Available online at www.sciencedirect.com
SCIENCE DIRECT®
PERGAMON
Phytochemistry 64 (2003) 689–699
PHYTOCHEMISTRY
www.elsevier.com/locate/phytochem

Isolation and characterisation of phytase from dormant *Corylus avellana* seeds

Vasilios M.E. Andriotis[1], James D. Ross*

School of Plant Sciences, The University of Reading, Whiteknights, Reading RG6 6AS, UK

Received 30 January 2003; received in revised form 10 June 2003; accepted 13 June 2003

Abstract

Phytase (*myo*-inositol-1,2,3,4,5,6-*hexakis*phosphate phosphohydrolase, EC 3.1.3.26), which catalyses the step-wise hydrolysis of phytic acid, was purified from cotyledons of dormant *Corylus avellana* L. seeds. The enzyme was separated from the major soluble acid phosphatase by successive $(NH_4)_2SO_4$ precipitation, gel filtration and cation exchange chromatography resulting in a 300-fold purification and yield of 7.5%. The native enzyme positively interacted with Concanavalin A suggesting that it is putatively glycosylated. After size exclusion chromatography and SDS–PAGE it was found to be a monomeric protein with molecular mass 72 ± 2.5 kDa. The hazel enzyme exhibited optimum activity for phytic acid hydrolysis at pH 5 and, like other phytases, had broad substrate specificity. It exhibited the lowest K_m (162 μM) and highest specificity constant (V_{max}/K_m) for phytic acid, indicating that this is the preferred *in vivo* substrate. It required no metal ion as a co-factor, while inorganic phosphate and fluoride competitively inhibited enzymic activity ($K_i = 407$ μM and $K_i = 205$ μM, respectively).

Keywords: *Corylus avellana*; Betulaceae; Hazel; Protein purification; Phytase; Phytic acid; Dormancy; Seed

1. Introduction

The phosphatases constitute a class of enzymes responsible for the mobilisation of phosphate reserves. They exhibit great heterogeneity with respect to catalytic properties and physiological functions (Duff et al., 1994). Of particular importance are the phytases (*myo*-inositol-1,2,3,4,5,6-*hexakis*phosphate phosphohydrolases, acidic pH optimum, EC 3.1.3.26) which belong to a sub-class of the acid phosphatases (APases, EC 3.1.3.2) (Duff et al., 1994). Alkaline phytases have also been reported to be present in the pollen of *Lilium longiflorum* (Scott and Loewus, 1986), *Typha latifolia* (Hara et al., 1985) and in legume seeds (Scott, 1991). Contrary to the majority of APases, the phytases exhibit a quite clear preference toward phytic acid (IP6, *myo*-inositol-1,2,3,4,5,6-*hexakis*phosphate). The latter exists as the K^+, Mg^+, Ca^{2+}, Zn^{2+} salts of the free acid, collectively known as phytate and is present in seeds, pollen, tubers and vegetative organs (Lott et al., 1995). Phytase (acidic pH optimum) specificity, however, is also not absolute, for the enzyme can release inorganic phosphate (Pi) from a variety of phosphorylated esters (Duff et al., 1994), while the alkaline phytases possess a more absolute specificity (Baldi et al., 1988).

In seeds, IP6 is the major store of phosphate. It accounts for 1–5% of dry wt., representing 50–80% of the total P content. Hence, it is generally assumed that the major role of IP6 is in Pi storage (Lott et al., 1995). It is also regarded as a store of *myo*-inositol which, via the *myo*-inositol oxidation pathway is directed to cell wall polysaccharide biosynthesis (Loewus and Murthy, 2000), thus being important for cell wall elongation and growth. *Myo*-inositol has been also implicated in seed desiccation, auxin physiology, biosynthesis of raffinose and galactopinitol oligosaccharides important for osmoregulation and stress responses, in the synthesis of phosphoinositides, in protein anchoring to cellular membranes (for a detailed discussion on *myo*-inositol functions refer to Loewus and Murthy, 2000). Since IP6 is present as the salt with cations it may represent a pool of mineral reserves (Lott et al., 1995). It has been proposed that IP6

* Corresponding author. Tel.: +44-118-3-788761; fax: +44-118-9-314962.
E-mail address: j.d.ross@reading.ac.uk (J.D. Ross).
[1] Present address: The Sainsbury Laboratory, John Innes Centre, Norwich Research Park, Colney Lane, Norwich NR4 7UH, UK.

doi:10.1016/S0031-9422(03)00415-1

may act as a potent antioxidant by virtue of its capacity to chelate free iron, thus preventing the generation of hydroxyl radicals ($OH^{\cdot}$) (Graf et al, 1987), which could in turn explain the long viability of some seeds (Laboure et al., 1993). IP6 has been also suggested to act as a second messenger ligand (Sasakawa et al., 1995), play a role in ATP metabolism (Safrany et al., 1999), DNA repair (Hanakahi et al., 2000), RNA export from the nucleus (York et al., 1999).

During pollen and seed germination phytase activity markedly increases with a concomitant reduction of IP6 levels (Lin et al., 1987; Gibson and Ullah, 1988). Low levels of phytase activity are usually found in dry seeds (Laboure et al., 1993; Gibson and Ullah, 1988). However, it is not clear whether de novo synthesis of the protein or activation of pre-existing enzyme could explain the subsequent rise in phytase activity (Gibson and Ullah, 1990; Laboure et al., 1993).

Phytase has been studied in both microorganisms and plants (Greiner et al., 2000; Wodzinski and Ullah, 1996; Gibson and Ullah, 1990, and references therein). The major difficulty usually encountered is the separation of phytase from contaminating APases, since these enzymes share many properties in common, thus rendering isolation attempts problematic. Partial purification is often achieved or fairly crude preparations are used, so that any conclusions on enzymic properties should be treated with care since they could well be attributed to any contaminating APases (Gibson and Ullah, 1990). Additionally, most of the reports refer to germinating seeds or pollen, while little is known about IP6 mobilisation and its regulation during seed dormancy relief, despite the importance of this unique and critical transition phase in the life history of higher plants.

In the course of our investigations on IP6 and Pi mobilisation during hazel seed dormancy relief by chilling we have purified a phytase enzyme, the first time from dormant seeds. In the present communication we report the isolation procedure and biochemical characterisation of the purified enzyme, with the aim of obtaining information regarding its biochemical properties and to allow comparison with previously well-characterised phytases from other sources.

2. Results and discussion

2.1. Purification of phytase

The hazel phytase was purified from dormant seeds by successive ammonium sulphate precipitation, size exclusion and cation exchange chromatographies, as detailed in Table 1. When the 30–75% $(NH_4)_2SO_4$-saturated fraction was analysed by Native-PAGE and the gel stained for APase activity three major bands of activity appeared with R_f 0.24, 0.61 and 0.64. These correspond to the activity bands indicated in Fig. 1 b. Incubation of a duplicate gel in the presence of 20mM NaF, a known inhibitor of APases and phytases, showed no appreciable staining of enzymic activity. This indicated that the bands observed in the former were APases (results not shown).

Size exclusion chromatography of the 30–75% $(NH_4)_2SO_4$-precipitated fraction resulted in both phytase and APase being included in the Sephacryl S-200 column (Fig. 1a). Electrophoretic analysis of the eluted fractions under non-denaturing conditions and post-electrophoresis staining for enzymic activity revealed that the band with R_f 0.24 corresponded to the major APase, while the phytase peak corresponded to the bands with R_f 0.61 and 0.64 (Fig. 1b). This gel filtration step did not resolve the two enzymes (Fig. 1a) with the protocol utilised; changing parameters, such as flow rate and/or sample volume, did not enhance resolution.

