USA). The water was changed three times during the 2-h dialysis period. The obtained crude protein was finally freeze-dried and the powder kept at −20 °C until use.

Purification of Protein from the Rhizomes of *Z. ottensii*

The purification process was slightly modified from the method described by Tipthara [24] in that Tris–HCl buffer pH 7.2 and an SP sepharose (Amersham Pharmacia Biotech, UK)

column were used instead. Briefly, 100 mg of crude protein powder was dissolved in 10 ml of 20 mM Tris–HCl buffer pH 7.2. Then, the solution at 10 mg/ml was loaded into a 5-ml loop Fraction collector (AKTA prime, Amersham Bioscience, Sweden) connected to SP sepharose column and microcomputer. After equilibration with the same sample buffer, the sample was injected into the column. Conditions used for this anion-exchange chromatography purification were: buffer *A*=20 mM Tris–HCl pH 7.2; buffer *B*=20 mM Tris–HCl pH 7.2 with 1 M NaCl; stepwise elution was used at 25, 50, 75, and 100% buffer B; flow rate=2 ml/min; 10 ml per fraction collected; and 5 ml loop injector used.

Protein Determination

Protein contents were determined by Bradford's procedure as described by Bollag [25]. Bovine serum albumin (BSA) was used as standard, and a standard curve derived from the average of three determinations of a 5–30 μg/ml BSA concentration series was created for every determination. The working procedure began with 50 μl of samples which were twofold diluted with deionized water in a 96-well flat-bottom plate. The series of standard BSA solutions were then placed into the appropriate wells. After 50 μl of Bradford's reagent was added to each analyzed well, the plate was carefully shaken to mix and then left for 15 min. Finally, the plate was read at 595 nm using an ELISA plate reader. The optical density (OD) of the obtained samples was used to calculate the protein concentration using the linear equation computed from the standard curve. During the column chromatographic separations, the elution profiles of proteins were determined by measuring the absorbance at 280 nm.

Native and Reducing Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis

Denaturing (reducing) sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) electrophoresis was performed as reported [25, 26] using a 15% (*w*/*v*) acrylamide separating gel and a 5%(*w*/*v*) acrylamide stacking gel under 40 mA (double panels) current and 280 V applied voltage. Relative molecular weights were achieved by comparison with coresolved sample bands from molecular markers (low molecular weight SDS marker, 1704461, Amersham Bioscience, Sweden). Native polyacrylamide gel electrophoresis (PAGE) was performed as above except without the 2-mercaptoethanol and SDS in the sample buffer, gels, and electrophoretic buffers, and without boiling of the samples. Equipment used in all these analysis was a power supply (Amersham, model EPS 301, Pharmacia Biotech, UK) and Vertical Electrophoresis Chamber set (Hoefer model miniVE, Pharmacia Biotech, UK).

α-Glucosidase Inhibition Assay

The assay method was modified from that reported previously [1, 7], using α-glucosidase from baker's yeast (Sigma, G5003, Sigma-Aldrich Co., Ltd., USA) as the working enzyme and *p*-nitrophenyl-α-D-glucopyranoside (Sigma, N1377, Sigma-Aldrich Co., Ltd., USA) as the substrate. The amount of the enzyme and substrate used in the assay was optimized for every new lot of enzyme and substrate purchased and selected so as to give approximately 0.8 OD (after the reaction was stopped) within 30 min incubation at 37 °C. Briefly, 40 μl of the serially diluted sample was transferred to each well of a 96-well microtiter plate. Then, 40 μl of enzyme (0.05 U per well, in 50 mM sodium acetate buffer, pH 5.5) was added to each well and the plate was shaken (190 rpm) on an orbital shaker (Biosan, model OS-10,

Latvia) for 1 min to mix the reagents. After incubation at 37 °C for 30 min, 40 μl of the substrate (0.005 mM per well, in 50 mM sodium acetate buffer, pH 5.5) was added to each well. The plate was shaken again for 2 min and then incubated at 37 °C for 30 min. The reaction was then stopped by addition of 40 μl of 1 M sodium carbonate solution to each well and shaken to mix, and then the plate was read at 400 nm with an ELISA plate reader (Biotek© Instrument Inc, USA). Controls had 40 μl buffer added instead of samples, while blanks had 40 μl buffer added instead of the enzyme. The percent inhibition was calculated using the following equation:

$$\%\,Inhibition = \frac{(OD_{control} - OD_{blank}) - \left(OD_{sample} - OD_{blank}\right) \times 100}{(OD_{control} - OD_{blank})}$$

IC_{50} Determination

IC_{50} determination was performed as per the α-glucosidase inhibition assay described above, but the amount of inhibitor was varied from 1.0–100.0 μg/ml, the upper limit representing the limit of solubility of the protein. The partial purified protein fraction used in this determination was freshly diluted from lyophilized power.

