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MAJOR PROTEINS OF YAM BEAN TUBERS

ALDRIN V. GOMES, GRACE SIRJU-CHARRAN* and JUNOR A. BARNES†

Department of Biochemistry, Faculty of Medical Sciences, and * Department of Plant Science, The University of the West Indies, St. Augustine, Trinidad and Tobago

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Key Word Index—*Pachyrhizus erosus* L. Urban; Fabaceae; Mexican yam bean; storage proteins; proteases; protease inhibitor.

Abstract—The tuberous roots of the Mexican yam bean, jicama, (*Pachyrhizus erosus* L. Urban) contained large quantities of two acidic glycoproteins which accounted for more than 70% of the total soluble proteins (about 3 g per 100 g of tuber on a dry weight basis). The two major proteins, tentatively named YBG1 and YBG2, had apparent M_rs of 28 000 and 26 000, respectively, by SDS-PAGE. A third protein named YBP22 which accounted for 2–5% of the total soluble proteins had an M_r of 22 000. YBG1 and YBG2 exhibited great similarity on the basis of their amino acid composition and had identical *N*-terminal amino acid sequences. The first 23 amino acids in the *N*-terminal region of YBG2 were DDLPDYVDWRDYGAVTRIKNQGQ which showed strong homology with the papain class of cysteine proteases. YBG1 and YBG2 were found to bind to a Concanavalin A-Sepharose column and were also stained positively by a sensitive glycoprotein stain. Both glycoproteins exhibited cysteine proteolytic activity. In contrast, YBP22 showed sequence homology with several known protease inhibitors, and a polyclonal antibody raised against this protein cross reacted with soybean trypsin inhibitor. © 1997 Elsevier Science Ltd

INTRODUCTION

The Mexican yam bean or jicama (*Pachyrhizus erosus* L. Urban; Fabaceae) is now being rediscovered as a root crop of great economic importance. This yam bean is one of six species in the genus *Pachyrhizus* which occur in Central America and Southern Mexico, where it was cultivated by the ancient Mayas several centuries ago [1]. The tuber production capacity of the genus *Pachyrhizus* is reported to be the highest among the tuber-bearing legumes [1]. Although these tuberous roots have been utilized mostly for their low-caloried carbohydrate content, they have been reported to have, on a dry weight basis, three to five times the protein content of other root crops, such as potato (*Solanum tuberosum* L.), sweet potato (*Ipomoea batatas* Lam), cassava (*Manihot esculenta* Crantz), yam (*Dioscorea rotundata*) and taro (*Colocasia esculenta* L. Schote) [2]. The yam bean is taxonomically unrelated to the above mentioned commercially important tubers.

Plant storage proteins are of great nutritional value to mankind, and they also serve diverse functional roles (e.g. contribution to seed development [3], the supply of amino acids to developing sprouts [4], insecticidal properties [1, 5] and anti-proteolytic properties [6]). Reports of tuber storage proteins have been limited to sweet potato, potato and yam [4]. Very little is known about the constituent proteins of the yam bean tuberous roots. In this work we have identified two possible storage glycoproteins (YBG1 and YBG2 of M_r 28 000 and 26 000, respectively) of the yam bean, which also showed cysteine proteolytic activity. In addition, a third tuber protein YBP22 (M_r 22 000) was also shown to exhibit high sequence homology with several known protease inhibitors. A polyclonal antibody raised against the latter protein cross reacted with soybean trypsin inhibitor and a protein from yam bean seeds (this work) with the same M_r as YBP22. This finding suggests that YBP22 is not tuber specific. Studies have shown that plants have evolved both simple and complex biological systems to defend themselves against pathogens and herbivores [1, 5, 7]. Although plant storage proteins serve mainly as sinks for nitrogen, carbon and sulphur [5], some storage proteins serve secondary roles as protease inhibitors and seed lectins which are known to be active in plant defence mechanisms [7]. If YBG1 and YBG2 are indeed storage proteins they would be the first of this class of proteins which are shown to possess proteolytic capabilities. It is possible that these proteins would be involved in the response of tubers to pathogens.

† Author to whom correspondence should be addressed.

RESULTS AND DISCUSSION

Isolation of the major proteins in the Mexican yam bean tuber

The bulk of these proteins were precipitated in the 45% ammonium sulphate fraction [8]. Under reducing electrophoretic conditions two distinct bands (YBG1 and YBG2) with M_rs of 28 000 and 26 000, respectively, were resolved. When more than 10 μg of homogenate was loaded on gels the two major protein bands merged forming one major band. These two major bands account for more than 70% of the total soluble proteins (*ca* 3 g of YBG1 and YBG2 per 100 g of tuber on a dry weight basis) in the tuber as determined by densitometric analysis [8]. Under native electrophoresis conditions YBG1 and YBG2 were found to exist in a 1:1 ratio in 6–9-month-old tubers. The storage of harvested yam bean tubers for 3 months at room temperature resulted in a decrease in the amount of YBG1 and YBG2 found in these tubers compared with the amount of these proteins in freshly harvested tubers, suggesting that these proteins may be linked to the metabolic state of the cell. This observation is not unlike other types of tubers such as sweet potato in which the major protein called sporamin was observed to disappear after 1 year of storage [9].