Both phytase and APase were retained on a Mono S cation exchange column at pH 4.5 and were released by an increasing NaCl gradient. Phytase eluted at ca. 80mM NaCl (Fig. 2). This phytase preparation also exhibited activity toward *p*-nitrophenyl phosphate (*p*NPP), a substrate commonly used in assays of APase activity. As judged by the yield against *p*NPP in the final phytase preparation (Mono S step peak marked with *, 0.2%) and in the APase activity peak (Mono S step peak marked with closed arrowhead, 22.5%), the two enzymes can be considered as being resolved.

The procedure described allowed for the hazel phytase to be purified ca. 300-fold with a yield of 7.5% and specific activity 394 10^{-3} mmoles Pi mg^{-1}protein. After each purification step quite large losses of enzymic activity were evident. These were not prevented by addition of glycerol (Laboure et al., 1993), Triton X-100 (Nakano et al., 1999), or phytate (Hara et al., 1985) in the buffers employed during chromatographic separations. The possibility exists that the removal of some stabilising factor resulted in enzyme inactivation, however, such an agent is yet to be found. Nevertheless, it is known that phytases, and APases in general, are quite unstable in dilute solutions and suffer by surface inactivation.

2.2. Biophysical properties

The molecular mass of the purified phytase was estimated after gel filtration on a calibrated Sephacryl S-200 column. As judged by the elution profile of the Sephacryl column, the native enzyme has an apparent molecular weight (M_r) 72±2.5 kDa (Fig. 1a, inset). After SDS–PAGE three closely migrating bands were observed, a major one with M_r 71 kDa and two minor bands with M_r 72.2 and 73.4 kDa respectively (Fig. 3a). The same banding profile after SDS–PAGE was obtained both in the presence (Fig. 3a) and in the

Table 1
Purification scheme of the hazel phytase

Step	Total protein (mg)	Total activity (Units)	Specific activity (mmoles Pi mg^{-1} protein h^{-1})	Purification (*n*-fold)	Yield (%)	
					phytase	APase
Crude	174.0	226×10^{-3}	1.3×10^{-3}	1	100	100
30–75%-satd.-$(NH_4)_2SO_4$	62.5	106×10^{-3}	2×10^{-3}	1.5	48	64
Sephacryl S-200	10.5	71×10^{-3}	7×10^{-3}	5.3	31.5	59
Mono S	0.042	17×10^{-3}	394×10^{-3}	303	7.5	0.2[a] 22.5[b]

[a] Yield against *p*NPP in the final phytase preparation (activity peak marked with * in Fig. 2).
[b] Yield against *p*NPP in the fractions with the highest APase activity (activity peak marked with closed arrowhead in Fig. 2).

absence (data not shown) of β-mercaptoethanol. These combined results suggest that the hazel phytase may be a monomeric protein. The determined M_r of the hazel enzyme is of the same order of magnitude as values previously reported for phytases from other plant sources (Greiner et al., 2000; Duff et al., 1994; Gibson and Ullah, 1988). However, in maize seedlings (Laboure et al., 1993) and roots (Hubel and Beck, 1996) the enzyme was found to be a homodimer with subunit M_r 38kDa. The hazel protein did not cross-react with rabbit polyclonal antibodies raised against the purified phytase from maize seedlings (kindly provided by Dr. A.M. Lescure, INRA, France) after Western blotting (results not shown).

Two bands of activity were evident after both Native-PAGE (Fig. 3b) and Native-IEF (Fig. 3c). The estimated pI values were ca. pH 6.1 and pH 6.2. The observed banding profile under non-reducing conditions could indicate that the enzyme was either not electrophoretically pure or there exist isoforms. The preparative nature of the gel filtration chromatographic step did not enable us to elucidate whether multiple size isomers exist. Furthermore, the close pI values determined after Native-IEF could indicate that separation of possible charge isomers by an ion exchange chromatographic step, such as the Mono S, would not be adequate. Nonetheless, multiple phytase forms have been reported (e.g., Hubel and Beck, 1996; Greiner et al., 2000). Gibson and Ullah (1988) in germinating soybean determined two peaks of phytase activity after chromatofocusing with estimated pI values at pH 5.5 and 5.0, although the latter was attributed to charge modification of the major pH 5.5 peak.

2.3. Glycosylation

It was found that the hazel enzyme interacted with Concanavalin A-Sepharose 4B (ConA) and could be subsequently affinity eluted from the column with α-methyl-D-glucopyranoside (Fig. 4). Non-specific interaction of the phytase protein with the chromatographic resin is ruled out by the high ionic strength of the buffer used. Also, a carry-over due to a possible carbohydrate-binding domain in the phytase molecule, which could facilitate an interaction with carbohydrate moieties of other glycoproteins (Faye et al., 1989), should also be dismissed due to the presence of high levels of salt in the buffer.

No staining indicative of carbohydrates was observed with periodic acid–Shiff (PAS) after Native-PAGE, while the major APase stained positively (results not shown). However, the PAS staining of carbohydrates is not a very sensitive method of glycoprotein detection after PAGE (Dubray and Bezard, 1982). Since the carbohydrate content of glycoproteins is usually low it is often necessary to over-load the gel to obtain an accurate positive or negative result (ibid.). This could explain both the results of the present study and those previously reported for purified phytases from other sources (Laboure et al., 1993; Gibson and Ullah, 1988). The latter authors reported that ovalbumin at concentration equivalent to the purified phytases was stained positively with PAS. However, ovalbumin has a high carbohydrate content e.g., 2% mannose and 1.2% glucosamine (Dubray and Bezard, 1982; and references therein).

The positive interaction of the hazel phytase with ConA is regarded as an indication for its putative glycosylation status, while further studies are required to fully elucidate this. However, such a post-translational modification could have resulted in the banding profile of the purified protein as observed after SDS-PAGE and Native-PAGE/IEF. Hegeman and Grabau (2001) have also isolated a phytase from soybean seeds with sequence similarity to the purple APases, this enzyme also interacting positively with ConA. Phytases from microorganisms have been shown to be glycoproteins, for example the *Aspergillus niger* phytase (Han and Lei, 1999; Ullah et al., 1999). Glycosylation of the *A. niger* phytase was essential for increased biosynthesis and higher thermostability of the protein when expressed in *Pichia pastoris* (Han and Lei, 1999). Yamagata et al. (1980) have purified and characterised an APase from rice aleurone particles which both positively interacted with ConA and stained with PAS. This enzyme, however, could be tentatively identified as a phytase on grounds of substrate specificity and kinetic parameters.

