

Full Length Research Paper

An alpha-glucosidase inhibitory activity of thermostable lectin protein from *Archidendron jiringa* Nielsen seeds

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Inhibitors of α -glucosidase from natural resources that inhibit the digestion of carbohydrate polymers into monosaccharides in the gut are used in the treatment of insulin-independent diabetes mellitus type 2. *Archidendron jiringa* belongs to pea family of leguminous plants, some of which are a source of interesting bioactivities, including α -glucosidase inhibitory (GI) activity. A novel GI lectin was enriched from the seeds of the Djengkol bean, *A. jiringa*, to apparent homogeneity by 90% saturation ammonium sulfate precipitation and Con A-Sepharose affinity column chromatography. This lectin had an IC_{50} value for GI activity of 0.031 ± 0.02 mg/ml, an estimated molecular mass of 35.7 kDa, of which 15.8% was carbohydrate, was thermostable up to 80°C for 70 min, showed an optimum activity within the pH range of 8.0 to 10.0 and a high activity with some divalent cations such as copper (Cu^{2+}) and high levels (50 to 100 mM) of zinc (Zn^{2+}) and iron (Fe^{2+}). The sequence of an internal 16 amino acid fragment of the protein showed 100% identity to the mannose-glucose specific lectin precursor of *Dioclea guainensis*. The GI lectin had a high specific interaction with α -glucosidase (affinity constant = $9.3773 \times 10^{-7} s^{-1}$, $K_s = 0.0241 s^{-1}$, $K_a = 2.39 \times 10^3 s^{-1}M^{-1}$ and $K_d = 0.0117 M$).

Key words: *Archidendron jiringa*, α -glucosidase inhibitors, lectin.

INTRODUCTION

Diabetes mellitus (types 1 and 2) is recognized as a serious global health problem, often resulting in substantial morbidity and mortality. Type 2 diabetes mellitus is a group of disorders characterized by hyperglycemia and associated with microvascular (retinal, renal and possibly neuropathic), macrovascular (coronary and peripheral vascular) and neuropathic (autonomic and peripheral) complications. Unlike type 1 diabetes mellitus, the patients are not absolutely dependent upon insulin

for life, even though many of these patients ultimately are treated with insulin. The management of type 2 diabetes mellitus often demands combined regimes, including diet and/or medicines. The regulatory drugs administered in such cases include sulfonylurea and biguanide, as well as insulin. Besides the use of multiple approaches, α -glucosidase inhibitors (GIs) are one of the alternative therapeutic approaches. The inhibition of intestinal α -glucosidases delays the digestion and absorption of complex carbohydrates and consequently suppresses postprandial hyperglycemia (Puls et al., 1977). Furthermore, other benefits of GIs, such as reducing triglycerides (Lebowitz, 1998) and postprandial insulin (Johnston et al., 1994) levels and anti-human immune

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virus (HIV) activity (Bridges et al., 1994; Fischer et al., 1996a, 1996b) have been reported.

Herbal medicine, also called botanical medicine or phytomedicine, refers to the use of any plant's seeds, berries, roots, leaves, bark or flowers for medicinal purposes and has long been practiced outside of conventional medicine, in folklore (herbal) treatments. Such ancient remedies are becoming of more interest to conventional medicine as up-to-date analysis and research show their value in the treatment and prevention of disease. Indeed, plants have been used for medicinal purposes long before recorded history. Recently, the World Health Organization (WHO) estimated that 80% of people worldwide rely on herbs for the prophylactic or remedial treatment of at least some serious ailments. Increasing public dissatisfaction with the cost of prescription medications, combined with an interest in returning to natural or organic remedies, has led to an increase in the use of herbal medicines (Bruneton, 1995).

α -Glucosidase is a very important enzyme responsible for the hydrolysis of dietary disaccharides into absorbable monosaccharide in microbial system and in small intestine of animal digestive system. Glucosidases are not only essential for carbohydrate digestion but it is also very important for processing of glycoproteins and glycolipids and are also involved in a variety of metabolic disorders and other diseases such as diabetes (Jenkins et al., 1981). Inhibition of α -glucosidase activity in animal guts decreases the blood glucose levels via delaying digestion of poly- and oligo-saccharides to the absorbable monosaccharides (McCulloch et al., 1983). Thus, GI testing is useful for screening plants that could be used for blood glucose treatment. Previous studies have revealed that the GI activity of cyanidin-3-galactoside, a natural anthocyanin, can be used in combination with acarbose for the treatment of diabetes (Adisakwattana et al., 2009). Some GI active substances have been developed from bacterial sources to pharmaceutical applications, such as acarbose (glucobay[®]) from *Actinoplanes* sp. 5 (Shinoda et al., 2006), voglibose (basen[®]) from *Streptomyces hygrosopicus* var. *limoneus* (Chen et al., 2006) and miglitol (glyset[®]) from *Streptomyces roseochromogenus* (Lee et al., 2002). Thus, considerable effort has been made to search for more effective and safe GIs from natural materials to develop physiologically functional foods to treat diabetes mellitus.

The treatment goal of diabetes patients is to maintain near normal levels of glycemic control, in both the fasting and post-prandial states. Many natural resources have been investigated with respect to the suppression of glucose production from dietary carbohydrates in the gut or glucose absorption from the intestine (Matsui et al., 2007). α -Amylase catalyses the hydrolysis of α -1,4-glucosidic linkages of starch, glycogen and various oligosaccharides whilst α -glucosidase further breaks

down the disaccharides into simpler monosaccharides that are then readily available for intestinal absorption. The inhibition of the activity of these enzymes in the digestive tract of humans is considered to be an effective means to control this type of diabetes by diminishing the absorption rate of glucose through reducing the conversion rate of complex carbohydrates by these enzymes (Hara and Honda, 1990). Therefore, effective and nontoxic inhibitors of α -amylase and α -glucosidase have long been sought. In addition, GIs have wide applications for the treatment of other carbohydrate mediated diseases in addition to diabetes (Fujisawa et al., 1991), such as cancer (Humphries et al., 1986; Pili et al., 1995), Alzheimer disease (Kivipelto et al., 2005) and certain forms of hyperlipoproteinemia and obesity (Mahley et al., 1999). Since α -glucosidase is required for the breakdown of carbohydrates to absorbable monosaccharides at the intestine, then GIs are usually used to prevent or medically treat type 2 diabetes (non-insulin-dependent diabetes mellitus (NIDDM)). These inhibitors combine with the intestine alpha-glucosidase and block the uptake of postprandial blood glucose (Holman, 1998).

Archidendron jiringa Nielsen (Fabaceae: Mimosoideae), known as the Jenkol bean or Luk Nieng tree, is a leguminous tree that is found in Indonesia, Malaysia and Thailand, and is economically important with diverse uses, including as a vegetable (young shoots) and pulse or food flavoring agent (seeds), medicine (leaves), source of dye for silk (pods) and timber for craft work and firewood (Ong and Norzalina, 1999). Given the abundance of this commercial species, and especially the abundant seed production (1,000-4,000 seeds per tree per year), then the aim of this research was to study the GI activity of *A. jiringa* seeds in relation to their proteinaceous content.

MATERIALS AND METHODS

Biological material

The fresh seeds of *Archidendron jiringa* were purchased from the local market in Bangkok, Thailand. Thus, the exact cultivar, geographical location and season of cultivation are not known and so the effect of such variations within the species in enzyme isoforms or levels is not addressed here. Plants were identified and voucher specimens (BKF130216) deposited at Botany Section Bangkok Herbarium. The human blood was obtained from the blood donation office of The Thai Red Cross Society, Bangkok, Thailand. All other non-human animal blood was supplied from the Division of Production and Supply, National Laboratory Animal Center, Mahidol University, Nakhon Pathom, Thailand.