Determination of Kinetic Parameters

Briefly, 500 μl of each of the three concentrations (0, 1.0, and 2.0 μg/ml) of fraction F50, solvated, and diluted in 50 mM acetate buffer pH 5.5, was added into separate 15-ml glass test tubes containing 500 μl of 0.1 U/ml α-glucosidase in the same pH buffer. Then, all tubes were mixed thoroughly with vortex mixer and left in a 37 °C water bath for 30 min. The K_m and V_{max} values of the enzyme including the inhibitor's K_i value were determined from corresponding Lineweaver–Burk plots.

pH Resistance Determination

The evaluation of pH resistance was carried out as follows. Each 200 μl of fraction F50 (50 μg/ml) solvated and diluted in deionized water was separately transferred into a 1.5 ml Eppendorf tube and dried using a SpeedVac Concentrators. The protein was then resolvated in 200 μl of the appropriate buffer or deionized water set to the desired pH and incubated at 37 °C for 1 h prior to assaying for α-glucosidase inhibitory activity as described above. The pH buffers used in this experiment were all at 50 mM concentration and were composed of glycine–HCl (pH 2.0, 3.0, and 4.0), sodium acetate (pH 4.0, 5.0, and 6.0), potassium phosphate (pH 6.0, 7.0, and 8.0), Tris–HCl (8.0, 9.0, and 10.0), and glycine–NaOH (10.0, 11.0, and 12.0). All buffers and the non-buffered pH solutions were adjusted to the final pH using 1 N NaOH or 1 M HCl, as appropriate.

Temperature Resistance Determination

Briefly, each 200 μl of fraction F50 (50 μg/ml) protein in 20 mM Tris–HCl buffer pH 7.2 was aliquoted into a 1.5 ml Eppendorf tube in triplicate and incubated at the desired temperature as follows; freezer (<4 °C), cooling bath (15 °C), laboratory room (25 °C), water bath (35, 45, 55, 65, 75, 85, and 95 °C), for 1 h. After centrifugation at 15,000×*g* for

15 min at 4 °C to pull the liquid down to the bottom of the tube these samples were evaluated for α-glucosidase inhibitory activity as described above.

Internal Amino Acid Sequence of Protein by LC/MS/MS

The amino acid sequence of an internal fragment of the purified protein from *Z. ottensii* rhizomes was performed by in-gel trypsin digestion of the selected SDS-PAGE resolved protein band as reported [27] and sequencing of the different tryptic peptides by LC/MS/MS mass spectrometry. The selected Coomassie-stained protein band was excised from the SDS-PAGE gel and washed with 3% (*v*/*v*) hydrogen peroxide. The protein was in-gel-reduced, alkalated, and digested with trypsin as reported [20]. After digestion, the peptides were subjected to two sequential extractions from the gel with 50% (*v*/*v*) acetronitrile/0.1% (*w*/*v*) TFA at 200 μl/g gel (wet weight), the two extracts then being pooled and air-dried. The extracted trypic peptides were then subjected to LC-nano ESI/MS/MS. All collected LC/MS/MS data were processed and submitted to a MASCOT search of an in-house NCBI database. The following criteria were used in the Mascot search: trypsin cleavage specificity with up to three missed cleavage sites, cysteine carbamidomethyl fixed modification, methionine oxidation variable modifications, ±0.2 Da peptide tolerance, and MS/MS tolerance, and ESI-TRAP fragmentation scoring.

Results and Discussion

The extraction ratio, a 1:4 (*v*/*v*) ratio of minced rhizome: 20 mM Tris–HCl buffer pH 7.2, was found to be suitable as the blended residual solid phase was approximately one third of the total volume under the liquid phase (data not shown). A lower buffer ratio might allow the residue to pack too fast, and the mixture would become more viscous and be harder to mix. In turn, a higher buffer ratio would decrease the extracted protein concentration. The rhizome contains a large proportion of coarse starch that settled down very quickly, as seen in the bottom of container, after the mixture was poured out. Thus, the residue quickly stuck whenever it was left for any time.