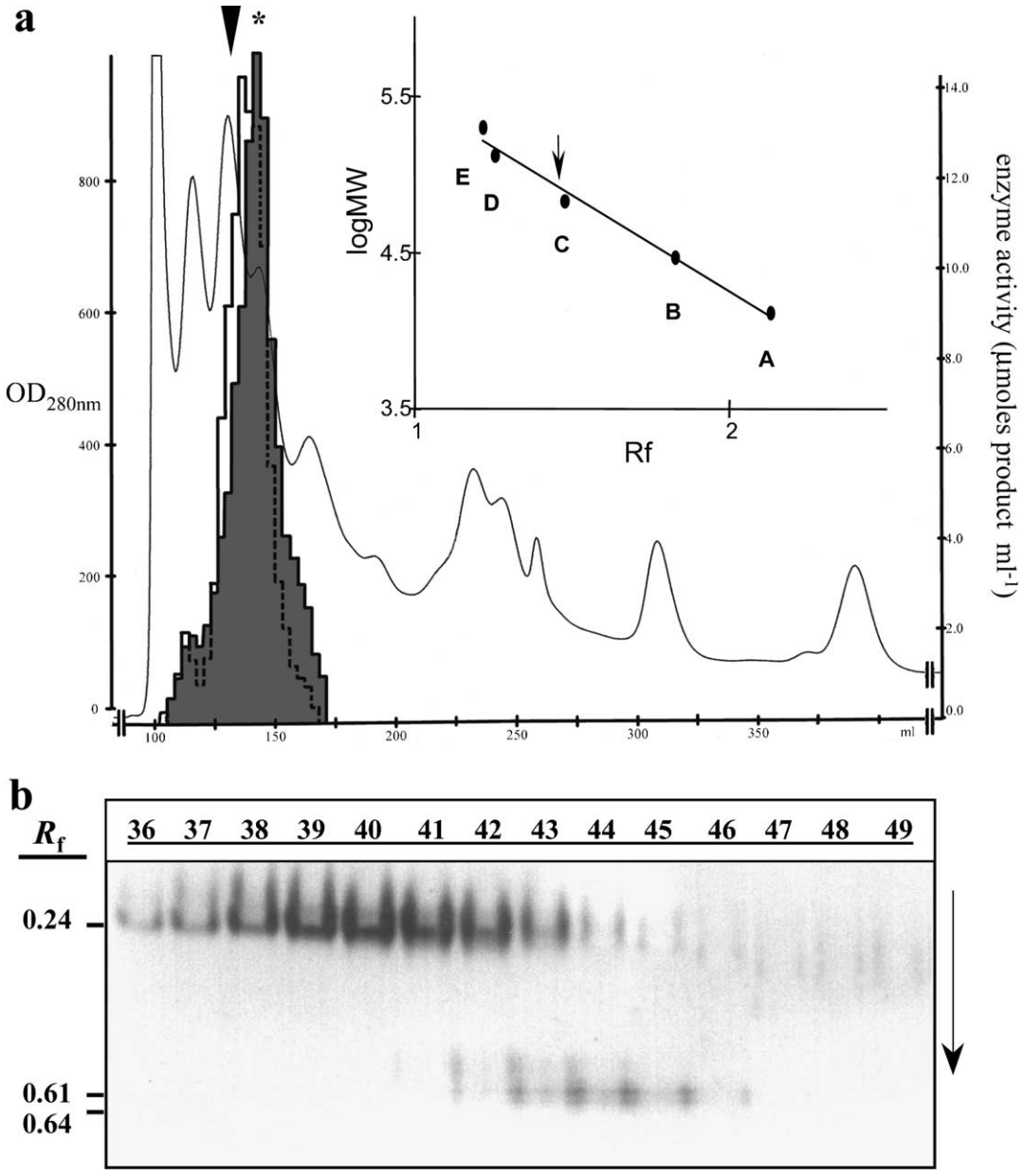

Fig. 1. (a) Size exclusion chromatographic separation of ammonium sulphate precipitated proteins (30–75% cut) from cotyledons of dormant hazel seeds on a HiPrep 26/60 Sephacryl S-200HR column. Fractions (3 ml/fraction) were analysed for protein (OD_{280nm}) and enzyme activity (phytase and total APase) which was expressed as μmoles product ml^{-1} and was normalised for clarity in plotting. Phytase activity corresponds to the peak marked with (*) and that of total APase activity to the peak marked with the closed arrowhead. The void volume of the column was ca. 100 ml as determined with Blue Dextran. *Inset*: plot of relative mobility (R_f) of standard proteins against the logarithm of their molecular size (logMW). The arrow indicates the relative elution volume of the native hazel phytase. Letters A–E correspond to cytochrome C, carbonic anhydrase, bovine serum albumin (monomer), bovine serum albumin (dimer), β-amylase respectively. (b) Electrophoretic analysis on 7.5% Native-PAGE of fractions 36–49 collected from the HiPrep 26/60 Sephacryl S-200HR column. The analysed fractions correspond to ca. 125–170 ml of the chromatographic run in (a). The gel was post-electrophoresis stained for APase activity with α-naphthyl phosphate and Fast Garnet GBC. Numbers on the left denote the relative mobility (R_f) of the observed bands of enzymic activity, while the arrow on the right indicates the direction of electrophoresis. Results are representative of at least three separate experiments.

Some of the functions of *N*-linked glycans include their role in protein localisation in cellular membranes, in seed protein storage bodies and facilitating the tight packing of proteins within these organelles (Lamport, 1980; Spencer, 1984). Phytases are usually associated with protein storage bodies of seeds and pollen (Gabard and Jones, 1986; Baldi et al., 1988), which was also shown for the hazel enzyme (Smith, 1990). Glycosylation may protect the protein backbone against proteolytic attack, while it is regarded as responsible for proper folding, thermal stability, and biological activity of glycoproteins (Rayon et al., 1998; Faye et al., 1989,

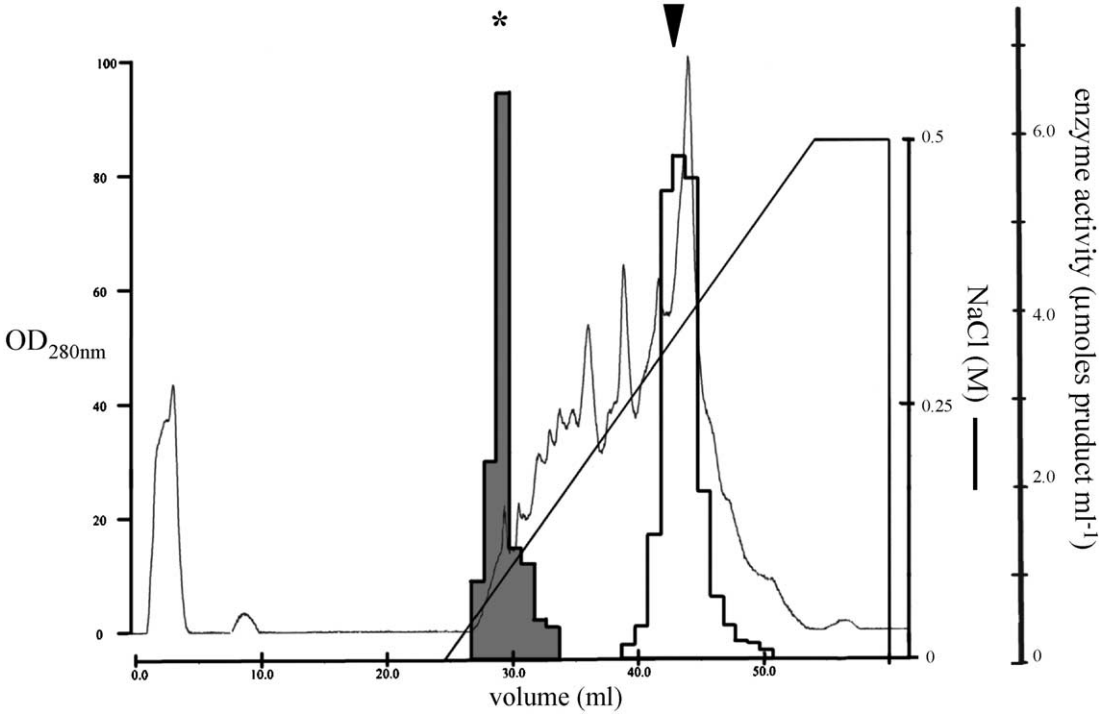

Fig. 2. Cation exchange chromatography of the concentrated phytase active pool from the gel filtration step on a Mono S 5/5HR column at pH 4.5. Eluted fractions were analysed for protein (OD_{280nm}) and enzyme activity (phytase: *; total APase: closed arrowhead) and expressed in μmoles product ml^{-1}. Enzymic activity was normalised for clarity in plotting. Results are representative of at least three separate experiments.

