

The interaction of the 11S globulin-like protein of kiwifruit seeds with pepsin

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Abstract

In a search for aspartic proteinase inhibitors (APIs) in kiwifruit seeds, we observed pepsin inhibitory activity (PIA) in an abundant globulin fraction extracted in high salt buffer with a Mr of ~148 kDa by gel-filtration. On a SDS-polyacrylamide gel, a major protein band of 54 kDa was observed under non-reducing conditions. This band was largely replaced by two subunits of Mr 33.5 and 20 kDa under reducing conditions. N-terminal sequencing of the smaller subunit, which had associated PIA, revealed a β -subunit of the 11S globulin-like protein (11S-GLP), legumin. After trypsin, chymotrypsin or papain digestion, the α -subunit of the kiwifruit legumin (11S-GLP) was degraded to varying degrees but there was no effect on the β -subunit, or on the PIA. This 11S-GLP also appeared to inhibit bovine spleen cathepsin D, *Candida albicans* secreted aspartic proteinases (SAPs) 1, 2 and 4, the plant fungus *Glomerella cingulata* SAP as well as apple seed aspartic proteinase, but to a much lesser extent kiwifruit seed aspartic proteinase. Through kinetic analysis of pepsin inhibition, the 11S-GLP and the purified β -subunit were found to fit a Michaelis–Menten model for competitive inhibition rather than a tight-binding model characteristic for typical proteinase inhibitors. Extrapolated complete inhibition was only obtained at an 11S-GLP concentration ~900 times that of the pepsin. Further investigation revealed that the 11S-GLP and the β -subunit acted as weak alternative substrates for pepsin. As the 11S-GLP or the β -subunit were degraded, the PIA activity declined in parallel. We discuss these results in terms of the substrate recognition site of pepsin compared with other proteinases.

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1. Introduction

Large (11S–15S) globulin-like proteins (11S-GLPs) also named legumin-like proteins, have been reported as storage proteins from a wide range of seeds including wheat [1,2], lupin [3], *Ginkgo biloba* [4], faba bean [5], alfalfa [6], buckwheat [7], rye and corn [1]. They are trimers or hexamers with Mr between 300 and 400 kDa. 11S globulin subunits consist of two polypeptide chains linked by at least one S–S bridge between cysteine residues at highly conserved positions in the acidic α -chain and basic β -chain. Both chains are post-translationally

generated from a common precursor protein, which is the product of one member of a multigene family [8].

Aspartic proteinases are widely distributed in living organisms including viruses, fungi, plants and animals [9]. They are a class of endopeptidases, usually with an acidic pH optimum and inhibited by pepstatin, a pentapeptide from streptomyces [10]. Aspartic proteinase activity has been found in seeds from a broad variety of plant species [11–15].

Tight-binding proteinaceous inhibitors of proteolytic enzymes are ubiquitous in nature. They generally bind at a 1:1 molar ratio and at low proteinase and inhibitor concentrations often show slow binding kinetics taking minutes to completely inhibit the target enzyme. The proteinase:inhibitor complex is stable and the inhibitor is rarely degraded by the target proteinase, except in some serine PIs where the bait sequence is cleaved [16]. Some inhibitors modulate the activity of endogenous proteinases operating in various physiological processes, while others are defence proteins with protective action against harmful endogenous or pathogenic proteinases [17–19].

Abbreviations: AP, aspartic proteinase; API, aspartic proteinase inhibitor; GLP, globulin-like protein; LSE, low salt extract; Mr, molecular mass; PIs, protease inhibitors; PIA, pepsin inhibitory activity; SAP, secreted aspartic proteinase; TCA, trichloroacetic acid; WAPI, wheat aspartic proteinase inhibitor

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Compared with cysteine and serine PIs, a very limited number of tight-binding proteinaceous aspartic proteinase inhibitors have been found. In plants, measured aspartic proteinase inhibitory activity (API), and a corresponding diagnostic gene sequence, have been described from potato and tomato [20–24] and squash phloem [25,26], while API activity has been reported from soft wheat bran [27]. Only two gene families have been shown to possess API activity, the Kunitz family and the squash API [16].

In this paper, we report on the isolation of an 11S-GLP from kiwifruit seeds that exhibits apparent API activity. Kinetic analysis and SDS-PAGE revealed that it is not a tight-binding inhibitor of pepsin, but a weak alternative substrate.