Chemicals and reagents

Concanavalin A Sepharose (ConA Sepharose) was purchased from Sigma Chemicals Co. (USA). Methyl- α -D-glucopyranoside was

purchased from Fluka (Germany). The reagents used in SDS-PAGE were obtained from Plusone Pharmacia Biotech (Sweden), except the low molecular weight calibration kit, used as standard molecular weight marker proteins, which was purchased from Amersham Pharmacia Biotech (UK). All other biochemicals and chemicals used in the investigation were of analytical grade.

Extraction of GI from seeds of *A. jiringa*

One kilogram of *A. jiringa* seeds was homogenized in and defatted in acetone at 4°C (200 ml aqueous acetone per 10 g seed). The insoluble material was then removed by vacuum filtration and extracted overnight at 4°C with 20 volumes of TBS (20 mM Tris-HCl buffer, pH 7.2, plus 150 mM NaCl). The suspension was then clarified by filtration through double-layered cheesecloth followed by centrifugation at 15,000 × g for 30 min. The clarified supernatant was harvested and ammonium sulfate added, with stirring, to 90% saturation and left with stirring overnight at 4°C. The precipitate was harvested by centrifugation at 15,000 × g for 30 min, discarding of the supernatant, and dissolved in TBS prior to being dialyzed against 3 changes of 5 L of water and then freeze-dried.

GI activity

The assay method was modified from that reported previously (Boonmee et al., 2007). GI activity was evaluated at every step of the enrichment procedure. Twenty microliter (20 µl) of α-glucosidase (1 U/ml) in TB (20 mM Tris-HCl buffer, pH 7.2) was mixed with 10 µl of the test protein sample and 60 µl of TB and then incubated at 37°C for 10 min before 10 µl of 1 mM *p*-nitrophenyl-α-D-glucopyranoside (PNPG) in TBS as substrate was added. After incubation at 37°C for 35 min, the reaction was stopped by the addition of 100 µl of 0.5 M Na₂CO₃. The GI activity was determined by measuring the release of the yellow *p*-nitrophenol at 400 nm, and calculated as follows:

$$\% \text{ Inhibition} = \frac{(\text{OD}_{\text{control}} - \text{OD}_{\text{blank}}) - (\text{OD}_{\text{sample}} - \text{OD}_{\text{blank}}) \times 100}{(\text{OD}_{\text{control}} - \text{OD}_{\text{blank}})}$$

Purification of GI from seeds of *A. jiringa*

Con A-Sepharose was pre-equilibrated with TBS and transferred to a 1.6 × 20 cm column. The ammonium sulphate cut fraction, resolvated in TBS, was then applied to the column (10 ml at a total protein concentration of 2 mg/ml) and run in at a flow rate of 1.5 ml/min. The column was then washed with TBS at the same flow rate, collecting 10 ml fractions, until the A₂₈₀ fell to <0.05. The bound proteins, including lectins, were then eluted from the column, using TBS supplemented with 0.2 M methyl-α-D-glucopyranoside as the competitor, at the same flow rate and collecting 10 mL fractions. Fractions were assayed for α-glucosidase inhibitory activity (assay for α-glucosidase inhibition activity section), and those found to contain α-glucosidase inhibitory activity were pooled, dialyzed (3.5 kDa cut-off tubing) against TB and concentrated by freeze dry to 50 mg/ml ready for further analysis.

Protein concentration

The protein content was determined by Bradford's procedure (Bradford, 1976). Bovine serum albumin (BSA) was used as the standard with four different concentrations between 5 to 20 µg/ml to construct the calibration curve. Each sample was serially two-fold

diluted with deionized water and 50 µl aliquots of each dilution were transferred into each well of a microtiter plate to which 50 µl of Bradford's reagent was added per well. The plate was shaken for 5 min and then left for 10 min before reading the absorbance at 595 nm using an enzyme linked immunosorbent assay (ELISA) plate reader. The obtained OD was calculated for the protein concentration using the linear equation computed from the BSA standard curve. During the column chromatographic separations, the elution peak profiles of proteins were determined by measuring the absorbance at 280 nm.

Carbohydrate determination

The phenol-sulfuric acid technique was slightly modified from the reported procedure (Dubois et al., 1956), by scaling up and using glucose as the standard. The enriched GI fraction was serially diluted and 500 µl aliquots of each dilution was transferred into 15 ml glass tubes, to which 500 µl of a 4% (w/v) phenol solution was added, thoroughly mixed and then left at room temperature for 5 min. Next, 4 ml of conc. H₂SO₄ was added into each tube, carefully mixed using a vortex mixer and 100 µl aliquots transferred into the well of a microtitre plate and the absorbance read at 492 nm. The obtained data was used to calculate the sugar content as glucose equivalents using the standard curve developed from five different concentrations of glucose (range 10 to 50 µg/ml) analyzed by the same procedure. Glucose (50 µg/ml) in deionized water and deionized water alone were used as the positive and negative controls, respectively, in the assay.

IC₅₀ determination

The half maximal inhibition concentration (IC₅₀), as the concentration of the protein sample that inhibited 50% of the maximal α-glucosidase enzyme activity was evaluated by using two-fold dilutions of each test protein sample and then proceeding as described aforementioned.

Hemagglutination assays

Serial two-fold dilutions of the purified lectin in TBS (50 µl) were incubated with 50 µl of rabbit erythrocyte suspension in 96-well U-shaped microtiter plates and the agglutination was scored after 1 h at room temperature. The hemagglutination unit (HU) was expressed as the reciprocal of the highest lectin dilution showing detectable visible erythrocyte agglutination and the specific activity was calculated as HU/mg protein. The hemagglutination activity was assayed separately, in the same manner as aforementioned, against erythrocytes from rabbits, rats, mice, guinea pigs, geese, sheep and the four human ABO blood groups.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Gels for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) were prepared with 15% and 5% (w/v) acrylamide separating and stacking gels, respectively. Tris-glycine buffer pH 8.3 containing 0.1% (w/v) SDS was used as the electrode buffer. Discontinuous SDS-PAGE in reducing conditions was performed according to the procedure of Laemmli (1970). Samples to be analyzed were treated with reducing sample buffer and boiled for five min prior to application to the gel. Electrophoresis was performed at a constant current of 20 mA per slab at room-temperature in a Mini-Gel Electrophoresis unit. Molecular weight

standards (10 mg/lane) were co-resolved in adjacent lanes and used to determine the subunit molecular weight of the purified protein(s). After electrophoresis, proteins in the gel were visualized by staining with Coomassie Brilliant blue R-250.

Effect of temperature on the GI activity and thermostable of the enriched GI fraction

The effect of temperature on the GI activity was determined by incubating the enriched GI protein fraction in TB at various temperatures (-20 to 90°C at 10°C intervals) for 30 min. The thermostable of the GI activity was investigated by incubating the enriched GI protein fraction sample at 70, 80 and 90°C in TB for the indicated fixed time intervals (10 to 120 min), cooling to 4°C and then assaying the residual GI activity with 100% and 0% activity controls, as described.

The pH-dependence of the GI activity of the enriched GI fraction

Incubating the enriched GI protein fraction samples in buffers of broadly similar salinity levels, but varying in pH from 2 to 14 was used to assess the pH stability and the pH optima of the GI. The buffers used were (all 20 mM) glycine-HCl (pH 2 to 4), sodium acetate (pH 4 to 6), potassium phosphate (pH 6 to 8), Tris-HCl (pH 8 to 10) and glycine-NaOH (pH 10 to 12). The enriched GI protein fraction was mixed in each of the different buffer-pH compositions, or TB as the control, and then left for 1 h at room temperature. Next, the samples were adjusted back to TB and assayed for GI activity. The activities attained were expressed as relative to that of the control, which was set as 100% activity.