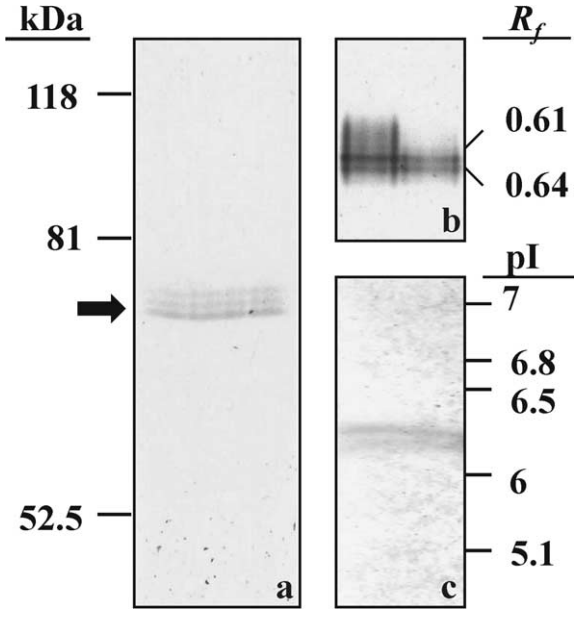

Fig. 3. Electrophoretic analysis of the isolated hazel phytase. (a) 10% SDS–PAGE of the final phytase preparation after Mono S chromatography. The arrow indicates the position of the 71kDa band. The molecular weight (kDa) of markers is shown on the left. (b) 7.5% Native-PAGE of the two fractions from the Mono S column exhibiting the highest phytase activity. Numbers on the right indicate the R_f values of the observed bands of enzymic activity. (c) pH 3–10 Native-IEF of the final phytase preparation after Mono S chromatography. pI values of markers are shown on the right. In (a) the gel was stained with silver nitrate. In (b) and (c) the gels were stained with α-naphthyl phosphate and Fast Garnet GBC. In all cases electrophoresis was toward the lower end of the gel. Similar results were obtained from at least three separate experiments.

1993). The presence of *N*-linked glycans may also improve the solubility properties of the glycosylated polypeptide chain by creating a hydrophilic "overcoat" (Lamport, 1980). In the case of hazel seeds, for which post-harvest drying induces secondary embryo dormancy that is subsequently alleviated upon chilling, the possibility exists that glycosylation, through its effect on protein solubility, could affect the activity of phytase in an environment with potentially limited water content.

2.4. Biochemical properties

2.4.1. pH optimum

The optimum pH for IP6 hydrolysis was found to be pH 5.0. Phytase activity rapidly decreased at the acidic side of the optimum (Fig. 5). No significant activity could be observed above pH 8. The pH values for half-maximal activity were determined at pH 3.5 and 6.5. Two main types of plant phytases have been identified to date: acidic enzymes (pH optimum 4.5–6.0) and alkaline phytases (pH optimum around 8). The hazel enzyme belongs to the former category together with phytases from a range of seeds (Greiner et al., 2000; Laboure et al., 1993; Gibson and Ullah, 1988). In the second class belong phytases from the pollen of *Lilium longiflorum* Thunb. (Scott and Loewus, 1986; Baldi et al., 1988) and *Typha latifolia* L. (Hara et al., 1985) and detergent extracts of legume seeds (Scott, 1991). We have also determined a pH 8.6 optimum phytase activity

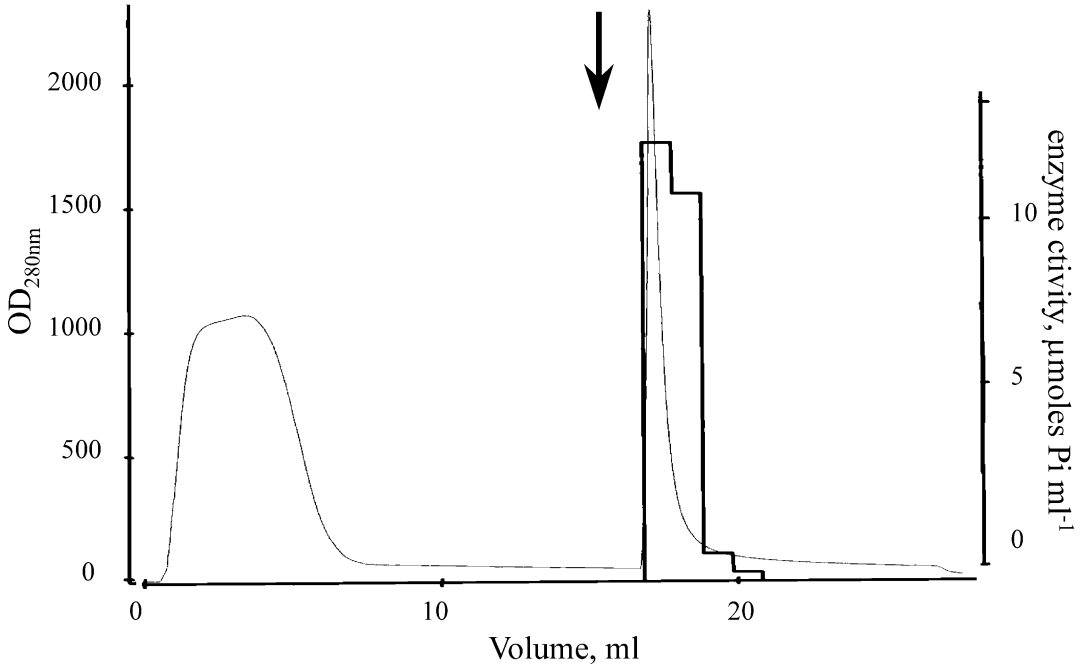

Fig. 4. Concanavalin A–Sepharose 4B (ConA) affinity chromatography of hazel glycoproteins. The concentrated phytase-active pool after the Sephacryl gel filtration step was loaded on a 5/5HR column packed with ConA (column volume ca. 1 ml), as described in Section 3.8. Eluted fractions (1 ml fraction^{-1}) were assayed for protein (OD_{280nm}) and phytase activity. The arrow indicates the application of α-methyl-D-glucopyranoside to affinity elute resin-bound proteins. No phytase activity could be detected in the flow through during sample application on to the column. The experiment was performed in triplicate with similar results.