2. Materials and methods

2.1. Plant material and seed preparation

Kiwifruit seeds were obtained from ripe fruit of *Actinidia deliciosa* (A. Chev.) C.F. Liang et A.R. Ferguson var. *deliciosa* ‘Hayward’, and *Actinidia chinensis* Planch. var. *chinensis* ‘Hort16A’. One hundred mature fruits were cut in half, the flesh scooped out and put in 5 l of water to which was added 1 ml of diluted (1:200, v/v) pectalase (Pectalase Rohapect, D5L special pectalase, Carter Associates). The solution was left at 20–25 °C for 2 days and the pulp washed through a sieve using a jet of water. The seeds were blotted-dry, frozen and ground in a cryo-mill followed by a mortar and pestle in liquid nitrogen to a fine powder. Other seeds were obtained either from commercial sources or directly from the fruit (apple).

2.2. Globulin extraction from seeds with high salt buffers

Finely powdered seeds (kiwifruit, buckwheat groats, pearl barley, *Arabidopsis thaliana* or apple) and wheat bran were extracted with (1:12, w/v) ice-cold acetone, the insoluble material separated by filtration on Whatman No. 1 filter paper and extracted using a modification of a published method for seed globulin extraction [28]. The delipidized powder was suspended at 4 °C in water (1/34, w/v) containing 10 mM CaCl_2 , 10 mM MgCl_2 and 1 mM PMSF, pH 8.0 to extract the albumin fraction of the proteins. This is the low salt extract (LSE). After stirring for 2 h at 4 °C, the suspension was centrifuged at $16,000 \times g$, 4 °C, for 1 h and the pellet was re-suspended overnight at 4 °C (1/34, w/v) in 0.1 M Tris–HCl buffer pH 7.5 containing 1.7 M NaCl, 1 mM PMSF, 10 mM EDTA and 10 mM EGTA. After centrifugation, the proteins in the supernatant were precipitated from solution by 85% saturation with ammonium sulphate, spun at $16,000 \times g$, 4 °C, for 1 h and the precipitate was dissolved in 50 mM Tris–HCl pH 7.5, spun at $10,000 \times g$ and dialyzed in water (the 11S-GLP precipitates out).

2.3. Gel-filtration of the 11S-GLP and size estimation

The globulin extract (see Section 2.2, before dialysis) was concentrated using Vivaspin 20 ml concentrators with 30 K

MWCO PES membrane (Sartorius) and applied to a Sephacryl S-300 (Amersham Biosciences; 60 cm $\times$ 1.6 cm) column equilibrated with 1 M NaCl in 20 mM Tris–HCl buffer pH 8.0. The column was calibrated using thyroglobulin (Mr 670 kDa), bovine serum albumin (monomer: 68 kDa; dimer: 135 kDa) and cytochrome *c* (12 kDa).

2.4. Purification of the β -subunit of the 11S-GLP

The insoluble proteins from *A. deliciosa* kiwifruit seeds, which appeared after dialysis (Section 2.2), were dissolved in 20 mM Tris–HCl buffer, pH 8.4, containing 8 M urea. The sample was applied to a 5 ml HiTrap Q anion exchange column equilibrated with the same buffer and the bound fraction eluted with 1 M NaCl in buffer. The unbound peak was buffer-exchanged, using a Vivaspin concentrator (Sartorius), to 25 mM formate, pH 4, containing 4 M urea, applied to a 1 ml HiTrap SP column equilibrated with this buffer and eluted with salt gradient (0–1 M NaCl) in buffer. The eluted protein peak fractions containing the PIA (Section 2.5) were pooled and applied to a Vydac C18 reverse-phase column (4.6 mm i.d. $\times$ 250 mm L) equilibrated with 10% acetonitrile in 0.1% TFA and eluted with an acetonitrile gradient (10–90% in 0.1% TFA). The protein peak inhibitory fractions of each run were combined and re-run. The main protein peak, which contained the PIA activity, was checked for purity on a SDS-polyacrylamide gel and submitted for N-terminal sequencing (see Section 2.10).