Effect of divalent metal ions on the GI activity

The effect of preincubation of the enriched GI protein fraction with six different divalent metal cations on the resultant GI activity was evaluated as follows. The enriched GI protein fraction (1 mg/ml) was incubated for 10 h with one of Ca^{2+} , Co^{2+} , Fe^{2+} , Mg^{2+} , Mn^{2+} and Zn^{2+} at one of the five different concentrations (5 to 100 mM), plus the control (0 mM divalent cation) in TB with continuous shaking and was then tested for GI activity as described using at least three replicates for each assay. The activities attained were expressed as relative to that of the control, which was set as 100% activity.

Mechanism of inhibition

To evaluate the inhibition mode of the enriched GI protein against α -glucosidase, the PNPG solution at one of 0.025 to 0.2 mM, as the substrate was added to the α -glucosidase (1 U/ml) in TB in the presence of 0, 0.05 and 0.075 mg/ml of the enriched GI protein fraction sample. The remaining α -glucosidase activity, and thus the GI activity, was determined as outlined aforementioned. The inhibition type was determined by Lineweaver-Burk plots, where v is the initial velocity and S is the substrate concentration used.

Internal amino acid sequence of GI by liquid chromatography/mass spectrometry/mass spectrometry (LC/MS/MS)

Each band in the electrophoretic gel was excised, cut into small

pieces (ca. 1 mm³) and washed with 100 μ l deionized water. The sample preparation process then followed the published method of Mortz et al. (1994), with the trypsinization using 100 ng of proteomics grade trypsin (Sigma) in 40 μ l of 50 mM NH_4HCO_3 at 37°C overnight. The supernatant was then harvested following centrifugation at 15,000 \times g for 1 min. The remaining peptides in the gel were extracted with a solution of 50% (v/v) acetonitrile containing 5% (v/v) formic acid for 10 min with shaking and subsequently pooled with the supernatant and taken to dryness. The likely amino acid sequence of each internal fragment of the trypsinized peptide was then analyzed by LC/MS/MS mass spectrometry. The extracted tryptic peptides were then subjected to LC-nano ESI/MS/MS. All collected LC/MS/MS data were processed and submitted to a MASCOT (<http://www.matrixscience.com>) search of the NCBI database (<http://blast.ncbi.nlm.nih.gov>). The following criteria were used in the Mascot search: (i) trypsin cleavage specificity with up to three missed cleavage sites, (ii) cysteine carbamidomethyl fixed modification, (iii) methionine oxidation variable modifications, (iv) \pm 0.2 Da peptide tolerance and MS/MS tolerance and (v) ESI-TRAP fragmentation scoring (Mortz et al., 1994).

Biospecific interaction determination by surface plasmon resonance (SPR)

Biospecific interactions, between the enriched GI protein from the seeds of *A. jiringa* and α -glucosidase enzyme were evaluated on an Autolab ESPRIT system. This is a biosensor system based on the principle of SPR. The various concentrations of protein sample in TB were coupled to a certified grade 11 MUA gold plate. The unreacted groups, on the surface of the gold, were then blocked with ethanolamine. All measurements were performed in the presence of 1 U/ml α -glucosidase enzyme.

RESULTS AND DISCUSSION

Enrichment of the GI activity in the seeds of *A. jiringa*

The present report represents the first investigation of the purification of a GI from *A. jiringa* seeds. *A. jiringa* seeds were homogenated and defatted to form a crude soluble extract and, after extraction in TBS followed by 90% saturation ammonium sulphate precipitation and dialysis, the GI was purified in a single step by Con A-Sepharose affinity column chromatography, yielding a single apparent GI at -3.3% (w/w) of the total starting seed weight. The Con A-Sepharose affinity column chromatography of the ammonium sulfate cut fraction resulted in two fractions, an unbound fraction that eluted with the TBS wash through and did not show any detectable GI activity, and a bound fraction that eluted with the presence of 0.2 M methyl- α -D-glucopyranoside that had a strong GI activity with an IC_{50} of 0.031 ± 0.02 mg/ml (Figure 1). Note that although the use of Con A-Sepharose affinity column chromatography assumes the GI component will be a lectin, in this case non-lectin GI components were excluded since the unbound fraction after affinity chromatography did not show any detectable GI activity.

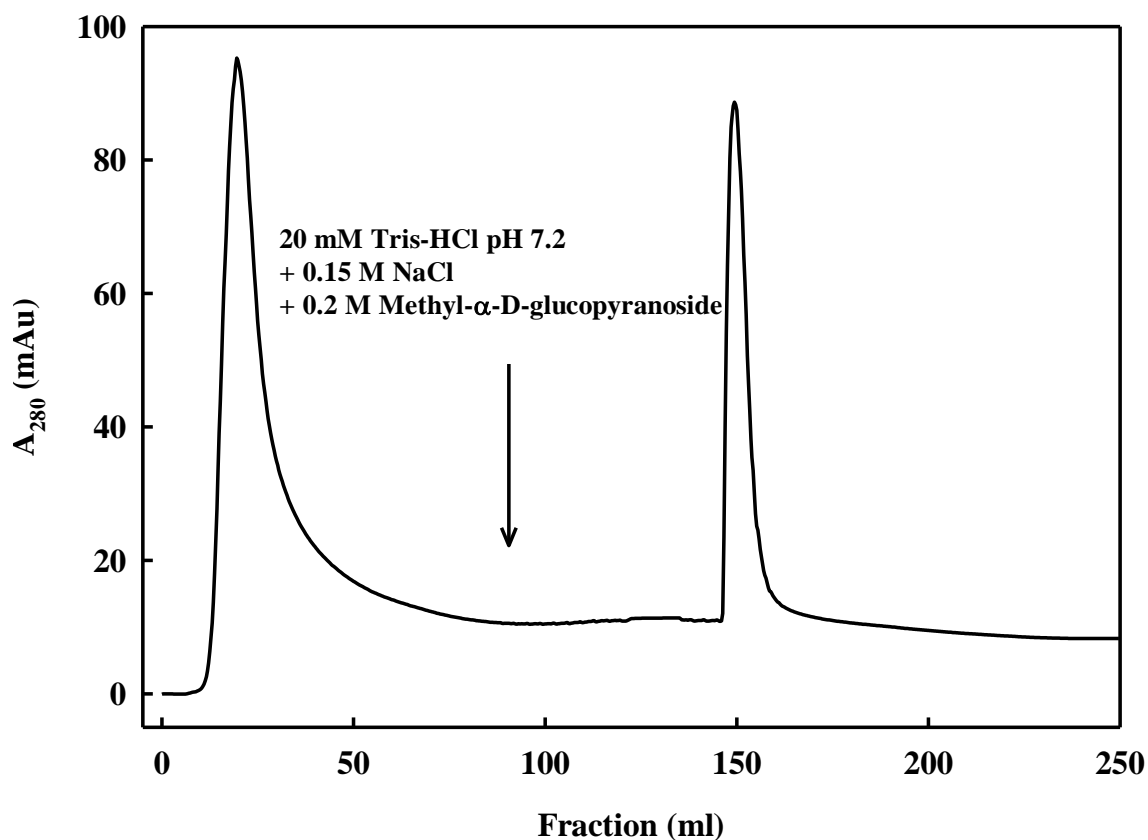


Figure 1. Affinity chromatogram showing the enrichment of GI active lectins from the ammonium sulphate cut fraction of proteins from *A. jiringa* seeds on a ConA-Sepharose column (1.6 × 20 cm) equilibrated and then washed (0-120 ml) with TBS. Lectin was then eluted with TBS containing 0.2 M methyl- α -D-glucopyranoside (120-250 ml) at a flow rate of 1.5 ml/min. The chromatogram shown is representative of three such repeats.