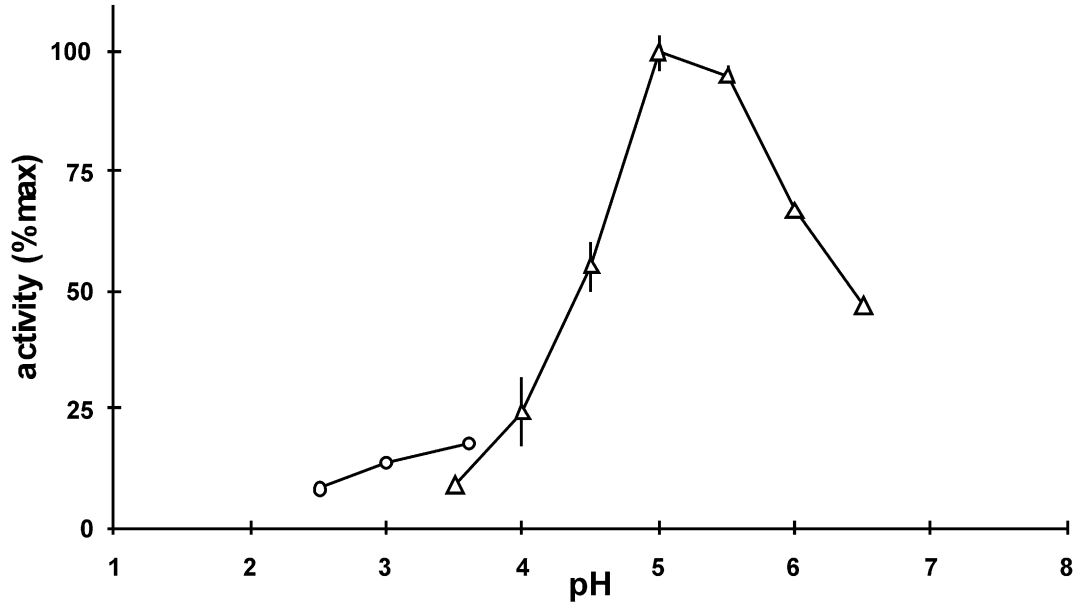

Fig. 5. Effect of pH on hazel phytase activity. Results are expressed as % of maximum activity. Buffers used were glycine/HCl (circles) and acetic acid/NaOH (triangles). Assays were performed in triplicate and bars represent standard errors.

in dormant and chilled hazel seeds, which is currently under further investigation.

The low pH optimum of phytase is characteristic of a vacuolar protein. Vacuoles in plant cells typically have acidic pH (Taiz, 1992) and in seeds may be formed by the fusion of protein body membranes, after digestion of the storage proteins (Pernollet, 1978). This is also evident in hazel embryonic axes during dormancy breakage by chilling (Ross, 1984). These vaculations are the primary site of IP6 accumulation in the cell (Lott et al., 1995) and among the enzymes shown to be associated with protein bodies are proteases, phytases and APases. For example, the major phytase in barley aleurone layers was associated with the protein bodies

(Gabard and Jones, 1986) a result also reported for the pH 5 enzyme from lily pollen (Baldi et al., 1988). Swanson and Jones (1996) showed that barley aleurone cells respond to the addition of gibberellic acid by acidifying their vacuoles through changes in the activity of tonoplast H^+ pumps. Moreover, a decrease in pH of the IP6-containing compartment, to a value as low as pH 4, was evident by ^{31}P NMR spectroscopy in germinating maize seeds (Barba et al., 1997). Acidic conditions appear to be essential for solubilisation of IP6 (ibid.) and for catalytic activity of both acidic proteases, APases and phytases. Therefore, a role of pH in the regulation of lytic metabolism in vivo is evident. This is also supported by the pH optimum of the hazel phytase, the acidic pH optimum of the hazel protease complement (Ratanakosum, 1986) and the fact that both enzymes were shown to be associated with hazel seed protein storage bodies (Ratanakosum, 1986; Smith, 1990).

2.4.2. Substrate selectivity

In order to determine the substrate selectivity of the hazel phytase several phosphorylated substrates were utilised for K_m and V_{max} determinations (Table 2). The hazel phytase exhibited typical Michaellis–Menten kinetics for all compounds tested. The enzyme had broad substrate specificity and IP6 was not the compound with the highest relative rate of hydrolysis. However, it exhibited the lowest K_m value for IP6 (160 μM). It has to be noted that after the initial hydrolysis of IP6 the system becomes multi-substrate, since phytases catalyse the release of Pi from IP6 in a step-wise manner. Thus, the reported kinetic parameters should be referred to as apparent values and can only be correct when the true initial rates are determined (Reddy et al., 1989). The K_m value for IP6 is significantly lower than the K_m estimates for the other compounds tested. Moreover, the hazel phytase exhibited the highest specificity constant (V_{max}/K_m) for IP6 compared to the various substrates tested, thus indicating that IP6 is the preferred substrate. The results presented allow us to classify the hazel phytase as a specialised APase with a clear, though not absolute substrate specificity. Alkaline phytases, conversely, exhibit almost absolute specificity (Baldi et al., 1988).

Table 2
Substrate specificity and kinetic parameters of the isolated hazel phytase

Substrate	Relative activity (%)	K_m (mmoles)	V_{max} (mmoles mg^{-1} protein h^{-1})	V_{max}/K_m
Phytic acid	100	0.16	0.7	4.4
ATP(5′)	250	0.55	1.3	2.4
Fructose 1,6-diphosphate	146	1.1	1	0.8
Ribose-5-phosphate	50	1.2	0.3	0.3
1-naphthyl phosphate	130	1.6	1	0.6
p-nitrophenyl phosphate	101	2.2	1.6	0.7
Glucose 6-phosphate	78.5	2.3	0.7	0.3
Na-Pyrophosphate	438.5	2.4	3.7	1.5

For determination of relative activity hydrolysis rate of phytic acid (2.5 mM) (dodecasodium salt from rice) was taken as 100%. For K_m and V_{max} estimation, phytase activity was assayed with increasing concentration of each compound and the data was subsequently transformed to double-reciprocal plots.

The kinetic parameters we determined for IP6 are of the same order of magnitude as those reported for the phytase from other plant sources (Greiner et al., 2000; Laboure et al., 1993; and references therein). Gibson and Ullah (1988) estimated a K_m value of 48 μM for the soybean phytase, which is among the lowest reported. The hazel enzyme exhibited substrate inhibition with activity being reduced ca. 20% at 5 mM IP6. Substrate inhibition at high IP6 levels could indicate a two-point attachment of the substrate to the enzyme. Irving (1980) also reported this for phytases from *Pseudomonas* sp., dwarf beans and corn endosperm-scutellar tissue. The broad substrate specificity of phytase in vitro implies that the enzyme would be able to catalyse the release of Pi from a wide range of phosphorylated substrates in vivo. This is of particular importance during the transition from the resting to the metabolically active and germinating seed as the demand in Pi, ATP and energy could be met by the hydrolysis of a wide range of phosphorylated substrates. Phytases and APases have been attributed such a role (Duff et al., 1994) however, the former would preferentially hydrolyse IP6.

2.4.3. Inhibition by inorganic phosphate

Inorganic phosphate, a product of IP6 hydrolysis, completely inhibited phytase activity at 5 mM. The nature of the inhibition was studied and found to be of the competitive type. A K_i of 407 μM was estimated. In soybean cotyledons the inhibition of phytase by Pi had a K_i of 28 μM (Gibson and Ullah, 1990), while in wheat bran and mung beans the reported K_i values were of the same order of magnitude as in the present report (ibid.). In the case of the hazel enzyme we suggest that the low V_{max} for IP6 hydrolysis and the competitive inhibition by Pi constitute a controlled mechanism of Pi release from IP6 hydrolysis during dormancy breakage, so that the embryonic axis is supplied with a constant amount of Pi. This would indicate that IP6 is broken down at a rate high enough to support growth during resumption of metabolic activity by the resting seed, while at the same time leaving appreciable amounts of Pi reserves to be utilised during seed germination and seedling growth.