The average protein yield obtained from the extractions of the rhizome batch reported here was 1.31±0.20 g/kg rhizomes, but it was observed that the amount of protein (per kilogram rhizome mass) obtained varied among the different rhizome batches. The *Z. ottensii* rhizomes used in this study was selected for identical size, thickness, flawless, and appearance. However, all were obtained from natural sources and so were not controlled or standardized for agrochemical, geochemical, and climatic variations in the environment, age, and cultivar genetics. Certainly, the rhizomes were noted to vary in color (purple) shades and numbers of bud or inner cavities, which may indicate differences in their ages and constituents. Whether these also reflect differences in the protein content in general or the specific protein of this report is unknown.

Preliminary testing found that solubility of the crude protein was 6.7–7.2 mg/ml in Tris–HCl buffer pH 7.2. All samples loaded into the chromatographic separation column were from the same extraction lot and at a 5 mg/ml concentration. Different proteins require different separation techniques in successive isolation and purification stages. For example, alocasin was isolated from giant taro (*Alocasia macrorrhiza*) rhizomes using 10 mM Tris–HCl pH 7.4 buffer without a $(NH_4)_2SO_4$ salting-out step, but the crude protein was only obtained at a low yield of 384 mg/kg rhizome, albeit after many chromatographic purification steps [28]. However, we used chromatography with SP sepharose gel

separation, a moderate anion-exchanger, to separate each protein in the crude extract using a 0 to 1 M NaCl gradient elution. However, the proteins eluted as a broad clump of bounded content with long tail eluting from about 0.27 M NaCl upwards (data not shown). This indicated that the extracted proteins have nearly similar negative charge density and that there was no clear partition (separation). It is possible that at the working pH condition (Tris–HCl buffer pH 7.2), the protein mixture, which can act as a buffer, had enough time to compensate gradually for the increase in the ionic strength from the gradient elution and retain the previous net negative charge. Thus, a stepwise elution was next performed, which resulted in much clearer and delineated peaks (Fig. 1).

From the stepwise elution, five distinct peaks were isolated, unbound, and F25, F50, F75, and F100 from the bound fractions named according to the percentage of buffer B used in their elution. It was found that the peak of fraction F50 was the highest, followed by unbound and F25, respectively. Quantification of the protein content in the five fractions revealed the highest protein content was found in F50 (Table 1), in accord with it having the largest peak in the SP sepharose chromatogram (Fig. 1). However, fractions F25 and U were ordered next in terms of protein yield (Table 1), in contrast to the chromatogram result (Fig. 1). This can be explained by two non-mutually exclusive assumptions. Firstly, the unbound peak may not all be protein, but may also be elevated by the presence of other small molecules which absorb light at 280 nm. Secondly, the protein in fraction U may contain less UV absorbing residues (tryptophan, phenylalanine, and histidine) than that in fraction F25. Both fraction F75 and F100 contained too low an amount of protein to be of use in any further investigation. Note, however, that from 50 mg of loaded protein, only 29 mg (58%) were recovered in the unbound and four bound peaks (Table 1), meaning that some 42% of the loaded protein was lost, perhaps still bound to the column. Since the crude extract had already been preliminary characterized and found to have α-glucosidase inhibition activity (97.5% inhibition using 50 mg crude protein/U α-glucosidase; Table 1), the unbound, F25, and F50 fractions were then evaluated for any α-glucosidase inhibitory activity, as well as for apparent purity by native and reducing SDS-PAGE analysis.