2.5. Aspartic proteinases and inhibitor assay

Assays were carried out in fluorescent micro-titer plates (Lia-plate, white, 96-well flat-bottom, medium binding, Greiner Labortechnik) either using Bodipy-casein, at 50 ng/ μl (EnzChekTM proteinase assay kit green fluorescence, Molecular Probes, Inc.) as previously reported [29] or the synthetic peptide MCA-Arg-Lys-Pro-Ile-Glu-Phe-Phe-Ile-Leu-Lys(DNP)-Arg-OH (Custom synthesized by Auspep, Parkville, Vic., Australia) at 0.2 mM. Substrate cleavage was followed in the Wallac Victor 1420 Multichannel Counter (Science and Technology, NZ) by measuring the increase in fluorescence (for Bodipy-casein see ref. [29]; for the synthetic peptide: 340 nm excitation and 430 nm emission). Rates were calculated from linear regression of the data [30].

Purified secreted aspartic proteinase (SAP) from *Glomerella cingulata* (GC) and SAPs 1–4 (EC 3.4.23.24) from *Candida albicans* were obtained as reported earlier [31]. Porcine pepsin A (EC 3.4.23.1) and bovine spleen cathepsin D (EC 3.4.23.5) were purchased from Sigma. Plant seed aspartic proteinases were purified as described below (Section 2.9). Pepsin was assayed in 0.75% lactate–HCl buffer, pH 2.2. Cathepsin D was assayed in 0.1 M citrate buffer, pH 5.0. Secreted aspartic proteinases from *C. albicans* and from *G. cingulata* were assayed in 0.05 M citrate at pH 3.7. Plant seed APs were assayed in 0.1 M citrate buffer, pH 5.5. All buffers contained 0.25 μl Tween 20/10 ml buffer. To measure inhibition, the aspartic proteinase and the serially diluted inhibitor were

incubated together for 5 min before assays were initiated with substrate addition.

When the reaction rate of pepsin with different substrates was investigated in a trichloroacetic acid (TCA)-based assay at room temperature, pepsin concentration was 2 µg/ml reaction mixture and the substrate was used at a final concentration of 1 mg/ml. At timed intervals, 400 µl of reaction mixture was added to 80 µl of 30% TCA on ice. After 30 min, the precipitated proteins were spun off. Product formation was followed by measuring absorbance of the supernatant at 280 nm.

2.6. Effect of papain, trypsin and chymotrypsin on 11S-GLP

The ability of these proteolytic enzymes to either digest or be inhibited by the 11S-GLP (Sephacryl S-300 protein peak fraction) or its β-subunit was measured. Assay conditions were for papain (EC 3.4.22.2): 0.1 M MOPs, 1 M NaCl, 2 mM DTT, pH 7, substrate: Phe-Arg-AMC; trypsin (EC 3.4.21.4): 0.1 M Tris, 1 M NaCl, 2 mM CaCl₂, pH 8.0, substrate: Z-Arg-AMC; chymotrypsin (EC 3.4.21.1) 0.1 M MOPS, 1 M NaCl, 2 mM CaCl₂, pH 8.0, substrate: Ala-Ala-Pro-Phe-AMC.

2.7. Time course of pepsin inhibition by the 11S-GLP

Pepsin was assayed, at 20 °C, in 1 ml of standard pepsin assay mixture at 0.2 nM pepsin and 25 µM synthetic pepsin substrate, in a Perkin-Elmer 50LS spectrofluorimeter with excitation at 323 nm and emission at 393 nm (2.5 nm slit). The reaction was started by the addition of pepsin and after 1–2 min, 11S-GLP (Sephacryl S-300 peak fraction) was added.

2.8. Time-course of the 11S-GLP digestion by pepsin

Pepsin was incubated at room temperature in lactate buffer, pH 2.2 with 11S-GLP concentrated peak fraction from Sephacryl S-300 column, at an initial concentration that inhibited 80–90% of the pepsin activity. At timed intervals, a sample was withdrawn and pepsin inactivated by heating at 90 °C for 5 min. Samples were checked for PIA activity as well as by SDS-PAGE.

2.9. Purification of aspartic proteinases from kiwifruit seeds

These were purified by affinity chromatography on a pepstatin A column. Affigel 15 (BioRad) was coupled to pepstatin A, using non-aqueous coupling according to manufacturer's instructions. The matrix was equilibrated in binding buffer (0.1 M sodium acetate, 1 mM EDTA, 0.5 M NaCl, pH 4.4) prior to proteinase binding. The LSE (Section 2.2) was precipitated with ammonium sulphate at 85% saturation. The precipitate was dissolved in the affinity gel binding buffer, mixed with the gel at a ratio of 6:1 (enzyme solution:gel, v/v) and left on the shaker at room temperature for 2 h. The unbound proteins were washed off with the same

buffer and the bound proteins eluted at pH 7.5. The eluted proteinases were stable under these conditions at 4 °C for few days.