2.4.4. Effect of metal ions

Different divalent cations and metal ions were included in the standard phytase assay in order to determine their effect on enzymic activity (Table 3). Among the

Table 3
Effect of ions, divalent cations, metals and metal chelators on hazel phytase activity

Agent	Concentration (mM)		
	0.1	1	2.5
Control	100	100	100
$FeCl_3 \cdot 6H_2O$[a]	83 (5)	71 (3)	32 (3)
NaF	Nd[b]	51 (3)	39 (2)
$CaCl_2$	95 (1)	95 (1)	101 (3)
$MgCl_2 \cdot 6H_2O$[a]	89 (0.5)	87.5 (0.5)	80 (4)
$MnCl_2 \cdot 4H_2O$	82 (1)	81 (0.5)	83 (2)
$Zn_2SO_4 \cdot 7H_2O$[a]	44 (1.5)	38 (1)	41 (0.5)
$CuSO_4 \cdot 5H_2O$[a]	80 (2)	40	30 (2)
$Na_2MoO_4 \cdot 2H_2O$	15 (1)	13	13
$Na_3Vo_4 \cdot 14H_2O$	21 (1)	15 (1)	10
EGTA	nd	nd	108 (2.5)
Tartrate	nd	nd	102 (2.5)
Citrate	nd	nd	116 (2)

Results are expressed as% of the control (no additive) and numbers in brackets are standard errors (±) ($n = 3$).
[a] Formation of visible precipitate.
[b] nd = not determined.

divalent cations, calcium had no significant effect, while Mg^{2+} and Mn^{2+} exhibited moderate inhibition. These results are different from those reported by Gibson and Ullah (1988) for the soybean-purified protein showing that enzymic activity was stimulated almost 50% over the control. Calcium was also reported to effect a slight activation of the maize phytase (Laboure et al., 1993). This could be attributed to the removal of Pi from solution as calcium phosphate, thus effectively reducing Pi levels in the in vitro assay mixture so that the enzyme would be able to act in a less inhibiting environment. Heavy metals such as copper, molybdenum and vanadium caused a reduction of enzymic activity between 70–90% at the 2.5 mM level. Fluoride was found to be a competitive inhibitor of the hazel enzyme ($K_i = 205\mu M$), typical of other phytases and APases (Duff et al., 1994). Metal ion chelators had a slightly activating effect on phytase activity. These results show that the hazel phytase does not require a metal as a co-factor, in line with Greiner et al. (2000) for the phytase from seeds of barley and other cereals. However, the present results could also reflect the ability of IP6 to act as a strong chelating agent. Thus the inhibition observed could be attributed to alteration of the substrate to an insoluble metal-IP6 form, effectively reducing the amount of IP6 for the enzyme to act upon.

2.5. Conclusion

In the present communication we report the purification of phytase for the first time from dormant seeds. The isolated enzyme appears to be a monomeric protein having biochemical characteristics similar to other phytases purified from various plant sources. However, it differs from the majority of plant phytases in that it interacts with ConA, thus suggesting that it is putatively glycosylated.

In a subsequent paper we will describe the purification and characterisation of three soluble hazel APase isozymes and compare them with the purified phytase. That we were able to separate these enzymic activities will facilitate future studies to be directed toward the isolation and identification of the intermediate products of IP6 hydrolysis during hazel dormancy alleviation. Lower phosphorylated esters of *myo*-inositol, particularly *myo*-inositol-1,4,5-*tris*phosphate (IP3) and *myo*-inositol-1,2,3,6-*tetrakis*phosphate (IP4), have been implicated in the induction of Ca^{2+} fluxes in mammalian and plant systems in response to environmental stimuli (Clark et al., 2001). Previous studies in hazel seeds have demonstrated that the Ca^{2+}-calmodulin system is operating early in the dormancy breakage period, in agreement with its proposed role as a signal transduction mechanism (Ross and Smith, 1992). Therefore, it is of great interest to determine whether IP3, such as that produced by the action of phospholipase C on phosphatidylinositol-4,5-*bis*phosphate, or some other biologically active compounds, such as IP4, are present as products of IP6 dephosphorylation.

3. Experimental

3.1. Plant material

Fruits of *Corylus avellana* L. were obtained from a commercial source. The lobed involucra was removed and the nuts left to dry to a constant wt. at room (ambient) temperature. They were then dry stored in airtight containers at 5 °C. When required, hazelnuts were removed from dry storage and either used directly, after pericarp and testa removal, or stratified at 5 °C for 0–6 weeks as previously described (Li and Ross, 1988).

3.2. Chemicals, other material and equipment

All chemicals used were of the highest purity available and purchased from Sigma-Aldrich (UK) and Bio Rad Laboratories (UK). The AKTA™-purifier 10/100 FPLC system and the HiPrep 26/60 Sephacryl S-200HR, Mono S 5/5HR and PD-10 (Sephadex G-25, coarse) columns were products of Amersham Pharmacia Biotech (UK).

3.3. Enzyme activity assay

The standard phytase assay contained, in a total volume of 300 µl, 0.1 M acetate buffer (pH 5), 2.5 mM phytic acid (dodecasodium salt from rice) and enzyme preparation. Reactions were held at 37 °C for 4 h and terminated by the addition of ice-cold trichloroacetic acid (TCA) to a final concentration 10% (w/v). Activity

was expressed in mmole Pi mg^{-1}protein h^{-1} and in the case of column chromatographies as μmoles Pi ml^{-1}.

APase was assayed with *p*-nitrophenyl phosphate (*p*NPP) as the substrate (5 mM) in 0.1 M citrate buffer (pH 5.6) and appropriately diluted enzyme preparation, to a total reaction volume of 1 ml. After 30 min at 37 °C reactions were stopped by adding 400 μl 0.5M NaOH and the liberated *p*-nitrophenol was quantified at 405 nm against appropriate enzyme blanks.

3.4. Protein and inorganic phosphate determination

Protein content was determined at 620 nm according to Sedmak and Grossberg (1977). BSA was used as the standard protein. Inorganic phosphate was determined according to Fiske and Subbarow (1925) with KH_2PO_4 as a standard.

3.5. Purification of phytase

Experiments with samples prepared from cotyledons of 6-week-chilled hazel seeds (non-dormant seeds) exhibited similar chromatographic patterns and no appreciable differences in catalytic properties compared with those from dry (dormant) seeds. Therefore, unless otherwise stated, the following purification protocol will be concerned with samples from dry (dormant) seeds, due to the relative ease of preparation of the experimental material.

Unless otherwise specified, subsequent operations were performed at 5 °C except for column chromatography that was carried out at room temperature. Acetone powders of cotyledonary tissue were prepared according to Loomis (1959) and stored at −20 °C with no loss of enzymic activity.