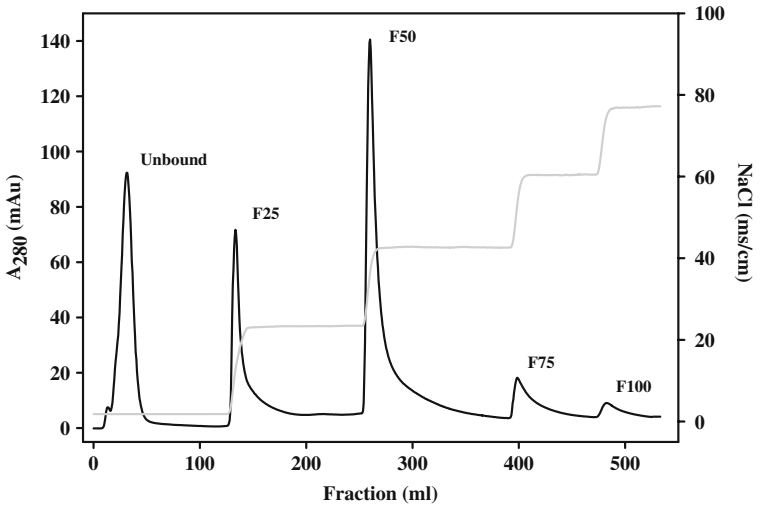

Fig. 1 SP sepharose chromatogram of the crude *Z. ottensii* rhizome protein extract (50 mg) with stepwise NaCl elution (0.25, 0.50, 0.75, and 1.00 M)

Table 1 The amount of protein in each purified fraction, after loading 50 mg total crude protein extract onto the column, and the α-glucosidase inhibitory activity.

Fraction	α-Glucosidase inhibitory activity	
	Protein yield (mg)	Maximal inhibition (%)
Crude extract	50.00	97.50±10.17
Fraction unbound	1.55±0.60	36.74±0.18
Fraction F25	4.20±3.00	28.54±4.22
Fraction F50	21.35±8.00	77.50±4.27
Fraction F75	1.15±0.80	Nd
Fraction F100	0.75±0.50	Nd

Nd not determined

The protein from each step of purification was analyzed for purity and protein pattern by native-PAGE (Fig. 2a), where, after SP sepharose affinity chromatography, a single protein from F50 band on the native-PAGE gel was observed, indicating that the purified protein obtained from the SP sepharose column should be a relatively pure protein. Reducing SDS-PAGE analysis revealed three bands, at about 32.5, 15.2, and 13.8 kDa were present in the F50 fraction (Fig. 2b). If fraction F50 contains a single protein, then it must consist of at least three subunits. Since the highest intensity bands were seen in the lower molecular weight, it is possible that the F50 fraction protein might consist of one large and at least two small subunits. However, further studies are required to ascertain which subunits are the active inhibitor of α-glucosidase and how many of the subunits are required to be bound to derive some or all of the inhibition activity. Each 40 μg of the unbound, F25, and F50 fractions, as dialyzed dried powders that were resolvated and subjected to an α-glucosidase

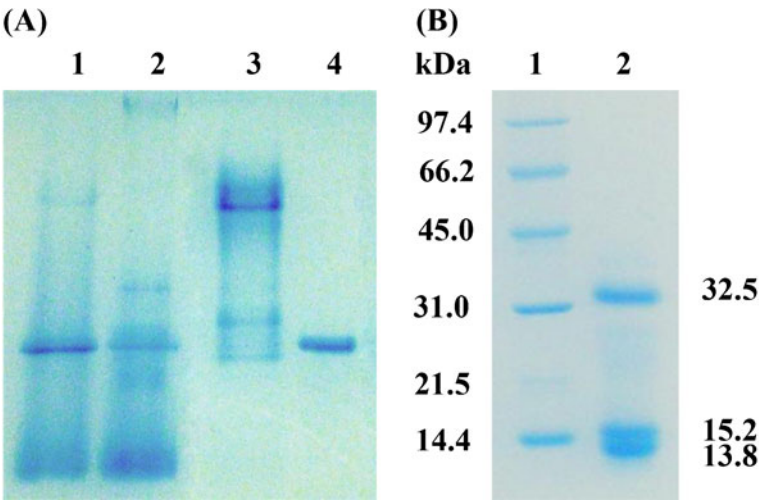

Fig. 2 **a** Coomassie Blue stained non-denaturing PAGE of the *Z. ottensii* rhizome protein from each step of purification where *lanes 1–4* show 20 μg of total protein from (1) the crude extract (homogenate), (2) the 80% ammonium sulfate precipitated and kept fraction, (3) the F25 bound SP sepharose fraction discarded, and (4) the F50-bound fraction kept. **b** Reducing SDS-PAGE analysis of the *Z. ottensii* rhizome protein purification. *Lane 1*, molecular weight standards; *lane 2*, fraction F50

inhibition assay, were found to exhibit inhibition activities of 36.74±0.81%, 28.54±4.22%, and 77.50±4.27%, respectively. Varying the concentration of each fraction revealed a dose-dependent inhibition of α-glucosidase for all three fractions. Thus, the F50 fraction was used in all further investigations due to its higher yield and inhibitory activity (Table 1).

