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ASPARTIC PROTEINASE LEVELS IN SEEDS OF DIFFERENT ANGIOSPERMS

GESINE VOIGT, BÖLE BIEHL, HEINRICH HEINRICHS and JÜRGEN VOIGT*†

Botanisches Institut, Technische Universität Braunschweig, Mendelssohnstraße 4, D-38092 Braunschweig, Germany

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Abstract—Aspartic proteinases have been detected in seeds of numerous angiosperms and of gymnosperms but their biological function is still unknown. We have analysed the specific activities of these endoproteinases in ungerminated seeds of different monocot and dicot plants. Rather similar aspartic endoproteinase levels were found in the seeds of most of the investigated plants but considerably elevated specific activities were measured in the recalcitrant seeds of *Hevea brasiliensis* and especially in the case of *Theobroma cacao*. In all the different plants, the levels of cysteine proteinases in the ungerminated seeds were lower than the specific activities of aspartic proteinases. Only small variations of aspartic proteinase activities were observed during germination. Copyright © 1997 Elsevier Science Ltd

INTRODUCTION

Aspartic proteinases (EC 3.4.23) are a class of endopeptidases that are active at acidic pH, inhibited by pepstatin A and show a conserved three-dimensional structure with two Asp residues in the active cleft [1, 2]. Plant aspartic endoproteinases have been detected in the seeds of monocot [3–5] and dicot [6–9] species as well as of gymnosperms [10], but have gained much less attention than the cysteine proteinases which play a major role in the degradation of storage proteins during germination [11]. Therefore, little is known about the biological function of plant aspartic proteinases. In tomato and tobacco, they are apparently implicated in the degradation of extracellular pathogenesis-related proteins [8]. On the other hand, aspartic proteinases have been reported to be associated with the intracellular protein bodies of buckwheat and hemp seeds [7, 12]. In ungerminated cocoa seeds, however, the intracellular aspartic proteinase did not co-sediment with the protein bodies [13], but was found to be involved in the post-mortem proteolysis of the 7S globular storage protein resulting in the generation of the cocoa-specific aroma precursors during fermentation of the cocoa seeds [14–16]. Therefore, we have compared the specific activities of aspartic proteinases in ungerminated and germinating seeds of different angiosperms.

RESULTS AND DISCUSSION

In order to comparatively investigate the aspartic proteinase levels, polyphenol-free acetone dry powders were prepared from the ungerminated seeds of different plant species and analysed for their proteolytic activities in the absence and presence of proteinase inhibitors using haemoglobin as the substrate and at a temperature of 45° since maximal proteolytic activities were measured under these conditions for all the species investigated. All incubations were performed at pH 3.5–4.0 because most aspartic proteinases were shown to exhibit their maximal activities in this pH range [1, 2]. In the absence of proteinase inhibitors, proteolytic activities between 1.7 (*Hordeum vulgare*) and 5.5 mU mg^{-1} protein (*Cucumis sativus*) have been determined for the ungerminated seeds of most monocot and dicot species (Table 1). However, considerably elevated proteolytic activities have been measured for the acetone dry powders prepared from the recalcitrant seeds of the tropical dicot plants *Hevea brasiliensis* (13 mU mg protein^{-1}) and especially *Theobroma cacao* (72 mU mg protein^{-1}). The major parts of these activites were sensitive to pepstatin A (52–100%) and may, therefore, be attributed to the action of aspartic proteinases (Table 1). On the other hand, only 0–23% of total proteolytic activities were sensitive to the cysteine proteinase inhibitor iodoacetic acid (Table 1). However, the iodoacetic acid-sensitive cysteine proteinases have been reported to exhibit maximal activities between pH 5.0 and 5.5 [11], the activities measured at pH 3.5–4.0 (Table 1) only representing about 20–30% of the maximal activities (data not

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*Author to whom correspondence should be addressed.

† Present address: Physiologisch-chemisches Institut, Universität Tübingen, Hoppe-Seyler-Strasse 4, D-72076 Tübingen, Germany.