Lectin GIs are known from kidney beans (*Phaseolus vulgaris*), with GI activity levels of 70.6 and 77.1% for the cooked and raw beans, respectively (Shi et al., 2007). This suggests a degree of thermostable, at least in the environment of the intact bean. Moreover, protein extracts of *Sesbania grandiflora* flowers prepared from 60% and 90% saturation ammonium sulphate precipitation revealed GI activity levels of 49.6% and 82.1%, respectively (Boonmee et al., 2007). Affinity chromatography presents advantages in relation to other conventional methods due to its specificity and consequentially the reduced number of enrichment steps, typically giving a higher product yield and purity (Goldenberg, 1989). As such, it is widely used in the purification of glycoproteins. For example, the mannose-glucose specific lectins from the tepary bean (*Phaseolus acutifolius*) and mulberry, *Morus* sp. (Rosales: Moraceae) seeds were purified by Con A-Sepharose based affinity column chromatography (Absar et al., 2005). However, in some contrast, there have been reports that affinity chromatography could not be applied successfully for the

purification of some lectins. For instance, the isolation of the lectin from *Dolichose biflorus* with *N*-acetylgalactosamine (NAG) immobilized to Sepharose was not successful, due to the substitution of the binding site at the C-6 hydroxyl group of carbohydrate in the matrix. Rather, these lectins were resolved by affinity electrophoresis, a combination of affinity and conventional chromatography (Borrebaeck and Etzler, 1980).

Modern isolation procedures generally employ affinity chromatography on an insoluble carbohydrate derivative (Goldstein and Hayes, 1978). Such insoluble, naturally occurring, or chemically modified substances, such as insoluble hog gestic musin (Etzier and Kabat, 1970), chitin (Shankar et al., 1976), arabinogalactan (Majumdar and Surolia, 1978), sephadex (Wang et al., 1974) and agarose or sepharose, have also been employed as affinity matrices for purification of interesting lectins. For example, the mannose-glucose specific lectins from the seeds of the tepary bean (*Phaseolus acutifolius*) and mulberry, *Morus* sp. (Rosales: Moraceae), seeds were purified by a Con A Sepharose based affinity

Table 1. The effect of divalent metal cations on the GI activity of the enriched GI lectin from *A. jiringa* seeds^a.

| Concentration (mM) | Metal salt | | | | | |
|--------------------|------------------|------------------|------------------|------------------|------------------|------------------|
| | Mg ²⁺ | Mn ²⁺ | Fe ²⁺ | Zn ²⁺ | Ca ²⁺ | Cu ²⁺ |
| 5 | 47.8 ± 0.028 | 45.0 ± 0.020 | 6.8 ± 0.013 | 49.9 ± 0.053 | No inhibition | 99.0 ± 0.015 |
| 10 | 42.4 ± 0.005 | 51.4 ± 0.701 | 21.8 ± 0.002 | 55.5 ± 0.115 | No inhibition | 98.8 ± 0.043 |
| 25 | 55.5 ± 0.750 | 49.1 ± 0.051 | 48.1 ± 0.007 | 67.6 ± 0.034 | No inhibition | 98.4 ± 0.025 |
| 50 | 41.5 ± 0.040 | 44.6 ± 0.045 | 116.3 ± 0.146 | 73.1 ± 0.004 | 52.0±0.005 | 94.8 ± 0.003 |
| 100 | 45.1 ± 0.065 | 56.4 ± 0.042 | 137.0 ± 0.127 | 74.4 ± 0.102 | 55.0±0.044 | 56.0 ± 0.112 |

^aData are shown as the mean ± 1 SD and are derived from 3 repeats. Means followed by a different superscript lower case letter are significantly different ($p < 0.05$; Kruskal Wallis tests).

chromatography column (Richard et al., 1990; Absar et al., 2005). Another example is the lectin from ground elder (*Aegopodium podagraria*) rhizomes which also could not be purified by Gal-NAG-Sepharose, but by an affinity chromatography of erythrocyte membrane protein immobilized on cross-linked agarose (Peumans et al., 1985).

Assay for hemagglutinating activity

A. jiringa GI showed no specificity in its ability to hemagglutinate erythrocytes from either different human blood groups (A, B, AB and O) or from rabbits, rats, mice, guinea pigs, geese and sheep. However, hemagglutinating activity against mouse and guinea pig erythrocytes were numerically the lowest, whilst that for human group O and sheep were the highest (Table 1). In this respect, it is similar to previous studies of lectin from Egyptian *Pisum sativum* seeds (Sitohy et al., 2007) and *Bauhinia monandra* (Coelho and Silva, 2000). Several lectins demonstrate a preference in agglutinating one or more types of human, or certain animal erythrocytes, such as *Sphenostyles stenocarpa* lectin that demonstrated a high agglutination of human blood type O (Machuka et al., 1999), *Hevea brasiliensis* lectin that preferentially agglutinated rabbit erythrocytes (Wititsuwannakul et al., 1998) and *Talisia esculenta* lectin that demonstrated a preference for human blood type AB (Freire et al., 2002).

The classical, and still the simplest way, to detect the presence of a lectin in biological material are to prepare an extract from the desired material and examine its ability to agglutinate erythrocytes. For agglutination to occur, the lectin must bind to the surface of the erythrocytes and form a cross-bridge between them. There is, however, no simple relationship between the amount of lectin bound and agglutination. This is because agglutination is affected by many factors, amongst them being the accessibility of receptor sites, and is also influenced by the external conditions of temperature, cell concentration and mixing. This difference in the

agglutination activity may be due to the nature of the glycoproteins protruding on the cell surface, which are weakly or not totally recognized by the lectin.

Molecular weight determination

The potential purity of the enriched GI lectin *A. jiringa* seeds α -glucosidase inhibitor proteins extract was evaluated at each step of the purification using SDS-PAGE under reducing conditions (Figure 2). The ammonium sulfate cut fraction showed many protein bands of a medium molecular weight from -14 to 45 kDa, including a band at about 35.7 kDa. After the Con A-Sepharose affinity chromatography purification step this 35.7 kDa band was absent in the unbound fraction, but in the bound fraction a significant increase in the intensity of the 35.7 kDa band (estimated size) was seen, and only this band, suggesting a high degree of likely purity. The 35.7 kDa band, as in this fraction, was subsequently found to contain 15.84% sugar by Dubois's method which is high compared to other reported GI lectins, such as those from the Chinese evergreen chinkapin lectin, *Castanopsis chinensis*, at 5.8% (Wong et al., 2008) or *Arundo donex* at 2.1% (Kaur et al., 2005). It remains plausible that during the enrichment procedures prior to and during Con A-Sepharose chromatography, residual endoglycanase activity, in conjunction with the preferential binding of the natural glycoprotein isoforms to the Con A resin, would select for purified glycoprotein of lower carbohydrate content than the real level. Conversely, we may have enriched for high carbohydrate content isoforms by the use of the Con A-Sepharose affinity chromatography.