From the inhibition data obtained using different concentrations of fraction F50, the data was processed, and the best-fit curve was derived using the commercial Sigmaplot 11 software (Fig. 3). The maximum inhibition and IC_{50} were calculated as 100% and 30.15 μg/ml, respectively. Cuminaldehyde, a small non-protein molecule isolated from *Cuminum cyminum* L. seeds, was reported to have an inhibition activity against Sprague–Dawley rat α-glucosidase with an IC_{50} of 0.5 mg/ml, which is 1.8 times lower than that of acarbose [29]. Although, different α-glucosidase sources might result in different relative acarbose inhibitory activities, for targeting human α-glucosidase the enzyme from baker's yeast was used as a preliminary testing tool. Anyway, it could be approximately deduced that the fraction F50 had a 16.1-fold and 8.9-fold greater inhibitory activity than cuminaldehyde and acarbose, respectively. Furthermore, the crude protein preparation from *Z. ottensii* reported here revealed a 97.5% maximum inhibition activity compared with the 82.07% reported for the crude protein (after 90% saturated $(NH_4)_2SO_4$ salting-out) from the flower extract of *Sesbania grandiflora* [30]. Moreover, after Superdex G200 and DEAE cellulose purification of the α-glucosidase inhibitory activity of reduced the maximal observed inhibition level to 74.0%, which concurs with our result here that after SP sepharose purification the F50 fraction revealed a 77.5% maximum α-glucosidase inhibitory activity compared with ~97% in the crude *Z. ottensii* rhizome extract. However, it could be seen that the inhibitory protein from *Z. ottensii* rhizomes was apparently slightly stronger than that from *S. grandiflora* flowers. In addition, the IC_{50} of F50 (30.15 μg/ml) was somewhat similar, given the differences in assays, to the IC_{50} obtained from the *M. alba* leaf extract (28.1 μg/ml) [14]. From the data obtained using fraction F50, the calculated K_i was 140 μmol while the K_m and V_{max} were 2.35 μmol and 0.11 mM/min, respectively (Fig. 4). In addition, F50 was characterized as a reversible competitive inhibitor where its linear curve intercepted the no-inhibit α-glucosidase curve.

No significant changes in the inhibition activity of fraction F50 was seen when pre-treated within the temperature range of 4–35 °C, while at 45 °C and, especially, 55 °C, the

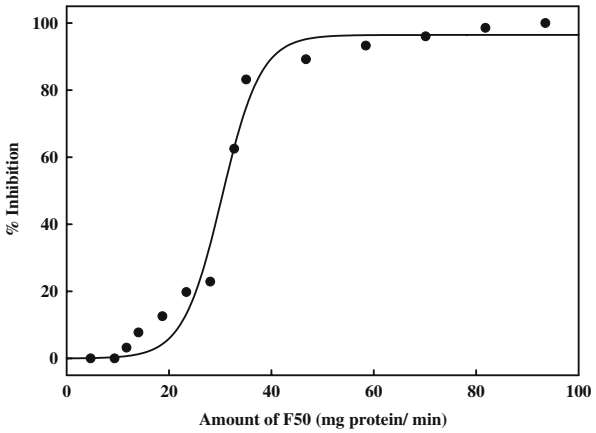

Fig. 3 Dose-dependent inhibition of α-glucosidase by the *Z. ottensii* rhizome fraction F50 protein