Table 1. Total proteolytic activities, aspartic proteinase and cysteine protease activities in ungerminated seeds of different monocotyledonous and dicotyledonous plants

Species	Protein (mg mg AcDP^{-1})	Total proteolytic activity* (mU mg protein^{-1})	Aspartic proteinase activity† (mU mg protein^{-1})	[%]	Cysteine proteinase activity‡ (mU mg protein^{-1})	[%]
H. vulgare‖	0.26±0.02	1.7±0.33	1.8±0.42 (1.6±0.36)	100 (73)	0	0
O. sativa‖	0.28±0.02	1.9±0.29	1.5±0.14 (1.2±0.08)	79 (71)	0.4±0.18	21
T. aestivum§	0.30±0.03	2.9±0.23	3.1±0.11 (2.4±0.08)	100 (86)	0	0
Z. mays§	0.29±0.03	3.0±0.37	1.8±0.17 (1.5±0.32)	60 (58)	0.7±0.20	23
A. hippocastanum§	0.39±0.04	4.5±0.55	3.0±0.28 (2.6±0.32)	67 (58)	0.2±0.10	4.4
C. sativus§	0.56±0.04	5.5±0.80	3.3±0.17 (3.0±0.40)	60 (66)	0	0
G. max‖	0.64±0.05	3.5±0.14	2.6±0.27 (2.3±0.22)	74 (66)	0	0
H. brasiliensis§	0.52±0.05	13.1±0.71	7.0±1.62 (4.3±0.95)	53 (33)	1.8±0.32	14
P. vulgaris§	0.44±0.03	2.9±0.39	1.5±0.35 (0.6±0.32)	52 (32)	0.5±0.23	17
T. cacao§	0.38±0.04	72.4±1.90	70.3±3.2 (61.5±2.55)	97 (85)	2.7±0.82	3.7

*Total proteolytic activities of polyphenol-free acetone dry powder were measured using haemoglobin as substrate as described in the Experimental.

†Proteolytic activities which could be inhibited by addition of pepstatin A (final concentration 5 μg ml^{-1}).

‡Proteolytic activity which could be inhibited by addition of iodoacetic acid (final concentration 200 μg ml^{-1}).

Determinations of proteolytic activities were performed at pH 3.5§ and pH 4.0‖, respectively. Values represent means (±S.D.) of at least four independent experiments.

shown). Therefore, in the case of *Phaseolus vulgaris*, *Oryza sativa* and *Zea mays*, the true cysteine proteinase levels were similar to the aspartic proteinase levels. In the seeds of the other plants, however, the aspartic proteinase activities were considerably higher than the corrected cysteine proteinase activities.

The apparent aspartic proteinase levels measured as the pepstatin-sensitive proportion of total proteolytic activities (Table 1) might be influenced by the action of other endo- and exoprotease activities. For this reason, we have also determined the aspartic proteinase activities by measuring the pepstatin-sensitive proteolytic activities in the presence of the endo- and exoprotease inhibitors EDTA, IAA (iodoacetic acid) and PMSF (phenylmethylsulfonyl fluoride). Essentially, the same pepstatin-sensitive (aspartic) proteinase activities were measured under these conditions (Table 1; values in brackets) as in the absence of EDTA, IAA and PMSF, with the exceptions of *H. brasiliensis* and *P. vulgaris*. Again, exceptionally high aspartic proteinase activities were found in the seeds of *H. brasiliensis* and especially *T. cacao* L.

Only small variations of the aspartic proteinase activities were observed during seed germination of *T. cacao*, *C. sativus*, *Glycine max*, *O. sativa*, and *Triticum aestivum* which could not be correlated with the degradation of storage proteins (days 6–10; data not shown). The rather high proportions of total proteolytic activities present in the ungerminated seeds of different monocot and dicot plants which could be attributed to the widespread aspartic proteinases indicate that this particular class of endopeptidases may have an important biological function during seed ripening which is, however, still unknown. This conclusion is corroborated by our recent observation that aspartic proteinase and globular storage proteins were co-ordinately accumulated during the development of *Theobroma cacao* seeds [17]. However, the question arises why the levels of aspartic proteinases are considerably elevated in the case of the ungerminated recalcitrant seeds of the tropical plants *H. brasiliensis* and especially *T. cacao*.

EXPERIMENTAL

Materials. The origins of the seeds used in the present study are as follows: *Aesculus hippocastanum* L. (genetically undefined seeds from the city area of Braunschweig, Germany); *C. sativus* L. (var. "Bastal" (F1 hybrid), from Carl Sperling & Co., Lüneburg, Germany); *G. max* L. Merr. (var. "Maple Arrow", obtained from Carl Sperling & Co., Lüneburg, Germany); *H. brasiliensis* L. (genetically undefined seeds provided by Dr M. W. Müller, CEPLAC, Salvador, Bahia, Brazil); *H. vulgare* L. (var. "Ca 700 604", from Carlsberg Research Center, Copenhagen, Denmark); *O. sativa* L. (var. "Basmati 370", from Lehnig, Braunschweig, Germany); *T. cacao* L. (genetically undefined pods provided by Dr S. Kamaruddin, MARDI, Cocoa and Coconut Division, Hilir Perak, Malaysia); *T. aestivum* L. (var. "Saratovskaya 29", from the Bundesforschungsanstalt für Landwirtschaft FAL; Braunschweig-Völkenrode, Germany); *Z. mays* L. (var. "ZW-1072", from the Institut für Pflanzengenetik und Kulturpflanzenforschung Gatersleben, Germany). The ungerminated seeds of *A. hippocastanum*, *C. sativus*, *G. max*, *H. brasiliensis*, *P. vulgaris* and *T. cacao* were shock-frozen in liquid N_2 after removal of testas and radicles and freeze-dried. The grains of *H. vulgare*, *O. sativa*, *T. aestivum* and *Z. mays*, however, were used without pretreatment.