2.10. Other protein techniques

Protein concentration was calculated by the method of Warburg and Christian [32] or by the Bradford assay using the Bio-Rad protein assay reagent with BSA as a standard. NuPAGE Bis-Tris Gels (Invitrogen) were run in NuPAGE MES-SDS Running Buffer (Invitrogen). SeeBlue Plus2 Pre-stained Standard (Invitrogen) was used for Mr determination and the gels were silver-stained using a standard protocol [29] or using Colloidal Coomassie Stain [33]. For electro-blotting, 10% SDS-tricine gels with 4% stacking gels were used for electrophoresis (anode buffer: 0.2 M Tris, pH 8.9; cathode buffer: 0.1 M Tris, 0.1 M tricine, 0.1% SDS, pH 8.25) followed by semi-dry blotting onto PVDF membrane (Immobilon) using the Trans-Blot SD Semi-Dry Transfer Cell (BioRad).

The N-terminal amino acids of the purified β-subunit were sequenced by the Protein Microchemistry Facility, Department of Biochemistry, University of Otago. Analyses were undertaken using narrow bore HPLC of PTC derivatives [34].

3. Results

3.1. Isolation of an aspartic proteinase inhibitor

Under high salt extraction conditions the main solubilised proteins are typically the globulins. The dialyzed ammonium sulphate-precipitated globulin fraction of kiwifruit seeds contained ~8 mg of protein/g seeds. Nearly all of this fraction was located in a Sephacryl S-300 protein peak of ~148 kDa (data not shown) and exhibited PIA. This fraction revealed a major band of 54 kDa on a SDS-NuPAGE gel under non-reducing conditions (Fig. 1A). Under reducing conditions, however, this major band was largely replaced by two main bands of 33.5 kDa (11S-GLP α-subunit) and 20 kDa (β-subunit) (Fig. 1B).

3.2. Further purification and identification of the API by N-terminal sequencing

Sequencing the electro-blotted major band (34 kDa) obtained after trypsin digestion of the 11S-GLP as well as the purified β-subunit of the 11S-GLP from *A. chinensis* with PIA gave the sequence GLEETI-TARL(V/H)ENIDS(R/P) or shorter versions of it. The missing residue (marked –) is most likely a C. This sequence shows strong homology to sequences of the β-subunit of 11S-GLP from other sources. For example, 14 out of 17 residues were identical with 11S-GLPs from *Cicer arietinum* and *Chenopodium quinoa*. This sequence is also fully compatible with the PROSITE PS00305 pattern N-G-X-[DE](2)-X-[LIVMF]-C-[ST]-X(11,12)-[PAG]-D last viewed on 16.12.2005 (<http://au.expasy.org/cgi-bin/get-prosite-entry?PS00305>). No other residues were readily identifiable from the