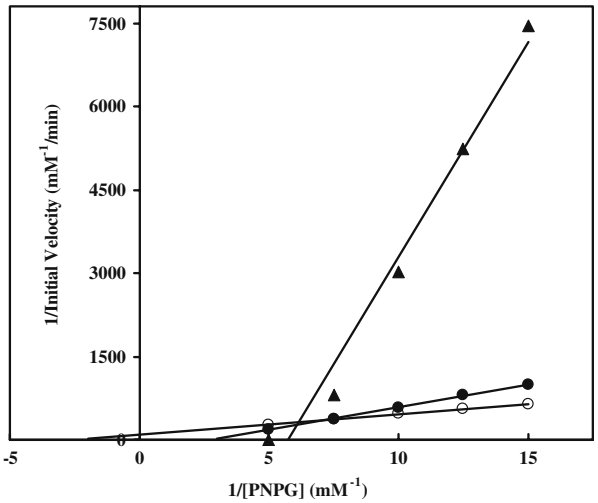

Fig. 4 Lineweaver–Burk plot of α-glucosidase kinetics with and without different concentrations of the *Z. ottensii* rhizome fraction F50 protein (*open circle*) 0 μg/ml, (*closed circle*) 1 μg/ml, and (*triangle*) 2 μg/ml. Data are shown as the mean±1SD and are derived from three repeats

observed inhibition activity was slightly decreased (Fig. 5). One possible reason was that the higher temperature range caused a change in the structure of fraction F50 (or even α-glucosidase enzyme) reducing its ability to bind to the enzyme active site. Thus, it can be deduced that the F50 fraction inhibitor is likely to be affected in the 45–55 °C temperature range. The inhibitory activity was maximal at preincubation at 65 °C and decreased markedly thereafter at higher temperatures. The maximal inhibition at 65 °C may, therefore, be regarded as the optimum temperature for this system. The baker's yeast enzyme used in this system had a poor activity at 65 °C, in common with other *S. cerevisiae*-derived α-glucosidases which are reported to have a maximal optima at close 30 °C [31], preventing

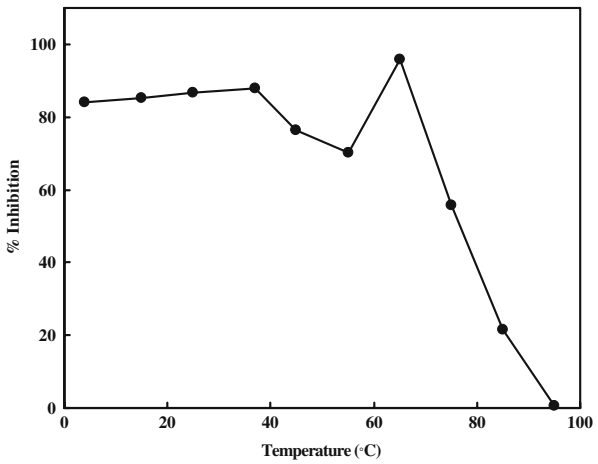

Fig. 5 Effect of preincubation of fraction F50 at different temperatures for the subsequent α-glucosidase inhibitory activity. Data are shown as the mean±1SD and are derived from three repeats

co-incubations of enzyme and inhibitor together. However, that the F50 α-glucosidase inhibitor could be used at a rather high temperature means that α-glucosidases from other sources that are more thermotolerant should be applied in future studies. The decrease in the inhibitory effect at temperatures over 65 °C could obviously be explained by degradation or denaturation of the F50 away from the active conformational state.

Changing the F50 protein preincubation buffer pH and salts revealed that the inhibition effect of fraction F50 was negated at pH 7.0 and steeply reduced at pH 10.0 (Fig. 6). The optimal pH for the *S. cerevisiae* α-glucosidase used was 6.8 (supplier's information sheet). In agreement with this, the optimum pH for baker's yeast (*S. cerevisiae*) α-glucosidase has been reported to be about pH 6.0 and 5.8–7.0, respectively [31, 32]. Thus, at pH at 7.0, the concentration of F50 used (50 μg/ml) might be too limited to overcome the strongest activities of the α-glucosidase enzyme. A higher amount of the F50 inhibitor would be needed in this case. However, the optimal pH of the F50 inhibitor may lie within the acidic range (pH 2.0–6.0) since high inhibition percentages (more than 90%) were obtained in this range. In which case, pH 7 is simply a suboptimal pH for the F50 inhibitor. Weaker inhibition activities (69.3–82.4%) were noted again as the pH increased to 8.0–9.0 before dropping off at pH 10 above the pI of fraction F50. The apparently high activity at pH 11.0 or 12.0 seen with buffer (Fig. 6), but not without (Fig. 7), should be ignored as an artifact. Although clear differential ion effects were seen (compare at pH 4, glycine acid vs. acetate; at pH 8, phosphate vs. Tris; and at pH 10, Tris vs. glycine base), this is more complicated as, in contrast to the preincubation of F50 at different temperatures, the preincubation at different pH values will also directly affect the α-glucosidase assay by changing the assay pH, since the volume and buffer capacity of the preincubation and assay mixture are equal on mixing or 1:2 after substrate addition.