Effect of temperature on the GI activity and thermostable of the enriched GI lectin

No significant changes in the inhibition activity of the enriched GI fraction was seen when pretreated for 30 min

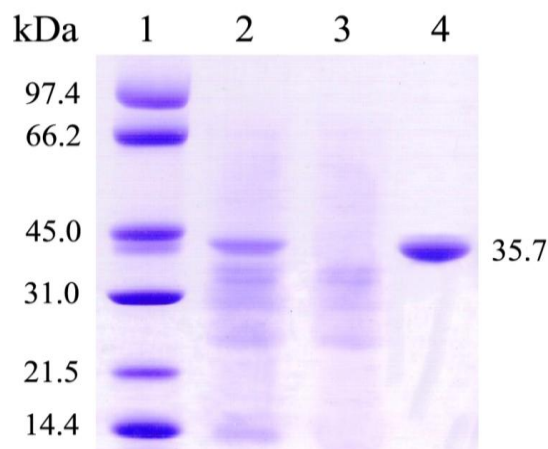


Figure 2. Reducing SDS-PAGE analysis of the enrichment of GI-active lectin from the seeds of *A. jiringa*. Lane 1, molecular weight standards (10 μ g); lane 2, 90% saturation ammonium sulphate cut fraction (10 μ g); lane 3, the non-bound ConA-Sepharose fraction (10 μ g); lane 4, the eluted ConA-Sepharose-bound fraction (10 μ g) as the enriched GI lectin fraction. Gels shown are representative of 3 independent enrichments.

within the temperature range of -20, -80°C, but at 90°C the observed GI activity was essentially abrogated (Figure 3A). This is a very broad temperature range for GI activity. Previously, the mannose/glucose-specific lectin from *C. chinensis* (CCL) was reported to be stable up to 60°C for 30 min, but above this temperature the activity declined (Wong et al., 2008). In accordance with the observed high levels of GI activity at up to 80°C, the thermal stability of this enriched GI lectin at 70, 80 and 90°C for up to 120 min was evaluated (Figure 3B). At 70°C the GI activity was stable for 20 min and then declined gradually to -30% after 120 min. At 80°C, an initial faster rate of loss of GI activity with time was seen in the first 10 min, but then it remained stable at -50% activity until 70 min before the declining to 30% activity after 120 min, the same level as at 70°C. However, in some contrast, the level of GI activity at 90°C declined rapidly to less than 20% at 20 min and was abrogated by about 100 min (Figure 3B). The thermal stability observed for this *A. jiringa* protein is comparable to that already reported for some other thermostable proteins treated under similar conditions (Konozy et al., 2003; Oliveira et al., 2002), but the only thermophilic lectin previously isolated to date is from *Momordica charantia*, which has a maximal activity at 55°C (Toyama et al., 2008).

Effect of pH on the GI activity

The enriched GI lectin fraction displays a high GI activity

at the relatively narrow alkaline pH range of 8 to 10, which would include the environment of the small intestine, with essentially no activity at pH 2 to 7, which include the stomach environment and pH 11 to 12 (Figure 4A). The stability of the GI activity of the enriched GI lectin preparation at pH 8 to 10 was then evaluated over a 120 min preincubation period, where no significant difference in the broad level of GI activity with time was noted over the 120 min at pH 8 and 9, and with only a slight decline at pH 10 after 100 min (Figure 4B). Thus, this lectin GI inhibitor has high stability at pH 8 to 10. The high thermostable temperature range, up to 80°C for 70 min, and pH optimum and stability or GI activity within the pH range of 8.0 to 10.0 (which is compatible with the site of gut alpha-glucosidase) suggests its good potential for therapeutic use in foods as well as a supplement oral pill.

Effect of divalent metal ions on the GI activity of the enriched GI lectin

The effect of divalent cations on the GI activity of the enriched lectin fraction from *A. jiringa* seeds was evaluated with six different divalent metal ions. Cu^{2+} at 5 to 50 mM, but not at 100 mM, and Fe^{2+} at 50 and 100 mM, but not below this, were found to support and stimulate, respectively, the GI activity. Zn^{2+} offered weak support for the GI activity, increasing slightly as its concentration increased over the evaluated range (5 to 100 mM), whereas Mn^{2+} and Mg^{2+} offered at best only weak support for the GI activity at all concentrations tested and Ca^{2+} was unable to support any, or was inhibitory to, GI activity at 5 to 25 mM and weak at 50 and 100 mM (Table 2). That some divalent cations appear to be essential for the GI activity of this protein, with good and weak support from Cu^{2+} and Zn^{2+} , respectively and Fe^{2+} being stimulatory at higher concentrations (50 and 100 mM), whilst others maybe inhibitory could be important in terms of potential therapeutic use of this lectin as a food additive, as well as in formulating oral administration pills.

Mechanism of inhibition

The catalytic kinetic studies for α -glucosidase activity, with different substrate and enriched GI lectin fraction concentrations were initially analyzed using Lineweaver-Burk plots (Figure 5) and then Eadie-Hofstee plots (data not shown). Both the maximal velocity (V_{max} , y-intercept) and the Michaelis-Menten constant (K_m , slope of the trend lines) decreased with increasing concentrations of the enriched GI lectin fraction, and so this GI acted as a non-competitive inhibitor of α -glucosidase. Non-competitive inhibitors do not compete with the substrate to bind to the active region of the free enzyme, but bind to

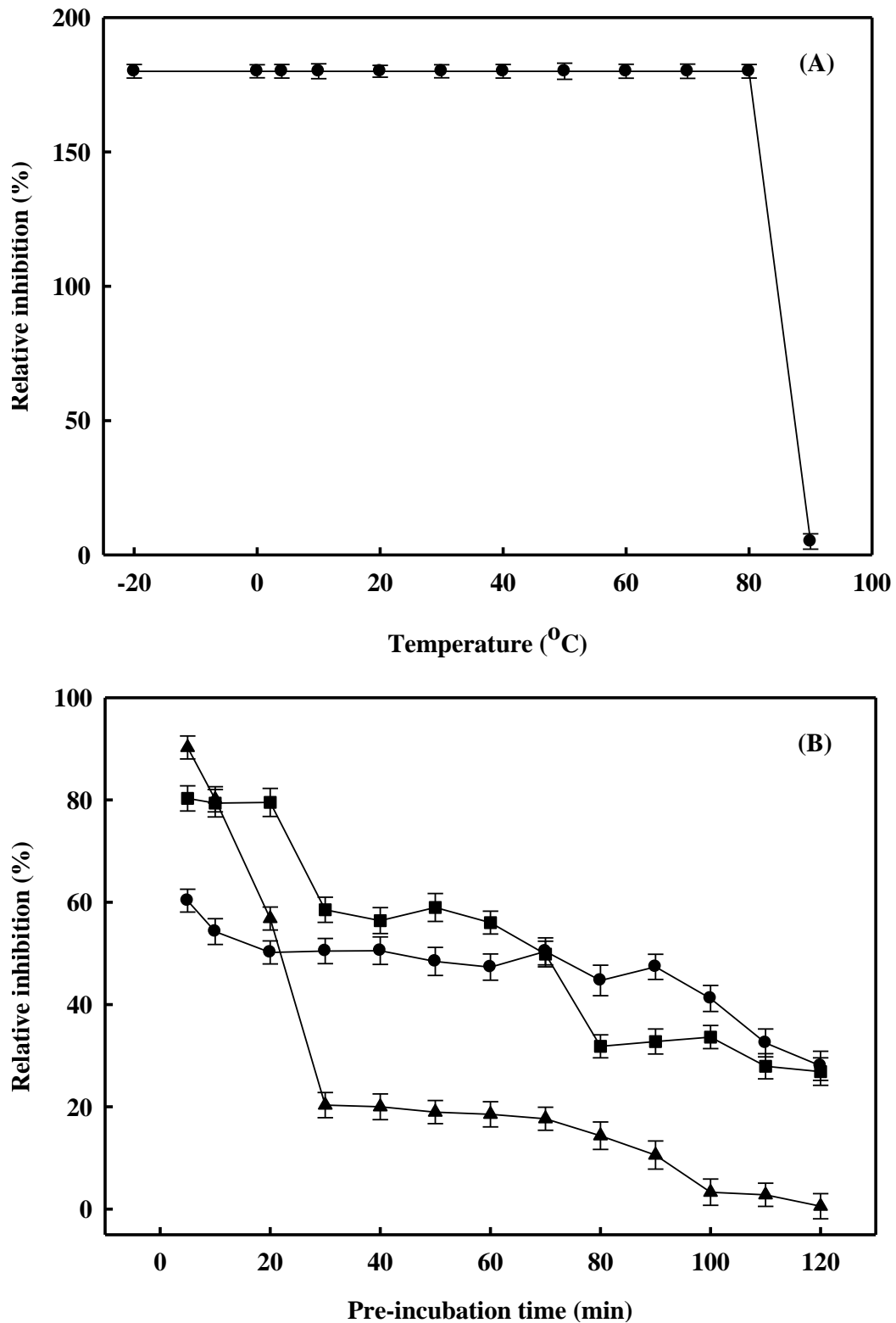


Figure 3. (A) Effect of the pretreatment temperature on the GI activity of the enriched GI lectin fraction towards α -glucosidase. (B) Thermostable with increasing pretreatment time of the enriched GI lectin fraction at (●) 70°C, (■) 80°C and (▲) 90°C on the subsequent GI activity against α -glucosidase. For both panels (A) and (B), the data are shown as the mean \pm 1 SD and are derived from three repeats. Means with a different lower case letter are significantly different ($p < 0.05$; Kruskal Wallis tests).