Soluble proteins were extracted with 0.1 M acetate buffer (pH 5.5), 1 mM DTT, 0.5 mM PMSF, 3% (w/w) PVPP (Buffer A) (1 g material/10 ml extraction buffer). The homogenate was mechanically shaken for 2 h and then filtered through 4 layers of muslin. The filtrate was centrifuged at 13,000×*g* for 20 min. Proteins precipitating between 30–75% with respect to $(NH_4)_2SO_4$ were collected after centrifugation and re-suspended in a small volume of 50 mM acetate buffer (pH 5.5), 100 mM NaCl (Buffer B). The sample was de-salted through a PD-10 column previously equilibrated with Buffer B and the eluate centrifuged as before to remove precipitated proteins. The pellet that had formed was found to contain no appreciable phytase activity. The clear sample was loaded onto a HiPrep 26/60 Sephacryl S-200HR gel filtration column equilibrated with Buffer B. The column, connected to an AKTA^{TM}-purifier 10/100 FPLC system, was developed with the same buffer at a flow rate of 1 ml min^{-1}. Fractions (3 ml) were collected and those exhibiting phytase activity were pooled and concentrated by means of $(NH_4)_2SO_4$ precipitation. Proteins were recovered by a further centrifugation as before and the pellet resuspended in 20 mM acetate buffer (pH 4.5, Buffer C) and de-salted through a PD-10 column equilibrated with the same buffer. The eluate was loaded onto a Mono S 5/5HR cation exchange column, pre-equilibrated with Buffer C, connected to an AKTA^{TM}-purifier 10/100 FPLC system. The column was washed with Buffer C for 25 min after sample application to remove unbound material and then developed with a linear gradient of 0–0.5 M NaCl in Buffer C at a flow rate of 1 ml min^{-1} within 30 column volumes (CV, 1CV ca. 1 ml). Fractions (1 ml) were collected and those exhibiting phytase activity were pooled and loaded onto a PD-10 column equilibrated with 0.1 M acetate buffer (pH 5). This final preparation showed no loss of enzymic activity, if subjected to experimental conditions within 48 h.

3.6. Biochemical characterisation of phytase

In order to study the biochemical characteristics of the purified phytase the standard activity assay was performed with the following modifications. For pH studies, two buffer systems were used at final concentration 0.1 M: glycine/HCl buffer for pH 2.5–3.6, acetic acid/NaOH buffer for pH 3.5–6.5. For pH stability determinations the purified enzyme was incubated in the above buffers for 4 h at 37 °C before performing the standard phytase assay. For kinetic studies, activity of purified phytase was assayed using *p*NPP, 1-naphthyl phosphate, Na-pyrophosphate, ATP, glucose 6-phosphate, fructose 1,6-diphosphate, ribose 5-phosphate alongside appropriate enzyme blanks, at final concentrations in the range 0–5 mM. Metal ions were also included in the standard phytase assay as the salt forms and at the levels indicated in Table 3.

3.7. Estimation of molecular weight

In order to estimate the molecular weight of the native hazel phytase a HiPrep 26/60 Sephacryl S-200HR column was calibrated with protein standards of known molecular weight (Sigma-Aldrich) consisting of cytochrome C (horse heart, 12.4 kDa), carbonic anhydrase (bovine erythrocytes, 29 kDa), bovine serum albumin (monomer: 66 kDa; dimer: 132 kDa), β-amylase (sweet potato, 200 kDa). The void volume of the column was estimated with Blue Dextran (2000 kDa). The column was operated as described above and the elution volume of the hazel protein was determined after assaying the fractions collected for enzymic activity.

3.8. Interaction of phytase with Concanavalin A

Ammonium sulphate concentrated proteins after the Sephacryl S-200 gel filtration step were loaded onto a PD-10 desalting column previously equilibrated with 0.1 M sodium acetate/acetic acid (pH 6) buffer containing

0.5 M NaCl, 1 mM $CaCl_2$, 1 mM $MnCl_2$ (ConA binding buffer). The eluate was centrifuged at 13,000×*g* for 20 min at 4 °C (SS 34 rotor) and the clarified supernatant was loaded, at a flow rate of 0.3 ml min^{-1}, to Concanavalin A- Sepharose 4B packed in a 5/5HR column (Amersham Pharmacia Biotech). The column was connected to an AKTA™-purifier 10/100 FPLC system and equilibrated with ConA binding buffer. The column was washed with the same buffer until the absorbance at 280 nm returned to baseline, in order to remove unbound material. Resin-bound proteins were affinity eluted with 0.5 M α-methyl-D-glucopyranoside in ConA binding buffer. Fractions (1 ml) were collected and assayed for phytase activity.

3.9. Protein electrophoresis

SDS–PAGE was performed in 0.75 mm thick 10% separating gels with 4% stacking gels according to Laemmli (1970). Electrophoresis was performed with a Mini-PROTEAN® II Electrophoresis Cell (Bio Rad Laboratories) at 50 mA constant current until the bromophenol blue tracking dye had reached ca. 1 cm from the bottom of the gel. Gels were stained for protein with silver nitrate according to Wray et al. (1981) and calibrated with SDS–PAGE standards (broad range, Bio Rad Laboratories).

Native-PAGE was performed in 7.5% separating gels as for SDS–PAGE but omitting SDS at 100 V at 5 °C until the bromophenol blue dye had reached ca. 1 cm from the bottom of the gel. Native-IEF was carried out in pH 3–8 IEF Ready Gels (BioRad) according to the manufacturer's instructions. For *p*I estimation the IEF gels were calibrated with pI markers (broad range: *p*I 4.45–9.6 IEF standards, Bio Rad).

Staining for enzymatic activity after Native-PAGE and Native-IEF was performed according to Hubel and Beck (1996) using 1-naphthyl phosphate and Fast Garnet GBC (sulphate salt).

Acknowledgements

The authors would like to express their gratitude to Dr. C.B. Johnson for reviewing the manuscript, to Mrs. Sue Mott and Mrs. Moy Robson for their most willing and efficient technical assistance and to Mr. Mike Taylor for providing research facilities including the AKTA™-purifier 10/100 FPLC system and chromatographic columns. Financial support by Mr. M.V. Andriotis and Mrs. E.N. Skordidou-Andrioti is gratefully acknowledged.

References

Baldi, B.G., Scott, J.J., Everard, J.D., Loewus, F.A., 1988. Localisation of constitutive phytases in lily pollen and properties of the pH 8 form. Plant Science 56, 137–147.

Barba, I., Gasparovic, C., Cabanas, M.E., Alonso, J., Murillo, I., San Segundo, B., Arus, C., 1997. Measurement of intracellular pH of maize seeds (*Zea mays*) during germination by ^{31}P nuclear magnetic resonance spectroscopy. Cellular Molecular Biology 43, 609–620.

Clark, G.B., Thompson Jr, C., Roux, S.J., 2001. Signal transduction mechanisms in plants: an overview. Current Science 80, 170–177.

Dubray, G., Bezard, G., 1982. A highly sensitive periodic acid-silver stain for 1,2-diol groups of glycoproteins and polysaccharides in polyacrylamide gels. Analytical Biochemistry 119, 325–329.

Duff, S.M.G., Sarath, G., Plaxton, W.C., 1994. The role of acid phosphatases in plant phosphorus metabolism. Physiologia Plantarum 90, 791–800.

Faye, L., Johnson, K.D., Sturm, A., Chrispeels, M.J., 1989. Structure, biosynthesis, and function of asparagine-linked glycans on plant glycoproteins. Physiologia Plantarum 75, 309–314.

Faye, L., Fitchette-Laine, A.C., Gomord, V., Chekkafi, A., Delaunay, A.-M., Delaunay, A.-M., Driouich, A., 1993. Detection, biosynthesis and some functions of glycans N-linked to plant secreted proteins. In: Battey, N.H., Dickinson, H.D., Hetherington, A.M. (Eds.), Post-Translational Modifications in Plants. Society for Experimental Biology Seminar Series 53, pp. 213–242.

Fiske, C.H., Subbarow, Y., 1925. The colorimetric determination of phosphorus. Journal of Biological Chemistry 66, 375–400.

Gabard, K.A., Jones, R.L., 1986. Localisation of phytase and acid phosphatase isoenzymes in aleurone layers of barley. Physiologia Plantarum 67, 182–192.