To evaluate the potential role of differential ion effects from the different buffers and limit the above changes to the assay pH, the pH tolerance assay was repeated only using a non-buffer system for the F50 protein preincubation (distilled water with pH set by HCl or

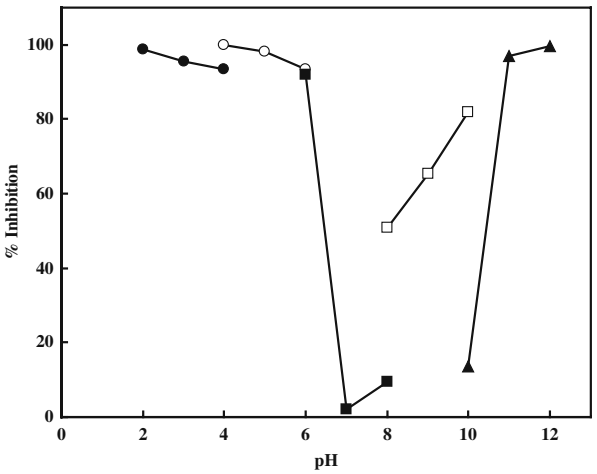

Fig. 6 Effect of preincubation of fraction F50 in different pH and buffer solutions on the subsequent α-glucosidase inhibitory activity. Pretreatment was with (*closed circle*) 20 mM glycine–HCl (pH 2-4), (*open circle*) 20 mM sodium acetate (pH 4–6), (*open square*) 20 mM potassium phosphate (pH 6–8), (*closed square*) 20 mM Tris–HCl (pH 8–10), and (*triangle*) 20 mM glycine–NaOH (pH 10–12). Data are shown as the mean±1SD and are derived from three repeats

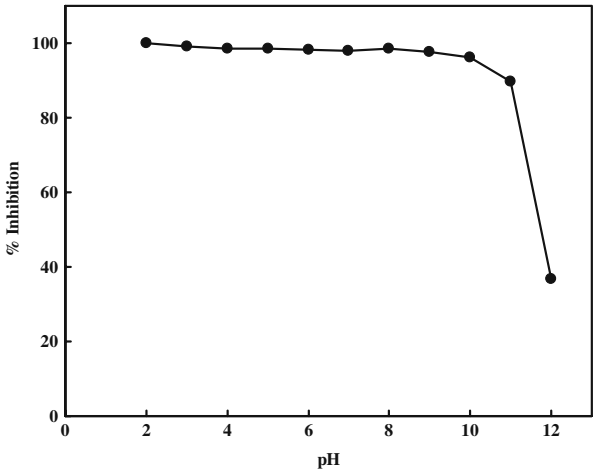

Fig. 7 Effect of preincubation of fraction F50 in different pH (buffer absence) solutions for the subsequent α-glucosidase inhibitory activity. Data are shown as the mean±1SD and are derived from three repeats

NaOH addition only). Under these conditions, no significant decrease in the observed level of α-glucosidase inhibition was seen within a pH range from 2.0–10.0 (Fig. 7). However, the activity sharply declined at pH values above 10. By comparison between Figs. 6 and 7, three points could be inferred. The first point is that there were ion effects evident with different buffers at the same pH as discussed above. The second is that pH values over 10.0 are not assayable, which is in agreement with the observation that α-glucosidase activity rapidly declined as the pH increased above 7.0 [31]. Finally, the decline in inhibition activities at pH 7.0 and 10.0 (Fig. 6) was the result from buffering ions. Thus, some inhibitor–ion interactions might block or slow down the inhibitor activity at such pH values.