YBG1 and YBG2 were also purified by anion-exchange chromatography on a DEAE-Sepharose CL-4B column (see Experimental) and these purified fractions were used for the investigations of proteolytic activity. The purity of YBG1 and YBG2 were such that they were not contaminated by any other protein in significant amounts, as judged by SDS-PAGE (Fig. 3). Attempts to purify YBG1 from YBG2 were unsuccessful. This is not surprising since both the M_r and isoelectric point (pI) for each of these proteins were close. We were unable to separate YBG1 and YBG2 from each other except by electrophoresis and electrotransfer.

Characterization of YBG1 and YBG2

The yam bean tuber was found to have a high relative percentage nitrogen (16.0%), as determined by the Kjeldahl method [31], which is typical of other tubers [9]. The three major proteins observed in the 45% ammonium sulphate fraction were all subjected to amino acid analysis and *N*-terminal sequencing. The amino acid compositions of the three proteins YBG1, YBG2 and YBP22 were all found to have high contents of acidic amino acids (Table 1), with the amino acid composition of YBG1 and YBG2 being very similar. Moreover, the *N*-termini of YBG1 and YBG2 were found to be aspartic acid in both cases and there was also a significant amount of acidic amino acids in the *N*-terminal sequence of both polypeptides (Fig. 1). The samples that were used for amino acid composition and *N*-terminal sequencing were obtained from transblots of two-dimensional gels (Fig. 2). Under two-dimensional conditions two major bands with M_rs of 36 000 and 34 000 were observed (Fig. 2). The mobilities of these two major bands correspond to the mobilities observed for YBG1 and YBG2 which were run on SDS-polyacrylamide gels without prior heating. The two-dimensional gel also showed that YBG1 and YBG2 are resistant to denaturation in 8 M urea since in the

Table 1. Amino acid compositions of YBG1, YBG2, papain and YBP22

Amino acid	Amino acid composition (mol%) YBG1	YBG2	Papain	YBP22
Asx	6.62	8.42	8.96	8.48
Glx	10.96	10.71	9.43	7.02
Serine	12.71	9.79	6.13	6.22
Glycine	17.43	19.83	13.21	12.28
Histidine	1.22	0.74	0.94	1.26
Arginine	2.45	3.11	5.66	1.93
Threonine	3.64	3.18	3.77	5.59
Alanine	9.49	8.25	6.60	6.87
Proline	3.98	3.43	4.72	11.42
Tyrosine	3.65	4.23	8.96	3.26
Valine	10.45	10.23	8.49	15.15
Isoleucine	4.85	4.70	5.66	4.10
Leucine	7.13	6.43	5.19	7.62
Phenylalanine	2.85	3.11	1.89	5.05
Lysine	2.57	3.84	4.72	3.75
Methionine	ND	ND	0.00	0.20

ND—none detected.

Asx, sum of aspartic acid and asparagine.

Glx, sum of glutamic acid and glutamine. Cysteine and tryptophan were most likely destroyed during the acid hydrolysis of the protein samples. Also due to the hydrolytic procedure used (see Experimental) it was not possible to determine the methionine content in the protein samples with confidence.

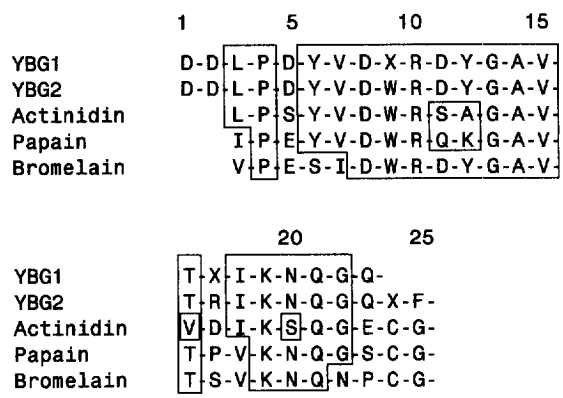

Fig. 1. *N*-terminal amino acid sequence of YBG1 and YBG2 and homology of these proteins to cysteine proteinases: actinidin (*Actinidia chinensis var. deliciosa* [11]), papain (*Carica papaya* [12]), and bromelain (*Ananas comosus* [13]). Amino acids are indicated by single-letter IUPAC nomenclature. X denotes that no amino acid derivative was detected by HPLC. The numbering is in accord with that of YBG1 and identical amino acids are boxed.