Germination. Ripe seeds of *T. cacao* from which pulp and testas had been removed, respectively, were set in moist vermiculite and incubated in the dark at 30°. Ten seeds were sampled at 0, 1, 3, 7 and 10 days, respectively, after imbibition. Seeds of *C. sativus*, *G. max*, *O. sativa* and *T. aestivum* were germinated in liquid cultures (tap water bubbled with air) at room temp. Seeds (20 g) corresponding to the initial dry weight were incubated for 0, 1, 3, 7 and 10 days, respectively. After removal of testas (in the case of dicot plants), roots and hypocotyls, the cotyledons and grains, respectively, were frozen in liquid N_2 and freeze-dried.

Preparation of acetone dry powders. The dry cotyledons and grains, respectively, were crushed in liquid N_2 and defatted by repeated extractions in a Soxhlet apparatus with petrol (bp 40–70°) at 45°. After solvent evapn, the materials were powdered and extracted again for 8 hr in the same manner. After extraction of fat, the powders were extracted with aq. Me_2CO essentially as described in ref. [18]. To remove the polyphenols, the defatted seed powders were extracted 3× with 80% (v/v) aq. Me_2CO containing 5 mM Na ascorbate and subsequently with 70% (v/v) aq. Me_2CO. The suspensions (20 ml aq. Me_2CO per 1 g seed powder) were stirred for 1 hr at 4° and the extracts removed by centrifugation (15 min at 13 000 *g*). After the final extraction step, the efficiency of polyphenol extraction was checked by heating an aliquot of the Me_2CO dry powder with 5 M HCl (red colour indicates the presence of residual polyphenols). After complete extraction of polyphenols, residual H_2O was removed by extraction with 100% Me_2CO. The residual solvent was removed by evapn under red. pres. and the Me_2CO dry powders (AcDPs) were stored at −20°.

Determination of protein. The proportions of protein concn present in the different Me_2CO dry powders were analysed by a modified biuret method [19] using bovine serum albumin as standard.

Enzyme solutions. Aliquots of the different Me_2CO dry powders corresponding to 50 mg of protein were homogenized with 1 ml of 20 mM Na_2HPO_4 adjusted to pH 6.8 by addition of citric acid. Where indicated, the protease inhibitors pepstatin A (final concn 5 $\mu g/ml^{-1}$), PMSF (final concn 900 $\mu g\ ml^{-1}$), iodoacetic acid (final concn 200 $\mu g/ml^{-1}$) and EDTA (final concn 3.75 mg ml^{-1}), respectively, were added from 200-fold stocks in 96% (v/v) MeOH (pepstatin A and PMSF) or in H_2O (iodoacetic acid and EDTA). The homogenates were preincubated for 2 hr at 0° in the absence or presence of protease inhibitors prior to the determination of protease activities.

Determination of protease activities. Protease activities were assayed at 45° and pH 4.5–4.0 in McIlvaine buffer (0.2 M Na_2HPO_4 adjusted to the desired pH value by addition of citric acid) using haemoglobin as substrate. Haemoglobin was dissolved in 0.2 M Na_2HPO_4 and the solution subsequently adjusted to pH 3.5 or 4.0 by addition of citric acid to get a stock soln of 1% (w/v). The substrate (900–990 μl) was mixed with 10–100 μl of the crude enzyme (final vol. 1 ml). The reactions were stopped immediately (= controls) or after a 1 hr incubation at 45° by addition of 0.2 ml of CCl_3COOH (25%, w/v). The precipitated protein was removed by centrifugation at 10 000 g for 15 min. Subsequently, proteolysis was measured colorimetrically by the trinitro-benzenesulphonic acid method as described in ref. [20]. One unit of protease is the activity which releases 1 μmol NH_2-groups per min at pH 3.5–4.0 (0.2 M McIlvaine buffer) and 45°.

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