The sequence analysis of a partial internal fragment of the purified protein (F50) from *Z. ottensii* rhizomes, obtained by in-gel digestion with trypsin and subsequent sequence analysis with LC/MS/MS, was performed. The products of the spectra were obtained, and peptide sequences were then determined according to a de novo sequencing algorithm to derive a short list of possible sequence candidates. These served as query sequences in a subsequent homology base search against the in-house Swiss-Prot non-redundant protein database using MS BLAST [33] to find likely candidates. As a result, the query sequence, AVANQPVSVTMDAAGR, NRNH, GCEGG, WPYR, and NWGESGYIR (Fig. 8), which was similar to those previously isolated for a cysteine proteinase from *Z. officinale* (ginger)

Accession number	Organism		Sequence	
	Zingiber ottensii (F50)		AVANQPVSVTMDAAGR NRNH GCEGG WPYR NWGESGYIR	
P82473	*Zingiber officinale* (Cysteine proteinase GP-I)	126	AVANQPVSVTMDAAGRDFQLYRNGIFTGSCNISANNYRTVGGRETGNDK-DYWIVKNSWGENWGESGYIR	194
Q5ILG7	*Zingiber officinale* (Cysteine protease gp2a)	267	AVANQPISVGIDASGRNFQLYHSGIFTGSCNTSLNHGVTVVGYGTENGN-DYWIVKNSWGENWGNSGYIL	335
Q5ILG5	*Zingiber officinale* (Cysteine protease gp3a)	267	AVANQPISVGIDASGRNFQLYHSGIFTGSCNTSLNHGVTVVGYGTENGN-DYWIVKNSWGENWGNSGYIL	335
Q40675	*Oryza sativa* (Cysteine protease precursor)	268	AVANQPVLVAVDASGSDFQFYSEGVFTGECGTDLDHGVAAVGYGTRDGTKYWIVKNSWGEDWGEKGYIR	337
P84346	*Jacaratia mexicana* (Mexicain)	214	AVANQPVSVVTDSRGRGFQFYKGGIYEGPCGTNTDHAVTAVGYGRT-----YLLLKNSWGPNWGEKGYIR	188
O22500	*Zea mays* (Cysteine proteinase Mir3)	258	AVANQPVSVAIDAAGTAFQLYSSGIFTGSCGTRLDHGVTAVGYGTENGKKDYWIVKNSWGESWGESGYVR	326
Q6F6A7	*Daucus carota* (Cysteine protease)	218	AVAMQPVSVAIDASGRAFQLYESGIFNGLCGTDLDHGVVAVGYGTENGKKDYWIVRNSWGEKWGEKGYIR	286
Q6F6A9	*Daucus carota* (Cysteine protease)	275	AVAMQPVSVAIDASGRAFQLYESGVFTGQCGTELDHGVVAVGYGSENGKKDYWIVRNSWGPEWGESGYIR	343
Q9SLY9	*Zea mays* (Cysteine protease)	258	AVANQPVSVAIDAAGTQFQLYSSGIFTGSCGTALDHGVTAVGYGTENGKKDYWIVKNSWGESWGESGYVR	326
Q6Y1E4	*Trifolium repens* (Cysteine protease 12)	251	AVANQPISVAIDASGSDFQFYKSGVFTGSCGTELDHGVTAVGYGTSNDGTKYWIVKNSWGTDWGEKGYIR	320

Fig. 8 Amino acid sequence from the tryptic fragments of the purified *Z. ottensii* rhizome fraction F50 protein. Comparisons are made with other cysteine proteinases from the cysteine proteinase family that showed the highest sequence homology in BLASTP searches of the NCBI and Swiss-Prot databases

(P82473) at amino acid residues 126 to 194, suggests that this protein could be a member of this cysteine proteinase family as well.

Conclusion

A protein was successfully purified from the rhizomes of *Z. ottensii* using a single-step moderate anion-exchange chromatography. The protein preparation was found to contain at least three components of 32.5, 15.2, and 13.8 kDa by reducing SDS-PAGE, and so, assuming purification to homogeneity, to contain these three subunits. In addition, the protein exhibited a strong α-glucosidase inhibition activity with maximum percent inhibition; IC_{50} and K_i of this protein were 77.5%, 30.15 μg/ml, and 140 μmol, while the K_m and V_{max} were 2.35 μmol and 0.11 mM/min, respectively. Further research is required to be conducted to characterize this protein in more detail, such as its inhibitory mechanism or molecular active site, interaction with other inhibitors, side effects, other target molecules, and related therapeutic possibilities. As an alternative approach, the evaluation into the potential for using this protein as a food additive for healthcare purposes could be pursued.

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