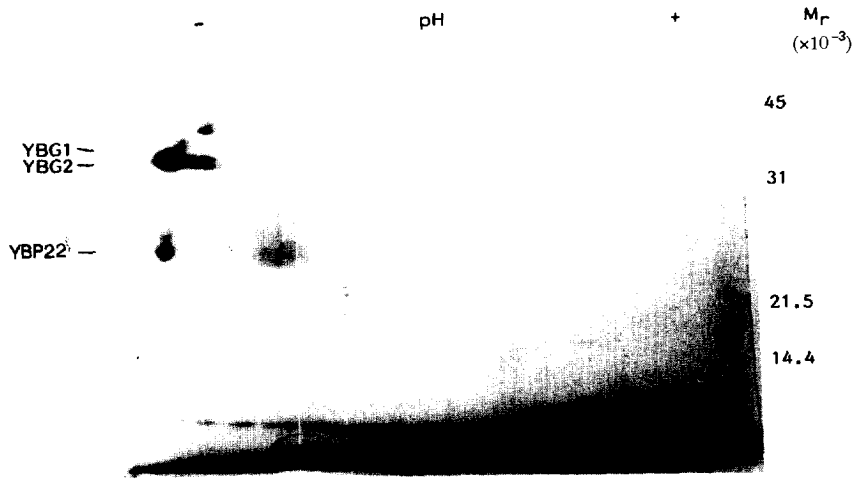

Fig. 2. Two-dimensional gel of yam bean tuber proteins (45% ammonium sulphate fraction). Separation of 60 μg of partially purified yam bean tuber proteins (45% ammonium sulphate fraction) in the horizontal dimension was achieved by isoelectric focusing in the pH range 3–10 on 6.7% polyacrylamide tube gels in the presence of 8 M urea, followed by separation in the vertical dimension by sodium-dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) using a 12% polyacrylamide gel.

first dimension isoelectric focusing was conducted in the presence of 8 M urea.

A database search for sequences with homology to YBG1 and YBG2 showed that these proteins are related to the papain class of cysteine proteases. Cysteine proteases have been isolated from a variety of sources including bacteria, plant and animal tissues. Papain, which is the most studied and best understood of these thiol proteolytic enzymes, showed 70% homology with residues 3–22 of the *N*-terminal sequence of YBG2 (Fig. 1). This high homology is typical of the high degree of sequence conservation in this class of proteases [10]. YBG1 had the same *N*-terminal sequence as YBG2 but we were unable to determine the amino acid residues at positions 9 and 17 of YBG1 (Fig. 1). However, it is likely that YBG1 contains Trp at position 9 and Arg at position 17 since these residues are generally difficult to observe during gas-phase sequencing. It is interesting to note that in nearly all other known cysteine proteases with a high degree of sequence homology to papain such as actinidin and bromelain, proline (Pro) is conserved at position 2. Pro is also conserved in YBG1 and YBG2 but occurs at position 4 (Fig. 1). This conserved Pro residue is believed to prevent undesirable *N*-terminal proteolysis [14]. Since neither the *N*-terminal sequence nor the amino acid composition can prove that YBG1 and YBG2 are proteins resulting from the expression of different genes coding for different isoforms, it is most likely that the difference between these proteins arise from post-translational modifications such as proteolysis or glycosylation.

The possibility that YBG1 and YBG2 are disulphide bonded subunits of similar amino acid sequence was investigated by native gel electrophoresis which showed that these proteins were distinct polypeptides under such conditions and therefore were not covalently linked to each other [Fig. 7(b)]. The possibility that one or both of these proteins contained intramolecular disulphide bonds was investigated by SDS-PAGE in the presence and absence of β-mercaptoethanol. In the absence of β-mercaptoethanol the mobilities of YBG1 and YBG2 on SDS-PAGE gels both increased when compared to their mobilities in the presence of β-mercaptoethanol thereby indicating the presence of intramolecular disulphide bonds (Fig. 3). Papain, which is known to contain intramolecular disulphide bonds, also showed increased mobility in the absence of β-mercaptoethanol (Fig. 3).

YBG1 and YBG2 were both found to be glycoproteins [Fig. 4(b)] by the use of the specific oxidation of carbohydrate by periodate prior to binding with a high-amplification colour generating reagent, biotin-hydrazide (Oxford Glycosystems). Ovalbumin was used as a positive control [Fig. 4(b), lane 2]. YBG1 and YBG2 were also found to bind to a concanavalin A-agarose column [Fig. 4(a)]. The presence of carbohydrates covalently attached to proteins can confer many functions to the glycoprotein, such as resistance to proteolytic degradation [15–16] and the transduction of information between cells [17]. Although papain and actinidin are not glycoproteins, some cysteine proteases such as bromelain are known to be glycosylated. The functional significance of the carbohydrate moieties on these proteases are not known at present. The highly acidic YBG1 and YBG2 glycoproteins were found to have isoelectric focusing points of 4.28 and 4.26, respectively, under native conditions (Fig. 5). In order to distinguish the difference in pI between YBG1 and YBG2, these proteins were individually electroeluted using a Bio-Rad electroeluter and subsequently run on an isoelectric focusing gel, pH range 4–6, in separate wells. The p*I*s obtained for YBG1 and YBG2 were clearly different from the published p*I* of papain (8.5) but is consistent with the lower content of Lys and Arg in YBG1 and YBG2