Gibson, D.M., Ullah, A.H.J., 1988. Purification and characterisation of phytase from cotyledons of germinating soybean seeds. Archives of Biochemistry and Biophysics 260, 503–513.

Gibson, D.M., Ullah, A.H.J., 1990. Phytases and their action on phytic acid. In: Morre, D.J., Boss, W.F., Loewus, F.A. (Eds.), Inositol Metabolism in Plants. Willey-Liss, New York, pp. 77–92.

Graf, E., Empson, K.L., Eaton, J.W., 1987. Phytic acid. A natural antioxidant. Journal of Biological Chemistry 262, 11647–11650.

Greiner, R., Jany, K.-D., Alminger, M.L., 2000. Identification and properties of *myo*-inositol hexakisphosphate phosphohydrolases (phytases) from barley (*Hordeum vulgare*). Journal of Cereal Science 31, 127–139.

Han, Y., Lei, X.G., 1999. Role of glycosylation in the functional expression of an *Aspergillus niger* phytase (phyA) in *Pichia pastoris*. Archives of Biochemistry and Biophysics 364, 83–90.

Hanakahi, L.A., Bartlet-Jones, M., Chappell, C., Pappin, D., West, S.C., 2000. Binding of inositol phosphate to DNA-PK and stimulation of double-strand break repair. Cell 102, 721–729.

Hara, A., Ebina, S., Kondo, A., Funaguma, T., 1985. A new type of phytase from pollen of *Typha latifolia* L. Agricultural and Biological Chemistry 49, 3539–3544.

Hegeman, C.E., Grabau, E.A., 2001. A novel phytase with sequence similarity to purple acid phosphatases is expressed in cotyledons of germinating soybean seedlings. Plant Physiology 126, 1598–1608.

Hubel, F., Beck, E., 1996. Maize root phytase. Purification, characterisation, and localisation of enzyme activity and its putative substrate. Plant Physiology 112, 1429–1436.

Irving, G.C.J., 1980. Phytase. In: Gosgrove, D.J. (Ed.), Inositol Phosphates: Their Chemistry, Biochemistry and Physiology. Elsevier Scientific Publishing Company, Oxford, pp. 85–98.

Laboure, A.-M., Gagnon, J., Lescure, A.-M., 1993. Purification and characterisation of a phytase (myo-inositol hexakisphosphate phosphohydrolase) accumulated in maize (*Zea mays*) seedlings during germination. Biochemical Journal 295, 413–419.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–685.

Lamport, D.T.A., 1980. Structure and function of plant glycoproteins. In: The Biochemistry of Plants. Vol. 3. Academic Press, London. pp. 501–541.

Li, L., Ross, J.D., 1988. Fructose 1,6-bisphosphatase in seeds of *Corylus avellana*. Phytochemistry 27, 1977–1980.

Lin, J.-J., Dickinson, D.B., Ho, T.-H.B., 1987. Phytic acid metabolism in lily (*Lilium longiflorum* Thunb.) pollen. Plant Physiol.ogy 83, 408–413.

Loewus, F.A., Murthy, P.P.N., 2000. *Myo*-inositol metabolism in plants. Plant Science 150, 1–19.

Loomis, W.D., 1959. Amide metabolism in higher plants. III. Distribution of glutamyl transferase and glutamine synthetase activity. Plant Physiol.ogy 34, 541–546.

Lott, J.N.A., Greenwood, J.S., Batten, G.D., 1995. Mechanisms and regulation of mineral nutrient storage during seed development. In: Kigel, J., Galili, G. (Eds.), Seed Development and Germination. Marcel Dekker, New York, pp. 215–235.

Nakano, T., Joh, T., Tokumoto, E., Hayakawa, T., 1999. Purification and characterisation of phytase from bran of *Triticum aestivum* L. cv. Nourin #61. Food Science and Technology Research 5, 18–23.

Pernollet, J.-C., 1978. Protein bodies of seeds: ultrastructure, biochemistry, biosynthesis and degradation. Phytochemistry 17, 1473–1480.

Ratanakosum, K., 1986. An investigation into protein mobilisation during dormancy breakage in hazel seeds (*Corylus avellana* L.). PhD Thesis, The University of Reading, Reading, UK.

Rayon, C., Lerouge, P., Faye, L., 1998. The protein N-glycosylation in plants. Journal of Experimental Botany 49, 1463–1472.

Reddy, N.R., Pierson, M.D., Sathe, S.K., Salunke, D.K., 1989. Phytates in Cereals and Legumes. CRC Press, Florida.

Ross, J.D., 1984. Metabolic aspects of dormancy. In: Murray, D.R. (Ed.), Seed Physiology. Vol. 2. Germination and Reserve Mobilisation. Academic Press, London, pp. 45–75.

Ross, J.D., Smith, S.B., 1992. Dormancy breakage in *Corylus avellana*: role of calcium. In: Come, D., Corbineau, F., (Eds.), Fourth International Workshop on Seeds. Basic and Applied Aspects of Seed Biology. Angers, France 20–24 July, 1992, Vol. 2, pp. 639–644.

Safrany, S.T., Caffrey, J.J., Yang, X., Shears, S.B., 1999. Diphosphoinositol polyphosphates: the final frontier for inositide research? Biological Chemistry 380, 945–951.

Sasakawa, N., Sharif, M., Hanley, M.R., 1995. Metabolism and biological activities of inositol pentakisphosphate and inositol hexakisphosphate. Biochemical Pharmacology 50, 137–146.

Scott, J.J., 1991. Alkaline phytase activity in nonionic detergent extracts of legume seeds. Plant Physiology 95, 1298–1301.

Scott, J.J., Loewus, F.A., 1986. A calcium-activated phytase from pollen of *Lilium longiflorum*. Plant Physiology 82, 333–335.

Sedmak, J.J., Grossberg, S.E., 1977. A rapid, sensitive, and versatile assay for protein using Coomassie Brilliant Blue G250. Analytical Biochemistry 79, 544–552.

Smith, S.B., 1990. An investigation into the mechanism of dormancy breakage. PhD Thesis, The University of Reading, Reading, UK.

Spencer, D., 1984. The physiological role of storage proteins in seeds. Philosophical Transactions of the Royal Society of London B304, 275–285.

Swanson, S.J., Jones, R.L., 1996. Gibberrelic acid induces vacuolar acidification in barley aleurone. The Plant Cell 8, 2211–2221.

Taiz, L., 1992. The plant vacuole. Journal of Experimental Biology 172, 113–122.

Ullah, A.H.J., Sethumadhavan, K., Mullaney, E.J., Ziegelhoffer, T., Austin-Phillips, S., 1999. Characterisation of recombinant fungal phytase (*phy*A) expressed in tobacco leaves. Biochemical and Biophysical Research Communications 264, 201–206.

Wodzinski, R.J., Ullah, A.H.J., 1996. Phytase. Advances in Applied Microbiology 42, 263–302.

Wray, W., Boulikas, T., Wray, V.P., Hancock, R., 1981. Silver staining of proteins on polyacrylamide gels. Analytical Biochemistry 118, 197–203.

Yamagata, H., Tanaka, K., Kasai, Z., 1980. Purification and characterisation of acid phosphatase in aleurone particles of rice grains. Plant Cell Physiology 21, 1449–1460.

York, J.D., Odom, A.R., Murphy, R., Ives, E.B., Wente, S.R., 1999. A phospholipase C-dependent inositol polyphosphate kinase pathway required for efficient messenger RNA export. Science 285, 96–100.