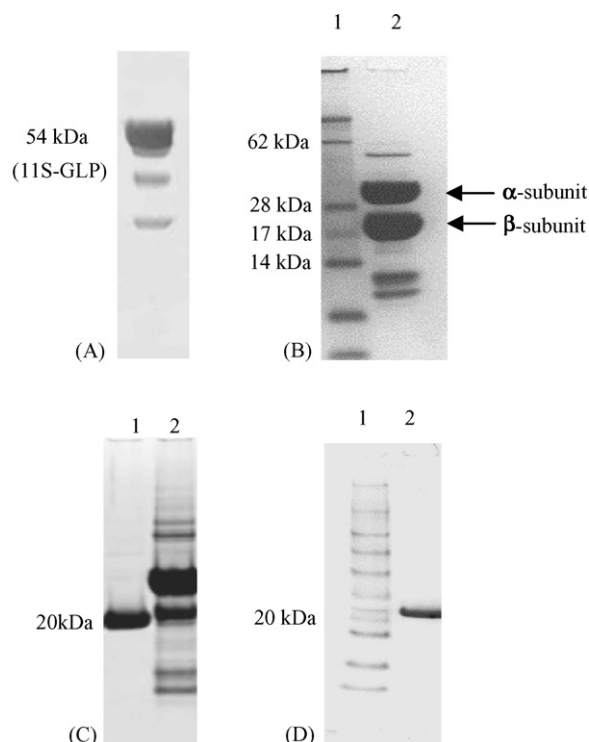


Fig. 1. Separation of partially purified or purified *A. chinensis* 11S-GLP or its subunits using SDS-NuPAGE. (A) Under non-reducing conditions showing an 11S-GLP (major protein band) at 54 kDa. (B) Under reducing conditions showing the 33.5 and 20 kDa α - and β -subunits of 11S-GLP (Lane 2) with SeeBlue Plus 2 Prestained Standard in Lane 1. (C) HiTrap Q column fractions of 11S-GLP; Lane 1 unbound fraction (β -subunit) and Lane 2 bound fraction. (D) Lane 2 Vydac C-18 column protein peak fraction of the β -subunit.

microsequencer trace files as secondary and so other minor peptide components could not be identified.

The 11S-GLP from *A. deliciosa* when fractionated on a HiTrap Q column (Section 2.4), separated into one bound and one unbound fraction, both possessing PIA. SDS-NuPAGE gel revealed that the unbound fraction contained predominantly the β -subunit while the bound fractions contained both the α - and β -subunits (Fig. 1C). The unbound fraction was further purified on a HiTrap-SP column followed by two rounds of reverse-phase chromatography. The protein peak fraction showed a single ~ 20 kDa protein band (Fig. 1D) with PIA (data not shown). MALDI-TOF-MS revealed a mass of 20.427 kDa.

3.3. Characterisation of the API

Ammonium sulphate precipitated and dialyzed 11S-GLP was soluble in 0.75% (v/v) lactate buffer pH 2.2 and in 20 mM Tris-HCl buffer containing 1 M NaCl, pH 7.0–9.0. When heated in the latter buffer at pH 8.2, the PIA was stable for at least 40 min at 95 °C. The PIA was also retained in 8 M urea and was stable for months at pH 6–9 but was lost after a few days at pH 2.2 or after a few weeks at pH 3.7 at 4 °C, with concomitant loss of the α - and β -subunits as revealed by SDS-NuPAGE gel electrophoresis (data not shown). The 11S-GLP as well as the β -subunit was found to inhibit a range of APs (porcine pepsin A, bovine spleen cathepsin D, *C. albicans* secreted aspartic proteinases (SAPs) 1, 2

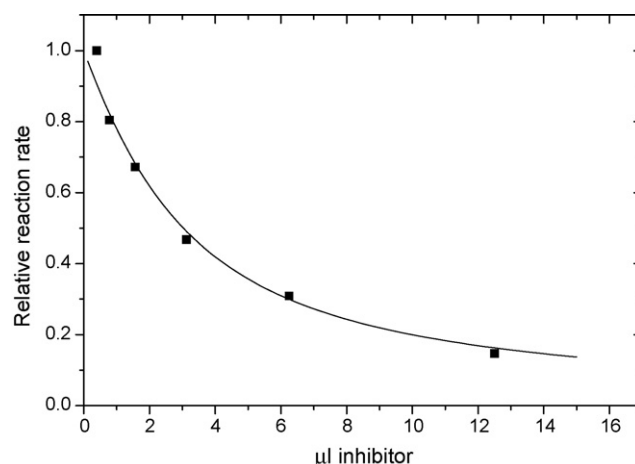


Fig. 2. Pepsin A inhibition by 11S-GLP (stock concentration 1.67 mM by UV measurement). 11S-GLP was incubated with pepsin in a final volume of 100 μ l of lactate buffer (75 mM) for 5 min before addition of 10 μ l of the substrate Bodipy-casein. Reaction rate was measured as the increase in fluorescence/min (Section 2.5).

and 4, the plant fungus *G. cingulata* SAP and apple seed aspartic proteinase). Purified kiwifruit seed AP was significantly less inhibited by the 11S-GLP than pepsin (data not shown).