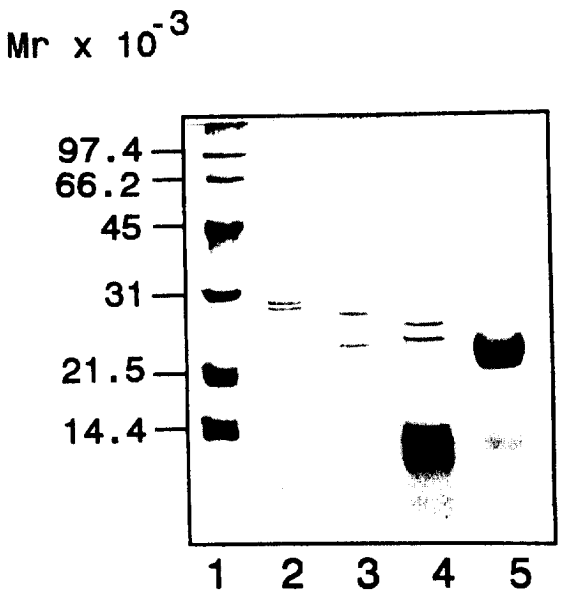

Fig. 3. Effect of β-mercaptoethanol on the electrophoretic mobility of YBG1 and YBG2. 12% SDS-PAGE of purified YBG1 and YBG2 (4 μg) in the presence (lane 2) and absence (lane 3) of β-mercaptoethanol. Lanes 4 and 5 contain papain in the presence (lane 4) and absence (lane 5) of 2-mercaptoethanol. All samples were boiled for 5 min before electrophoresis. Lane 1 contains Bio-Rad low M_r standards; phosphorylase b (97.4 k), bovine serum albumin (66.2 k), ovalbumin (45 k), carbonic anhydrase (31 k), soybean trypsin inhibitor (21.5 k) and lysozyme (14.4 k).

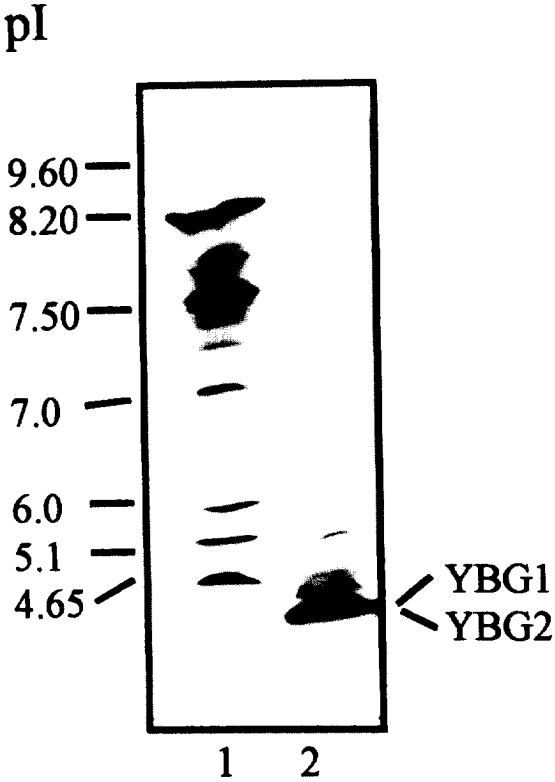

Fig. 5. Isoelectric focusing (IEF) of yam bean tuber proteins. The isoelectric points (pI) of native yam bean tuber proteins were determined by isoelectric focusing (IEF) under non-denaturing conditions (pH 3–10 gradient). Lane 1 contained 2 μl of Bio-Rad (pI 4.6–9.0) IEF standard proteins. Lane 2 contained 30 μg of the 45% ammonium sulphate fraction of yam bean tuber proteins.