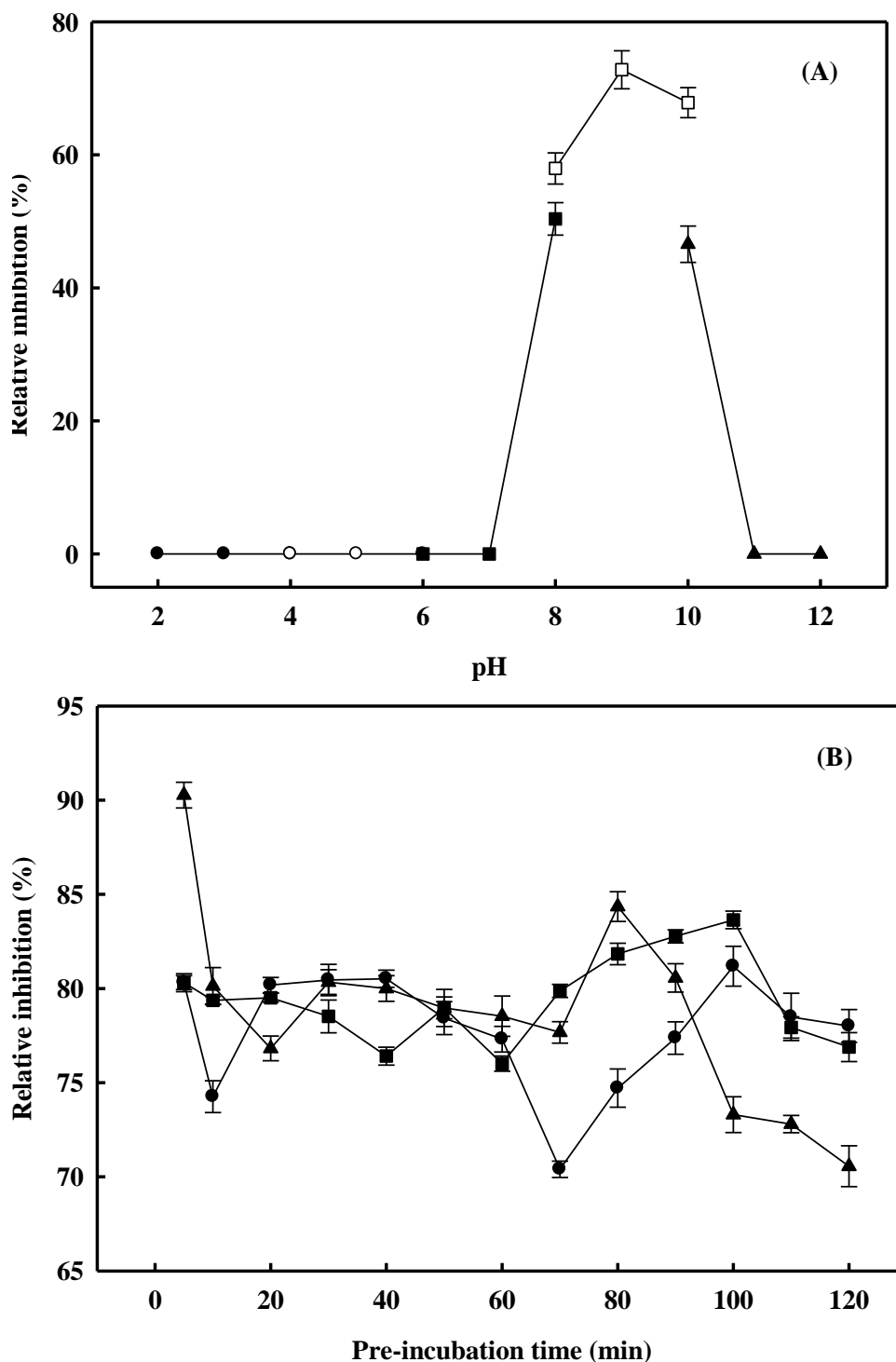


Figure 4. The effect of pH pretreatment on the GI activity of the enriched GI lectin fraction against α -glucosidase. The following buffer systems (all 20 mM) were used: (●) glycine-HCl (pH 2.0-4.0), (○) sodium acetate (pH 4.0-6.0), (■) potassium phosphate (pH 6.0-8.0), (□) Tris-HCl (pH 8.0-10.0) and (▲) glycine-NaOH (pH 10.0-12.0). (B) The pH stability with increasing pretreatment time of the enriched GI lectin fraction at pH (●) 8.0, (■) 9.0 and (▲) 10.0 on the subsequent GI activity against α -glucosidase. For both panels (A) and (B) the data are shown as the mean \pm 1 SD and are derived from three repeats. Means with a different lower case letter are significantly different ($p < 0.05$; Kruskal Wallis tests).

Table 2. Hemagglutinating activity of *A. jiringa* seed GI against human and animal erythrocytes.

| Erythrocyte source | Agglutination (titer) ^a |
|--------------------|------------------------------------|
| Mouse | 2 ³ |
| Rat | 2 ⁴ |
| Guinea pig | 2 ³ |
| Goose | 2 ⁴ |
| Sheep | 2 ⁵ |
| Rabbit | 2 ⁴ |
| Human Type A | 2 ⁴ |
| Human Type B | 2 ⁴ |
| Human Type O | 2 ⁵ |
| Human Type AB | 2 ⁴ |

^aTiter is defined as the reciprocal of the end point dilution causing detectable agglutination of erythrocytes. The initial amount of *A. jiringa* GI used in these assays was 100 µg and was serially diluted 1:1 (v/v) for all subsequent dilutions. Data shown are the mean ± 1 S.D and are derived from 3 repeats.