3.4. Is the β -subunit of 11S-GLP a tight binding inhibitor or a competitive substrate for APs?

The purified β -subunit was quantified for protein content by the Bradford assay and was also titrated with pepsin. The inhibitor present was quantified by protein assay and also calculated by extrapolation of the initial linear part of the inhibition curve to complete inhibition, assuming that the inhibitor (Mr 20 kDa) was tight binding and equating that to the amount of pepsin present in the assay (Fig. 2). The calculated inhibitor/pepsin mole ratio was 890, far higher than would be

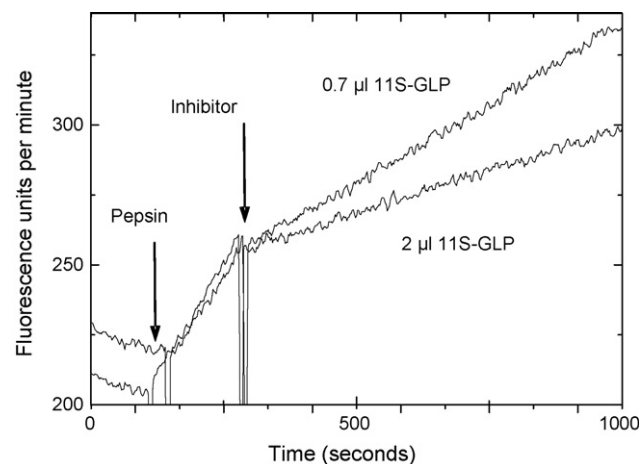


Fig. 3. Time-course of pepsin inhibition by 11S-GLP using the synthetic peptide substrate MCA-Arg-Lys-Pro-Ile-Glu-Phe-Phe-Ile-Leu-Lys(DNP)-Arg-OH. The reaction (1 ml) was started by the addition of pepsin (0.2 nM final concentration) to the substrate (25 μ M final concentration) in lactate buffer. 11S-GLP (stock concentration 1.67 mM by UV measurement) was added 1–2 min later (Section 2.7).

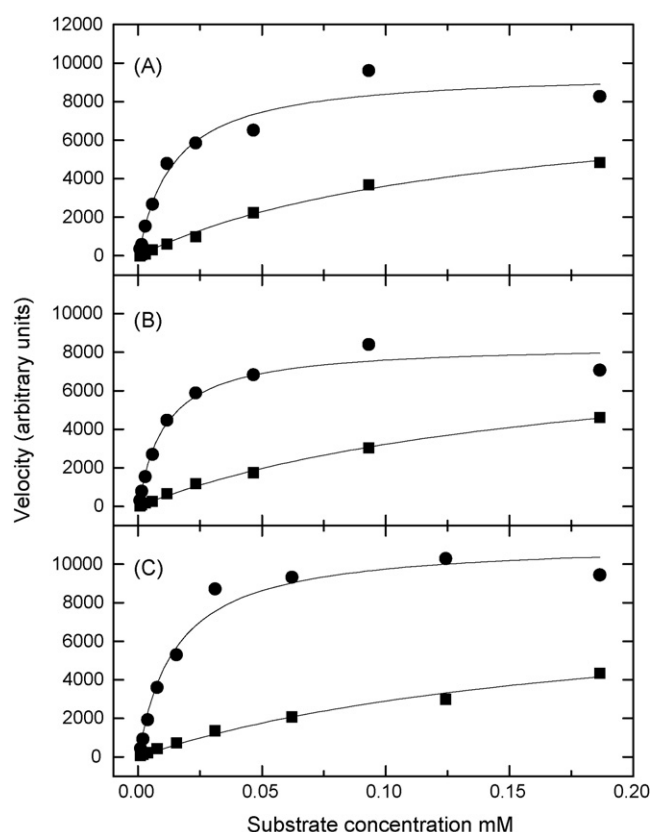


Fig. 4. Inhibition of pepsin reaction with the synthetic peptide substrate by (A) 11S-GLP, (B) the β -subunit of 11S-GLP and (C) BSA, all at 1 mg per ml.

observed for a tight binding inhibitor (usually expected to bind the proteinase at a 1:1 ratio [35]). This result could suggest that the inhibitor is a very minor component of the β -subunit preparation, but in a pepsin kinetic assay (Fig. 3) using the synthetic substrate, we observed rapid inhibition, inconsistent with tight binding kinetics.