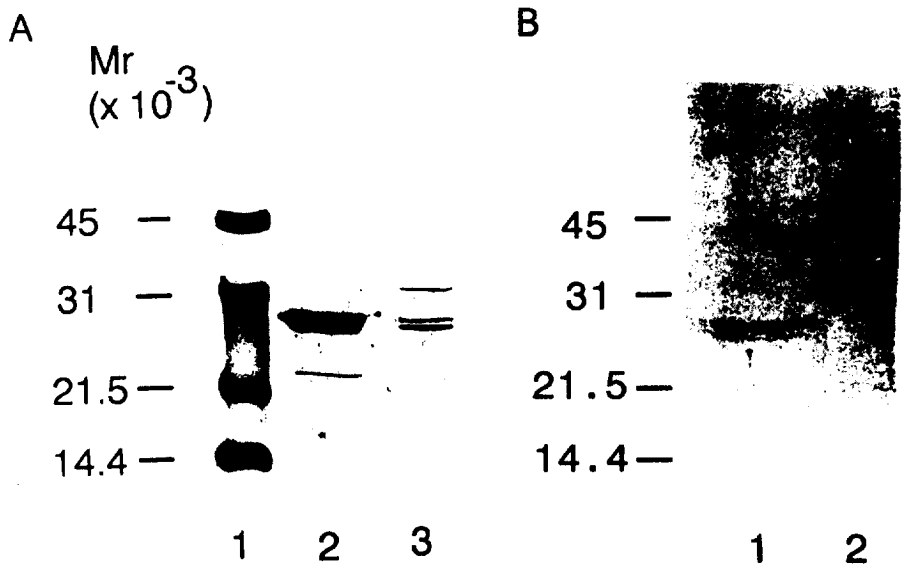

Fig. 4. Identification of YBG1 and YBG2 as glycoproteins: a) SDS-PAGE of Concanavalin A-agarose binding proteins on a 12% gel. Lane 1, Bio-Rad low M_r standards. Lane 2, total yam bean tuber homogenate. Lane 3, tuber proteins eluted from Concanavalin A-agarose column in the presence of 20 mM glucose; b) Transblot of purified YBG1 and YBG2 probed for carbohydrate moieties via the sensitive Oxford Glycosystems GlycoTrack™ glycoprotein detection kit method which uses a specific-carbohydrate oxidation reaction involving periodate prior to binding of a high-amplification colour development reagent. Lane 1, 5 μg of YBG1 and YBG2; lane 2, 1 μg of ovalbumin.

(Table 1). Isoelectric points of cysteine proteases are known to vary from 3.1 to 11.7 [10]. Treatment of the major proteins (YBG1 and YBG2) with SDS and/or β-mercaptoethanol (βME) or dithiothreitol (DTT) were found to cause degradation of these proteins into smaller polypeptides when the sample was incubated for more than 15 min at 37° or above. Similar phenomena were also observed with yam proteins subjected to similar treatments [9]. This suggests that these proteins are all unstable under these conditions.

The multiplicity of bands observed on the IEF gel and on two-dimensional gels are likely to have arisen from both charge and size heterogeneity within the protein sample. It is thought that the extensive charge heterogeneity of patatins may be due to glycosylation and other post-translational modifications [7]. Overall, the data suggest that the major yam bean proteins share some of the features of the storage proteins of other tubers [7, 9].

The papain family contains proteases exhibiting a wide variety of activities, including endopeptidase and exopeptidase activity [18]. Since there are also papain family members which show no catalytic activity [18] we decided to determine if YBG1 and YBG2 were indeed proteases. Investigation of YBG1 and YBG2 for putative protease activity using the protease substrate, azocasein, confirmed the proteolytic activity of these storage proteins (Fig. 6). Although substrate was still present in the assay mixture of YBG1 and YBG2 as evident from the papain assay, no increase in trichloroacetic acid soluble peptides was found after about 15 min, which suggested that YBG1 and YBG2 proteolysed fewer sites on azocasein compared to papain. The thiol proteolytic activity of YBG1 and YBG2 was confirmed with the use of the irreversible cysteine protease inhibitor E-64. E-64 (10 μM) significantly inhibited both papain (58 ± 4% inhibition) and YBG1 and YBG2 (63 ± 5% inhibition) activity suggesting that YBG1 and YBG2 are cysteine proteases. Since we were unable to separate YBG1 from YBG2 conveniently for enzymatic studies, a native gel containing gelatin was used to determine if either or both YBG1 and YBG2 possessed proteolytic activity. Both of these proteins possessed proteolytic activity via this method (Fig. 7). YBG1 and YBG2 were both able to digest the gelatin present in the gel, resulting in clear bands on the Coomassie Blue stained gel.

Most of the simple monomeric proteases of plants, animals and micro-organisms are cysteine proteases with an M_r in the range 20 000–30 000 [19, 20]. Many of them are extracellular enzymes which are easy to purify and assay [21]. In addition, these small, highly active monomeric proteases, when located intracellularly, may be partly responsible for protein turnover [22]. A major function of these proteases is the breakdown of stored proteins for nitrogen mobilization from leaves and storage organs such as the tubers and seeds. The proteases and anti-proteases present in the yam bean tuber are most likely important for physiological processes (i.e. mobilization of reserves, possibly by self digestion) and in defence. A comparison between the major yam bean tuber proteins YBG1 and YBG2 with the potato storage proteins, patatins shows that both groups of proteins have similar properties such as glycosylated residues, moderate to extensive charge heterogeneity, occurrence in large amounts with respect to other soluble proteins and the expression of residual enzymatic activity. These collective features suggest that the major storage proteins may have evolved along similar evolutionary lines.