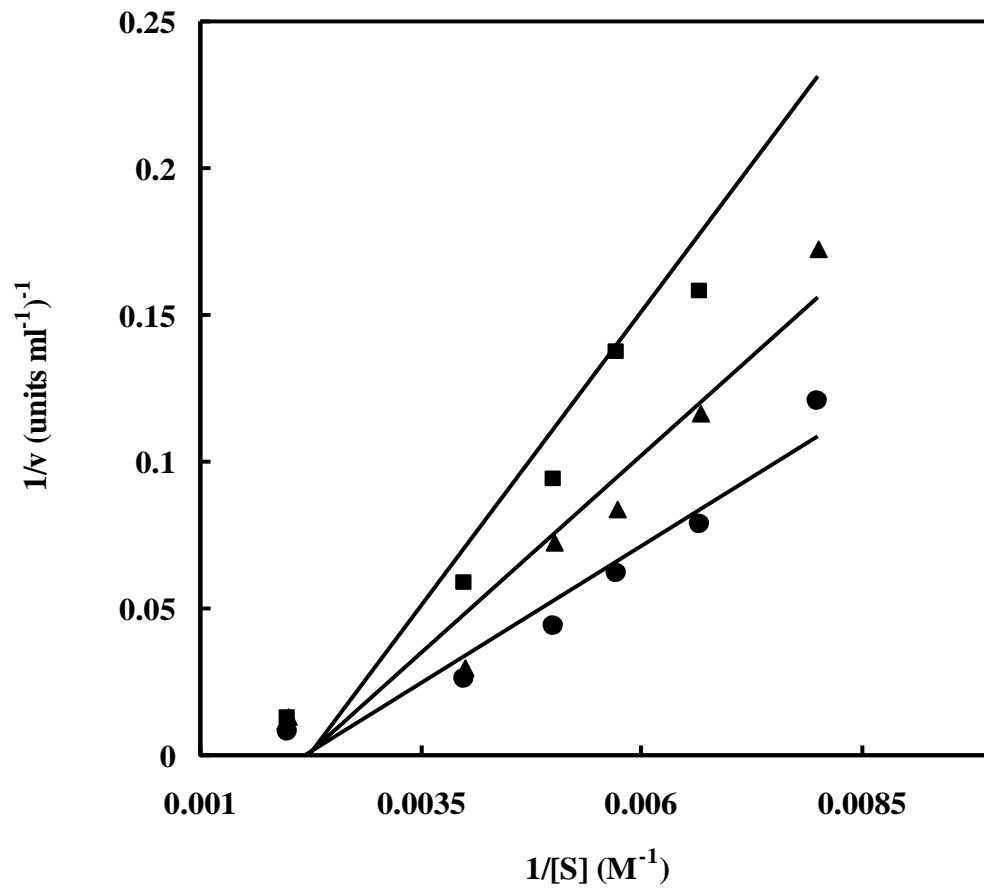


Figure 5. Lineweaver-Burk plots derived from the inhibition of α -glucosidase by the enriched GI lectin fraction from *A. jiringa* seeds. α -glucosidase was treated with each indicated concentration of PNP solution (one of 0.025-0.2 mM) in presence of the enriched GI lectin fraction at (●) 0, (■) 0.05 and (▲) 0.075 mg protein/ml. Data are shown as the mean ± 1 SD, derived from three repeats.

(A)

| | 1 | 5 | 10 | 15 | Accession Number | | | | | | | | | | | | | | |
|---|-----|---|----|----|------------------|---|---|---|---|---|---|---|---|---|---|---|---|-----|--------|
| <i>Archidendron jiringa</i> lectin | R | V | S | S | D | G | S | P | Q | G | S | S | V | G | R | A | | | |
| Lectin precursor (<i>Dioclea guianensis</i>) | 68 | R | V | S | S | D | G | S | P | Q | G | S | S | V | G | R | A | 83 | A9J248 |
| Concanavalin-A precursor (Con A) (<i>Canavalia gladiata</i> , Sword bean) | 69 | R | V | S | S | N | G | S | P | Q | G | S | S | V | G | R | A | 84 | P14894 |
| Concanavalin-A precursor (Con A) (<i>Canavalia ensiformis</i> , Jack bean) | 69 | R | V | S | S | N | G | S | P | Q | G | S | S | V | G | R | A | 84 | P02866 |
| Lectin alpha chain (<i>Dioclea guianensis</i>) | 159 | - | V | S | S | S | G | D | P | Q | G | S | S | V | G | R | A | 173 | P81637 |
| Lectin alpha chain (<i>Dioclea rostrata</i>) | 159 | - | V | S | S | S | G | D | P | Q | G | N | S | V | G | R | A | 173 | P58908 |
| Lectin alpha chain (<i>Cratylia floribunda</i>) | 158 | R | V | S | - | N | G | S | P | Q | S | N | S | V | G | R | A | 172 | P81517 |
| Mannose/glucose-specific lectin (<i>Cratylia mollis</i> , Camaratu bean) | 156 | R | V | S | - | N | G | S | P | Q | S | N | S | V | G | R | A | 170 | P83721 |
| Mannose-specific lectin (<i>Chimonanthus praecox</i>) | 101 | L | Y | S | S | Q | G | S | A | I | W | S | S | K | T | W | Q | 117 | A2SVT1 |

Figure 6. (A) Amino acid sequence of a 16-residue internal tryptic peptide of the enriched GI lectin from *A. jiringa* seeds. Comparisons are made with some of the other lectins from the mannose-glucose specific lectin family that showed the highest sequence homology in BLASTp searches of the NCBI and SwissProt databases. Shaded regions represent regions of identity. **(B)** LC/MS/MS spectra of the tryptic digest of the enriched GI lectin used to derive the data in (A) above.

enzyme-substrate complex, resulting in an enzyme-substrate inhibitor complex. For this reason, inhibition cannot be overcome by increasing the concentration of substrate. When the concentration of the GI lectin fraction was plotted against $1/V_{max}$ (observed), a K_i value for the GI lectin of 1.887 $\mu\text{g/mL}$ was obtained via non-linear regression using the least squares difference method.

The GI activity of different compounds is described in the literature (Kim et al., 2005; Shim et al., 2003; Tadera et al., 2006). α -Glucosidase was effectively inhibited by naringenin, kaempferol, luteolin, apigenin, (+)-catechin/-epicatechin, diadzein and epigallocatechin gallate (Tadera et al., 2006). These flavonoids exhibited a mixed and close to non-competitive type of inhibition on the yeast α -glucosidase. However, a combination of

non-competitive and uncompetitive inhibition was observed in the study of α -glucosidase inhibition of pine bark extract against yeast *S. cerevisiae* α -glucosidase (Kim et al., 2005), whilst non-competitive inhibition of α -glucosidase was reported for the *Rhus chinensis* extract, a Korean herb traditionally used in the treatment of type 2 diabetes in Korea (Shim et al., 2003).

Potential identification of the GI lectin

The sequence analysis of a partial internal fragment of the enriched GI lectin from *A. jiringa* seeds, obtained by in gel digestion with trypsin and subsequent sequence analysis with LC-MS/MS, revealed a peptide fragment with the likely sequence VSSDG SPQGS SVGR (Figure

6A). Comparisons to all protein sequences in the SwissProt database using BLASTp searching identified this fragment as a likely homolog of the lectin precursor from the mannose-glucose specific lectin family, with 16/16 (100%) identical amino acids to the lectin precursor from the common bean, *Dioclea guianensis* and 12 to 15/16 identical residues with other members of this lectin family (Figure 6B).

Biospecific interaction of the GI lectin with α -glucosidase

Biospecific interactions, between the enriched GI lectin from the seeds of *A. jiringa* and α -glucosidase were evaluated on an Autolab ESPRIT system. This biosensor system is based