We then undertook a classical Michaelis–Menten steady state kinetic analysis of the PIA. We measured the rate of hydrolysis of the synthetic peptide substrate at a range of concentrations of 11S-GLP and of the β -subunit, with BSA as a control (Fig. 4). All three proteins were found to behave as

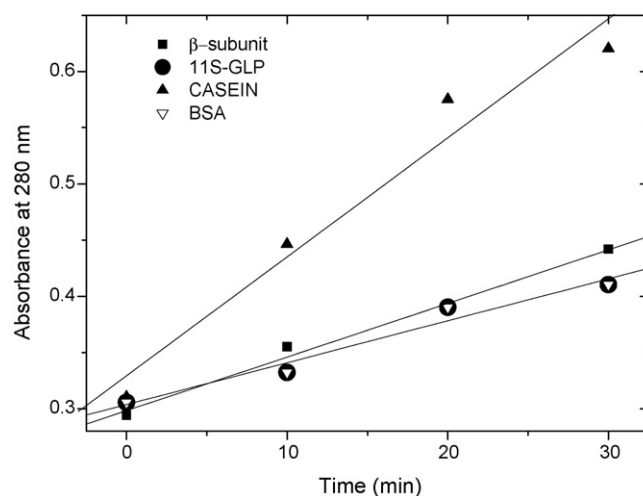


Fig. 5. Time-course of the reaction (TCA-based assay) of pepsin with different substrates: casein, BSA, 11S-GLP and the β -subunit of 11S-GLP.

competitive inhibitors raising the apparent K_m for the fluorescent synthetic peptide substrate and giving apparent K_i values in the μM range (Table 1). The E_i/K_i values (~ 0.01) obtained are typical for classical Michaelis–Menten steady state kinetic inhibition (Table 1).

We directly tested whether the various protein inhibitors were actually alternative substrates, using a simple trichloroacetic acid (TCA)-based assay. When the 11S-GLP or the purified β -subunit was used as the sole substrate for pepsin, a time response curve was obtained that was similar to that produced by BSA, but all were less effective substrates than casein (Fig. 5).

If 11S-GLP were actually an alternative substrate, we would expect the PIA to decline as the 11S-GLP was digested. The time course of 11S-GLP digestion with pepsin, with simultaneous measurements of the remaining PIA, showed that within 30 min the PIA decreased from an initial value of 85% inhibition to 60% and no intact α - or β -subunit of 11S-GLP was visible on the SDS-polyacrylamide gel. Only small bands of Mr 4–9 kDa were observed. After 16 h, the PIA decreased to 30%. At this time, the total globulin fraction degraded to faint silver stained bands of 5–7 kDa (Fig. 6).

Table 1

Kinetic parameters for the interaction of pepsin with BSA, *Actinidia chinensis* 11S-GLP and the purified β -subunit using the synthetic peptide substrate (MCA-Arg-Lys-Pro-Ile-Glu-Phe-Phe-Ile-Leu-Lys(DNP)-Arg-OH)

	Substrate only	Substrate + BSA	Substrate + 11S-GLP	Substrate + β -subunit
Apparent K_m (μM)	13.5 ± 2.7	180 ± 30	180 ± 20	140 ± 20
$V_{\max}$ (arbitrary F units/min)	9300 ± 520	10100 ± 900	8900 ± 600	8600 ± 800
Apparent K_i (μM)	–	0.7 ± 0.1	0.6 ± 0.09	1.6 ± 0.5
Pepsin (nM) in assay mixture	8.1	8.1	8.1	8.1
Inhibitor (nM) in assay mixture ^a	–	7100	8100	15000
Inhibitor/pepsin	–	887	1000	1852
E_i/K_i	–	0.012	0.014	0.005

K_m , K_i and $V_{\max}$ values were calculated using the standard Michaelis–Menten model for classical competitive inhibition. Estimated parameters using more complex models were not significantly different from zero. Values represent the estimated parameter with its asymptotic standard error ($n = 2$).

^a Measured by UV absorbance.

^b Pepsin in assay mixture.