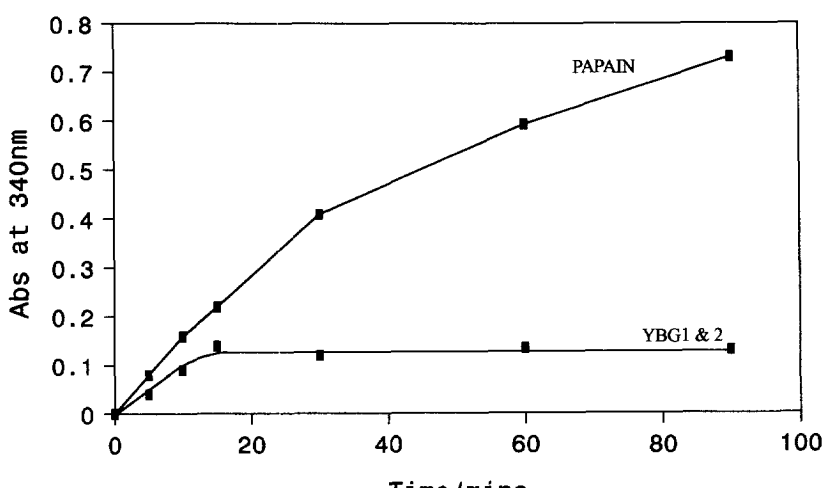

Fig. 6. Time course of the degradation of azocasein by YBG1 and YBG2 and papain. The glycoproteins YBG1 and YBG2 (1.5 μM) were incubated in sodium phosphate buffer (0.1 M, pH 6.5) at 37° with the substrate azocasein (1.8 mg ml^{-1}) in the presence of 2 mM DTT and 5 mM EGTA in a final reaction volume of 1.1 ml. The absorbance of trichloroacetic acid—soluble products were measured at 340 nm.

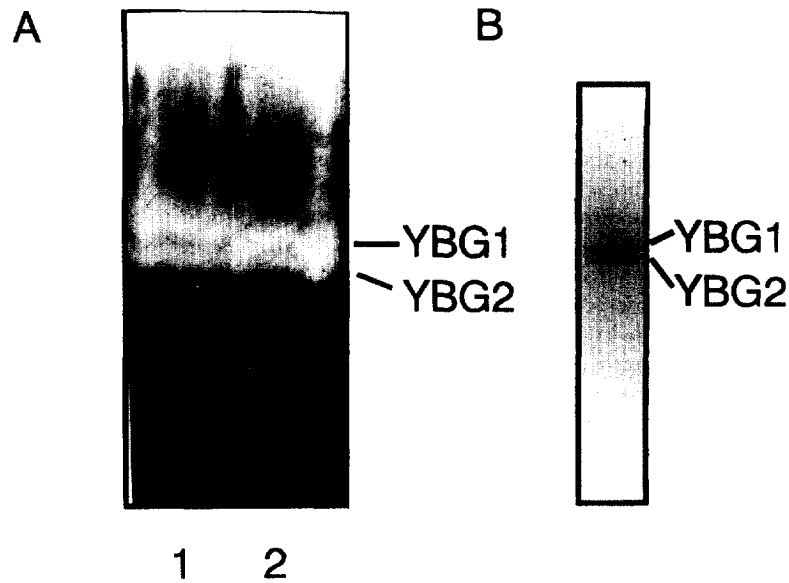

Fig. 7. Native PAG-gelatin gel electrophoresis of YBG1 and YBG2. Purified fractions from the DEAE-Sepharose column were run on a 12% native PAGE-0.2% gelatin gel and left for 12 hr in reaction buffer as described in methods: (a) lane 1 contained 6 μg of purified YBG1 and YBG2 while lane 2 contained 4 μg of purified YBG1 and YBG2; (b) a sample containing 4 μg of purified YBG1 and YBG2 which was electrophoresed on a 12% native gel without gelatin.

Characterization of YBP22

A comparison of the *N*-terminal sequence of YBP22 with other proteins in the Genepro data bank showed that YBP22 has marked sequence homology with several known protease inhibitors (including the Kunitz-type serine protease inhibitors of legumes [23], such as soybean trypsin inhibitor A (STIA)), Win3 gene product of poplar (a wound-inducible inhibitor) [24] and the taste modifying protein miraculin [25]. YBP22 *N*-terminal sequence showed homology with 10 out of 16 amino acids (62.5%) of STIA (Fig. 8). The amino acid analysis of YBP22 (Table 1) showed that it contained moderate amounts of Asp, Glu, Ser, and Lys which are all known to be significant in plant protease inhibitors [26–27]. Furthermore, the amino acid composition of YBP22 was significantly different from YBG1 and YBG2. Investigations utilizing an antibody raised against YBP22 showed cross reaction with soybean trypsin inhibitor (STI) suggesting that YBP22 may be a yam bean trypsin inhibitor (Fig. 9). Anti-YBP22 also cross reacted with several protein bands from the yam bean seed with the most intensely staining band being the same M_r as YBP22 (unpublished data). The fact that anti-YBP22 cross reacted with several other protein bands is consistent with the observation that seeds of the Fabaceae (Leguminosae) are rich sources of trypsin and other serine protease inhibitors [28]. Several inhibitors of trypsin-like enzymes provide weak inhibition of the cysteine pro-

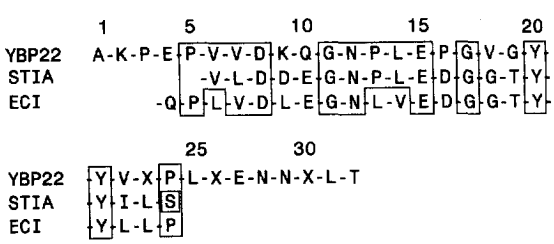

Fig. 8. *N*-terminal amino acid sequence of YBP22 and homology to protease inhibitors. Soybean trypsin inhibitor A (STIA, *Glycine max*) [26] and chymotrypsin inhibitor (ECI, *Erythrina variegata* var. *orientalis*) [27]. Amino acids are indicated by single-letter IUPAC nomenclature. X, no amino acid derivative was detected by HPLC. Identical amino acids are boxed and the numbering is in accord with that of YBP22.