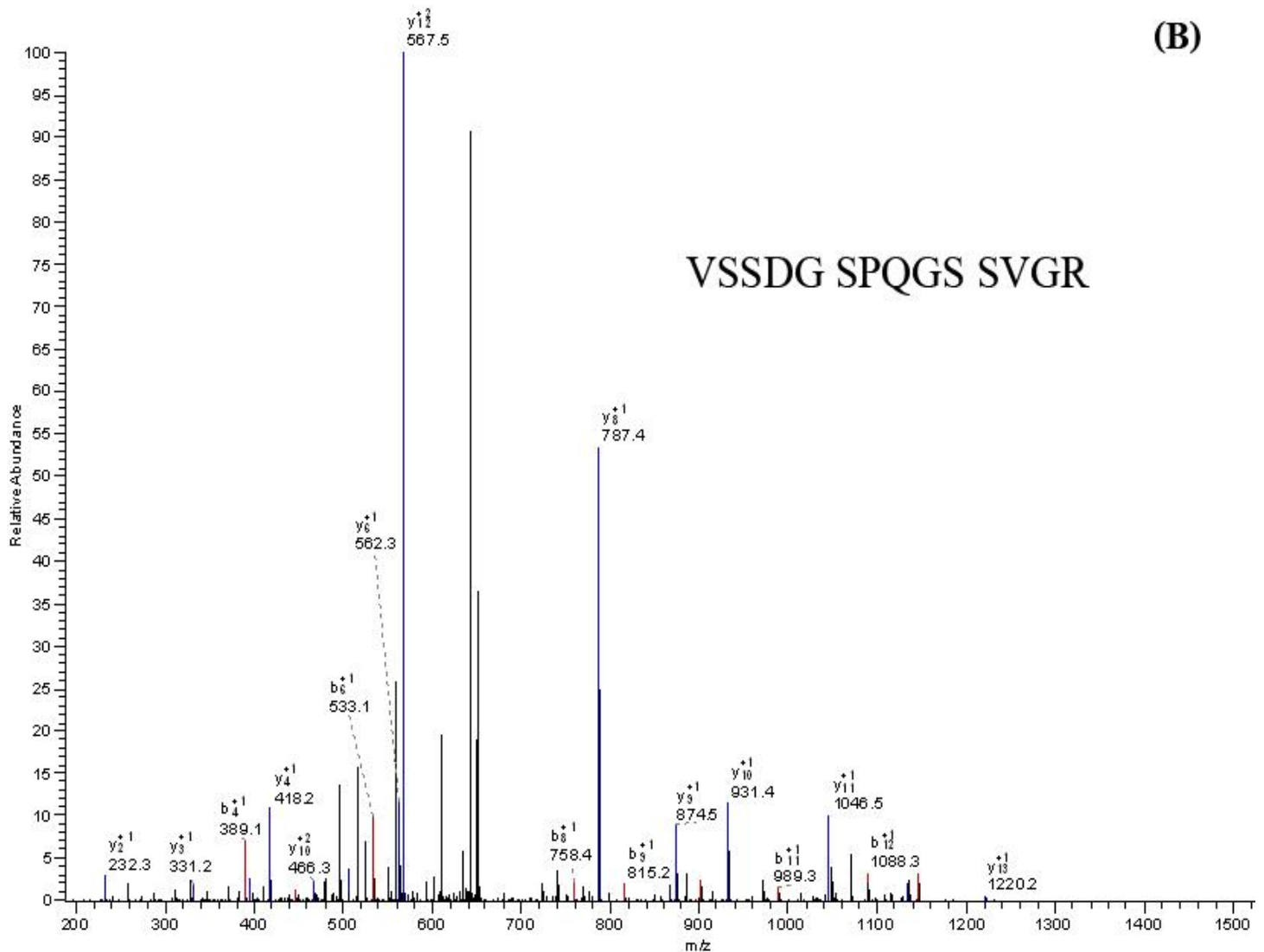


Figure 6. Contd.

on the principle of SPR. The various concentrations of the enriched GI lectin in TB were coupled to a certified grade 11 MUA gold plate and the unreacted groups on the surface of the gold were then blocked with ethanolamine. The blank channel used phosphate buffer as a control and all measurements were analyzed by 1 U/ml α -glucosidase enzyme. The GI lectin was found to have a specific interaction with α -glucosidase with an affinity constant = $9.3773 \times 10^{-7} \text{ s}^{-1}$, $K_s = 0.0241 \text{ s}^{-1}$, $K_a = 2.39 \times 10^3 \text{ s}^{-1}\text{M}^{-1}$ and $K_d = 0.0117 \text{ M}$ (Figure 7). The presented analytical system based on SPR is a valuable tool for the characterization of GIs and especially their binding-domains, which can be done by analyzing the initial binding rate and calculating the K_d value.

Conclusion

The high thermostable temperature range, up to 80°C for 70 min and pH optimum and stability or GI activity within the pH range of 8.0 to 10.0 (which is compatible with the site of gut α -glucosidase) of purified lectin from *A. jiringa* seed suggests its good potential for therapeutic use in foods as well as a supplement oral pill.

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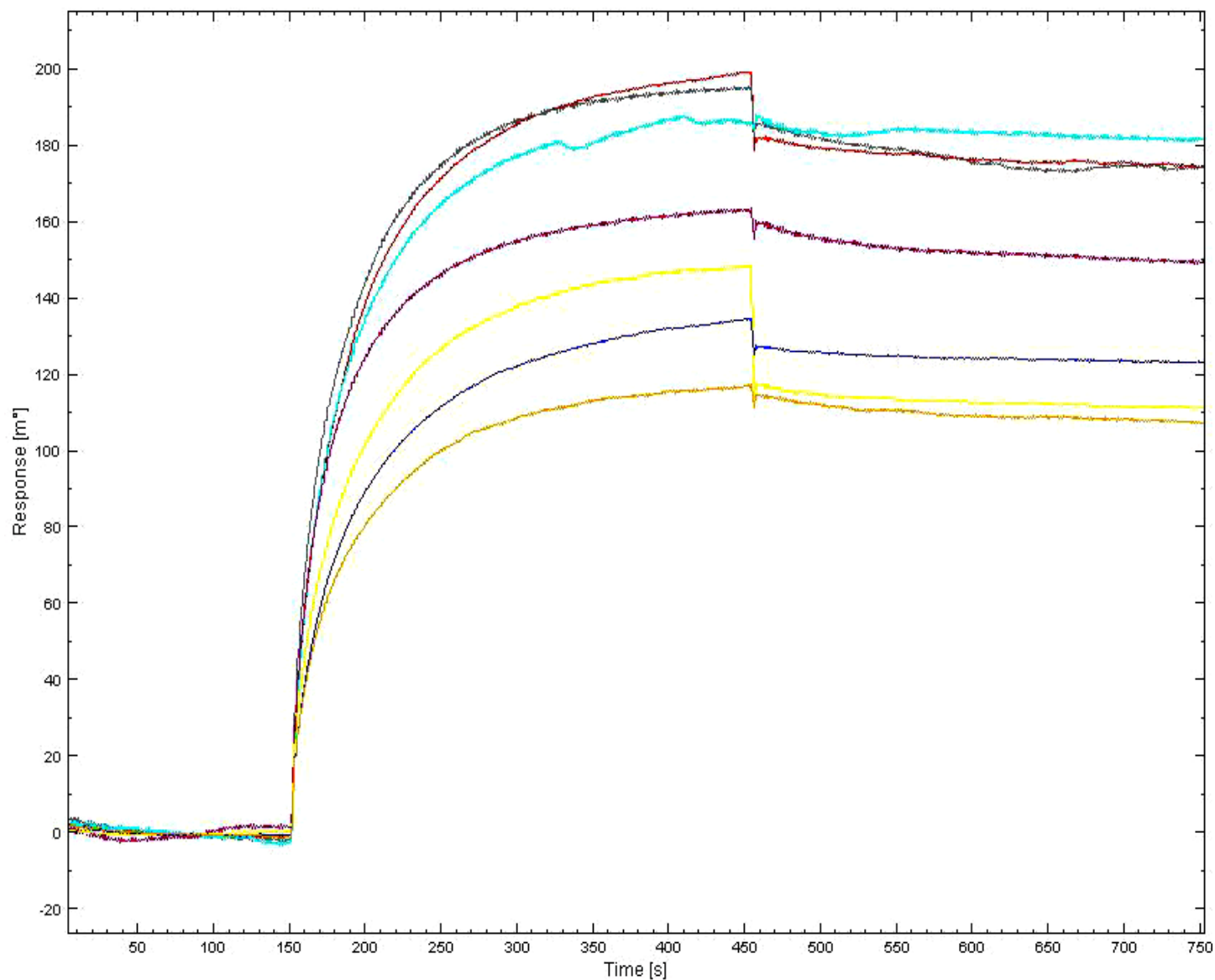


Figure 7. Representative sensorgram interactions of the enriched GI lectin sample at concentrations from 1.4 to 28 μg , and analyzed with the α -glucosidase enzyme as probe (1 U/ml)

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