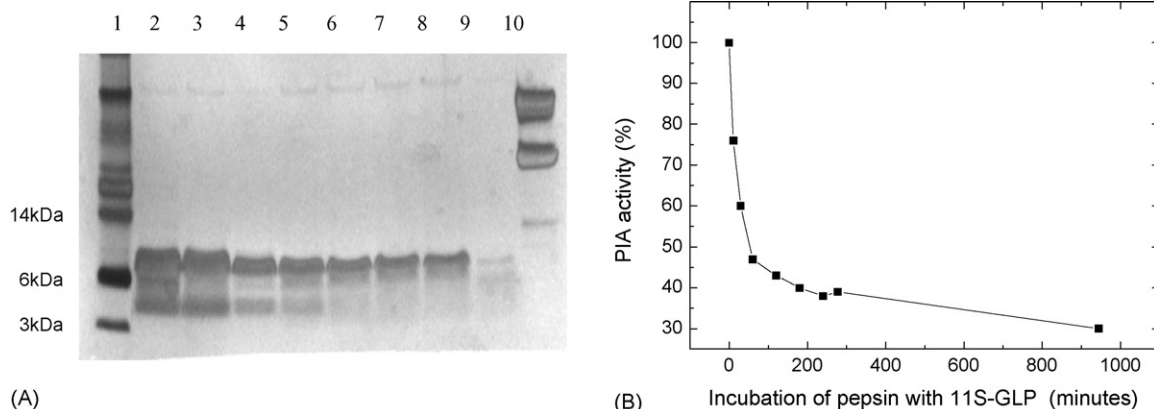


Fig. 6. Time-course of digestion of 11S-GLP with pepsin. (A) NuPAGE (4–12%) SDS gel; Lane 1: SeeBlue Plus 2 Prestained Standard, Lanes 2–9 pepsin digest after 16, 31 min, 1–4 h, 4 h 40 min and 16 h, Lane 10 11S-GLP before digestion. (B) Percent of measured PIA during incubation of 11S-GLP with pepsin.

3.5. Effect of the 11S-GLPs on other classes of proteinases

Studies using trypsin, papain and chymotrypsin, assayed with both casein and their synthetic peptide substrates, showed that these proteinases were not inhibited by the 11S-GLP or by BSA. Incubation of the 11S-GLP with chymotrypsin had no effect on the PIA as determined by titrating the inhibitor with pepsin before and after chymotrypsin digestion. Analysis by SDS-PAGE showed that the α -, but not the β -subunit, was digested to smaller peptides (data not shown). A similar observation was noted for papain and trypsin (data not shown).

4. Discussion

11S-GLPs, also called legumin-like, seed storage proteins have been reported from buckwheat [7], wheat [1], soft wheat bran [27] and *A. thaliana* seeds [36]. We also observed PIA from the globulin fraction of a range of plant sources including wheat bran, buckwheat groats, pearl barley, *A. thaliana* and apple seeds (data not shown).

The kiwifruit 11S-GLP shows similar heat and pH stability properties to aspartic proteinase inhibitor described from soft wheat bran (WAPI). However, while the 11S-GLP is an apparent inhibitor of a number of APs, it is a poor inhibitor of kiwifruit APs. A similar observation was also noted for WAPI towards the endogenous wheat AP [37]. The kinetics of pepsin inhibition by WAPI was also typical for classical Michaelis–Menten steady state kinetic inhibition as found in this work. In addition, WAPI was also found not to inhibit trypsin, chymotrypsin or papain, another observation shared by the kiwifruit 11S-GLP. We believe that the wheat inhibitor [27,37] might have also been an alternative substrate for pepsin rather than a true inhibitor.

Why this protein seems to inhibit APs and not cysteine proteinases may be explained on the basis of the substrate specificity shown by pepsin and other APs in general [9,38]. The active site of pepsin recognizes eight substrate residues [39]. The specificity determinants are the steric structures of the eight-substrate side chain binding sites in the active site cleft of

the enzyme. The major specificity recognition sites are the P1 and P1' subsites, which prefer large hydrophobic residues [40] while subsites distant from the P1 and P1', are in general less specific and can accommodate different types of residues. It seems that the 11S-GLP, its β -subunit and BSA, satisfy these criteria and hence serve as alternative substrates to the substrates used in the assay and appear to inhibit pepsin assayed with other substrates. Inhibition of pepsin in this work by 11S-GLP was shown to occur immediately, reflecting fast binding kinetics. Trypsin and chymotrypsin cleaved the α -subunit as has been observed in the case of lupin 11S globulin [3] but not the β -subunit and they did not appear to be inhibited by the 11S-GLP. It seems that the native β -subunit is not a substrate to these enzymes and the α -subunit cleavage does not affect the reaction rate with the synthetic peptide substrates, possibly because of a low binding affinity. This may also reflect the relatively small number of residues these enzymes recognize. It has been noted that at least in the case of trypsin and protein substrates, the binding event may be the rate-limiting step [38].

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