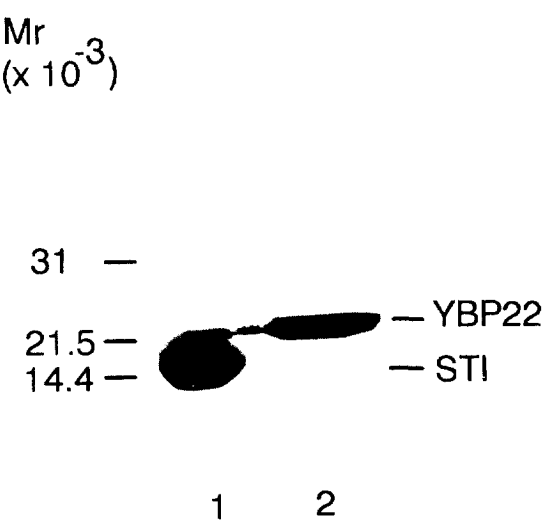

Fig. 9. Immunoblot of yam bean tuber proteins probed with anti-YBP22. Lane 1 contained 2 μg of soybean trypsin inhibitor while lane 2 contained 8 μg of the 45% ammonium sulphate fraction of yam bean tuber proteins. STI, soybean trypsin inhibitor. YBP22, 22 k yam bean tuber protein.

tease papain. Therefore, YBP22, which may be a trypsin-like protease inhibitor could be one of the endogenous inhibitors of YBG1 and YBG2.

Since the proteolytic activity of YBG1 and YBG2 may present a potential threat to the tuber, it is essential that their activity is strictly regulated. As such these proteases are likely to have co-evolved with intrinsic protease inhibitors. Further investigations are in progress to test the hypothesis that YBP22 could be an intrinsic inhibitor of YBG1 and YBG2. We were unable to detect any carbohydrate moiety attached to YBP22 with the aid of the periodate method of Oxford Glycosystems. In addition YBP22 did not bind to the Concanavalin A-agarose affinity matrix; these observations suggest that it is unlikely to be a glycoprotein.

EXPERIMENTAL

Materials. Mature tubers of *P. erosus* L. Urban were harvested from field-grown plots. The gel electrophoresis reagents including molecular markers were obtained from Bio-Rad. Papain was obtained from Boehringer-Mannheim. Soybean trypsin inhibitor (STI), azocasein, Concanavalin A-agarose were all obtained from Sigma. DEAE-Sepharose CL-6B was obtained from Pharmacia. The materials used for preparing buffers and salt solutions were Analar or ACS certified grade and purchased from either BDH Chemicals Ltd. (U.K.) or J. T. Baker Inc. (U.S.A.).

Isolation of soluble storage proteins. Yam bean tuber pith tissue (20 g) was homogenized with 50 mM Tris-acetate pH 7.5 and centrifuged according to the method of ref. [4]. The supernatant was fractionated with 35 to 50% solid $(NH_4)_2SO_4$. Pptd proteins were dissolved in 50 mM Tris-acetate buffer and exhaustively dialysed against 50 mM Tris-acetate buffer pH 7.5 containing 1 mM ethylenediamine tetra-acetic acid (EDTA). The 45% dialysates was aliquoted and stored at $-70°$ until required. All extraction procedures were performed at 4°. The homogenization buffer consisted of 50 mM Tris-acetate, pH 7.5, 1 mM EDTA, 1% ascorbate and 0.5 M sucrose.

DEAE-Sepharose CL-6B column chromatography. YBG1 and YBG2 were purified by a single step procedure utilizing a DEAE-Sepharose column (manuscript in prepn). Briefly, yam bean tuber proteins in the 10 000 *g* supernatant were loaded onto a DEAE-Sepharose column which was equilibrated with 20 mM Tris-HCl pH 7.4, containing 1 mM EDTA, 100 mg l^{-1} phenylmethylsulphonyl fluoride (PMSF) and 10 mM β-mercaptoethanol and proteins eluted with a 10–300 mM NaCl gradient. Then the NaCl concn was increased step wise to 0.4 M. YBG1 and YBG2 were eluted with 0.4 M NaCl soln.

Concanavalin A (Con-A)-agarose column chromatography. 5 mg of tuber proteins was loaded onto a 0.75×5 cm Con-A-agarose column equilibrated with 20 mM Tris-HCl pH 7.2 containing 1 mM $CaCl_2$, 1 mM $MnCl_2$, 1 mM $MgCl_2$ and 200 mM NaCl. The column was washed with equilibration buffer until no more protein was detected in the washings from the column and then con A-binding proteins were eluted with a step-wise gradient of 10–100 mM glucose in 20 mM Tris-HCl pH 7.2 containing 200 mM NaCl.

Cysteine protease activity. The glycoproteins YBG1 and YBG2 (1.5 μM) and papain (65 nM) were incubated in Na-Pi buffer (0.1 M, pH 6.5) at 37° with the substrate azocasein (1.8 mg ml^{-1}) in the presence of 2 mM DTT and 5 mM EGTA in a final reaction vol. of 1.1 ml. 0.25 ml of the mixt. was withdrawn at intervals, as indicated in Fig. 6, and added to 0.5 ml of 10% CCl_3COOH (TCA). After centrifugation at 12 000 rpm for 5 min the *A* of TCA-soluble products were measured at 340 nm. Suitable controls were carried out using boiled YBG1 and YBG2 and papain instead of native proteins and these values obtained subtracted from those obtained using the native enzymes. In experiments where E-64 was utilized 65 nM papain and 1 μM YBG1 and YBG2 were incubated in the buffer/azocasein mixt. as described above, with some tubes containing 100 μM E-64. The reaction was stopped after 30 min and the *A* at 340 nm determined.

Gel protease assay. Samples were mixed with $\times 4$ non-denaturing sample buffer (i.e. sample buffer without SDS or β-mercaptoethanol) to obtain a final $\times 1$ buffer concn and loaded onto a 12% non-denaturing polyacrylamide gel (PAG) containing 0.2% gelatin. After electrophoresis at 100 V (constant) in cold buffer, the gels were equilibrated in 10 mM Na-Pi buffer pH 6.5 containing 1% Triton X-100 for 1 hr with gentle shaking and then incubated for 0–24 hr in 50 mM Na-Pi buffer pH 6.5 containing 5 mM EGTA and 2 mM DTT at 25° with gentle agitation. The gels were then stained and destained as for SDS-PAGE gels.

Glycoprotein detection. Glycoprotein detection was carried out on transblotted yam bean samples using the Glyco Track™ glycoprotein detection kit (Oxford Glycosystems, U.K.) as described in the instruction manual. Glycoprotein staining was also performed according to the method of ref. [29]. Bovine serum albumin (BSA) and ovalbumin (Oxford Glycosystems, U.K.) were used as negative and positive glycoprotein standards, respectively.

Densitometric analysis. A Bio-Rad Model 620 video densitometer was used to quantify the amount of protein present in the samples relative to all the stained bands present in the gel.

Protein determination. Protein concn was routinely performed using the method of ref. [30] with BSA as standard.

Total nitrogen content. This was determined by the Kjeldahl technique according to the method of ref. [31].

Amino acid composition and N-terminal sequence determination. Samples for amino acid analysis were electrophoresed on a Bio-Rad mini gel according to the method of ref. [32]. After electrophoresis, the proteins were transblotted onto polyvinylidene difluoride (PVDF) membrane and stained for 5 min in 0.25%

Coomassie Blue R-250 in 40% MeOH. The membrane was then washed thoroughly with milli Q H_2O at room temp. air dried and the protein bands of interest excised and stored in Eppendorf tubes at −20°. Amino acid analysis was performed on an automatic Applied Biosystems model 420 amino acid analyser on replicate hydrolysates according to the manufacturers instructions. Sequential prepn of phenylthiohydantoin derivatives of amino acids from the amino-terminus was performed with an Applied Biosystems model 473 sequence analyser according to the directions of the manufacturer.

Polyacrylamide gel electrophoresis. Non-denaturing gel electrophoresis was performed at pH 8.8 on different percentage polyacrylamide slab gels according to the method of ref. [33]. Standard gel electrophoresis in the presence of 0.1% SDS at pH 8.3 (SDS-PAGE) was performed according to the method of ref. [32]. M_r determinations were based on Bio-Rad low M_r markers: phosphorylase b (97.4 k), bovine serum albumin (66.2 k), ovalbumin (45 k), carbonic anhydrase (31 k), soybean trypsin inhibitor (21.5 k) and lysozyme (14.4 k). Gels were stained for 30 min with 0.1% Coomassie Blue R-250 in fixative (40% MeOH, 10% HOAc) and destained overnight in the same fixative.

Two-dimensional gel electrophoresis. Protein samples were first sepd by isoelectric focusing on rod gels in the first dimension. The gels were then incubated for 45 min in SDS-PAGE sample buffer, then sealed to the surface on an SDS slab gel with 2% agarose in 62.5 mM Tris-HCl pH 6.8 buffer, before electrophoresis in the second dimension as described in ref. [34].

Isoelectric focusing. Isoelectric focusing of native protein samples was performed on 5% polyacrylamide mini gels containing 4% carrier ampholyte (pH 3–10) and 10% glycerol according to a modification of the method of ref. [35]. The pH 4–6 gradient was obtained by using pre-blended pH 4–6 carrier ampholytes (Bio-Rad). A 0.75 mm thick gel was used, which was pre-run for 10 min at 100 V and then 10 min at 200 V before loading samples. After loading the samples, the gel was run at 200 V for 1.5 hr and then at 400 V for a further 1.5 hr. The gels were then fixed in 12.5% TCA containing 30% MeOH for 15 min, rinsed with H_2O and stained with a 1% soln of Coomassie Brilliant Blue (R-250) for 20 min and destained in an MeOH–HOAc–H_2O (5:1:4) soln.

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