Characterization of a Novel Acid Phosphatase from Embryonic Axes of Kidney Bean Exhibiting Vanadate-Dependent Chloroperoxidase Activity

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Running title: Acid Phosphatase Exhibiting Vanadate-Chloroperoxidase Activity
†The nucleotide sequence(s) reported in this paper has been submitted to the GenBank database with accession numbers, AB116719 for cDNA and AB116720 for genomic DNA.
A novel colorless acid phosphatase (KeACP), which was distinct from the kidney bean purple acid phosphatase, was purified to apparent homogeneity and cloned from embryonic axes of kidney bean (*Phaseolus vulgaris* L. Ohfuku) during germination. When ortho-vanадate (*VO₄^{3-}* ) is added to the apo-form of the enzyme, KeACP uniquely exhibits the chloroperoxidase activity with loss of phosphatase activity. This is the first demonstration that KeACP is vanadate-dependent chloroperoxidase in plants to be characterized and suggests that KeACP may play a role in modifying a wide variety of chlorinated compounds that are present in higher plants. The enzyme is a dimer that presents three forms made up of combination of the dominant 56-kDa and the minor 45-kDa subunits, and both subunits contain carbohydrate. The full-length cDNA of the *KeACP* gene is 1,641 nucleotides, and this sequence is predicted to encode a protein having 457 amino acid residues (52,865 Da), including a signal peptide. The complete nucleotide sequence of the genomic DNA (3,228 bp) of KeACP consists of seven exons and six introns. Northern blot analysis demonstrated that the *KeACP* gene was expressed specifically in embryonic axes of the kidney bean, and its expression coincided with elongation of the embryonic axis during germination.

Key words: acid phosphatase, chloroperoxidase, vanadate, kidney bean, germination, embryonic axes.
Acid phosphatase (ACP)1 (EC 3.1.3.2) enzymes catalyze hydrolysis of phosphate monoesters at a pH range 4–7. The physiological role of these enzymes in cells is not well understood, partly because ACPs are widely distributed in nature and appear to be ubiquitous, exhibiting minimal substrate specificity (1). ACP has been implicated in the release, transport and recycling of inorganic phosphate. Recent comparative analysis of the structure of ACPs from higher plants and mammals has allowed the identification of conserved sequence and sequence motifs in this enzyme from many species (2, 3). Plant ACPs are present in various organs of germinating seeds and also in different cell compartments, suggesting that the enzyme is involved at various cellular metabolic and bioenergetic levels. Plant ACPs are also induced under various environmental and developmental conditions, including salt or drought stress, seed germination, flowering and pathogen infection (4–6). However, the in vivo function of plant ACPs remains largely unknown, although the stimulation of phosphatase activities in response to phosphate starvation is well documented, and ACPs play a role in the utilization of phosphate compounds (1).

Purple ACPs are commonly found in a wide range of plant species and belong to a family of non-specific ACPs containing a dinuclear Fe-Me center (where Me can be Fe or Zn) in their active sites (2). The purple ACP from the kidney bean was first characterized by Beck et al. (7). It is a dimeric enzyme with two identical subunits that can each be crystallized. The active site contains a Fe$^{3+}$-Zn$^{2+}$ system in both subunits. The subunits are connected by a disulfide bond, and the purple color is due to tyrosine-Fe$^{3+}$ charge transfer transitions (8). Many reports have indicated that purple ACPs belong to the metallo-phosphatase family of proteins, which also includes phosphoprotein phosphatase and other types of phosphomonoesterases (3, 9, 10).
Recently, the enzyme active sites of the ACP have been shown to be somewhat conserved in a completely different class of enzymes, the vanadium haloperoxidases (11, 12), suggesting that nature has developed the same type of binding site for phosphate and vanadium. The ACPs are classified into several different groups. Sequence motifs are shared between the membrane-associated phosphatases, including type 2 phosphatase and vanadate-dependent chloroperoxidase. Similarly, haloperoxidases have been reported to perform phosphatase reactions by substituting the vanadate group with phosphate (11, 13). Recently, del Pozo et al. (14) reported that purple ACP-like protein (AtACP5) from *A. thaliana* induced by phosphate starvation exhibited phosphatase activity and simultaneously catalyzed the peroxidation of luminal. This activity was probably dependent upon the ferrous moiety of the AtACP5.

In the present study, we found for the first time in a plant species an ACP enzyme that is able to catalyze chloroperoxidation by substituting the apo-enzyme with vanadate. This fact suggests that KeACP may play a role in modifying a wide variety of chlorinated compounds that are present in higher plants, because the halo-metabolites are mainly formed through oxidation of halides by haloperoxidase and H2O2 and by a subsequent reaction with a nucleophilic acceptor (15).

The first process in germination is the uptake of water by seeds during imbibition. A number of physical and biochemical changes are induced in response to this, including ACP activity, resulting in germination and subsequent elongation of the embryonic axis. Germination mechanisms, including the early stage of embryonic axis elongation, are still poorly understood, although the amount of ACP has been shown to increase significantly in seeds upon germination (16). Consequently, there is interest in the identification and characterization of enzymes that are actively transcribed and
synthesized during the germination process. Despite numerous reports that ACPs occur in a variety of plants and plant tissues, the exact physiological role of these enzymes has yet to be established. In the present work we describe the purification and cloning of KeACP from kidney bean embryonic axes, and biochemical characterization of this enzyme, which was distinct from purple ACP. Furthermore, we discuss the physiological role of KeACP in responses to germination of the kidney bean.

EXPERIMENTAL PROCEDURES

Plant materials – Kidney bean (Phaseolus vulgaris L. Ohfuku) dry seeds from the 2001 harvest were obtained from a farm in Hokkaido, Japan. They were sterilized in 30 ppm 2-(4-thiazolyl) benzimidazole for 15 min and rinsed with distilled water.

Purification of KeACP from embryonic axes of kidney bean – Sterilized kidney bean seeds were soaked with distilled water at room temperature for 1 hr. Imbibited seeds were then transferred onto a 0.56% agar plate containing 0.4 mM phosphorus and incubated at 25°C in darkness. After incubation for 72 hrs, the embryonic axes were removed from the germinated seeds and collected. Subsequent purification steps were carried out at 4°C or on ice. Embryonic axes (600 g) were homogenized with three volumes of 100 mM Tris-HCl (pH 7.5) containing 1 mM KCl, 1 mM phenylmethylsulfonyl fluoride, 5 mM EDTA and 5 mM 2-mercaptoethanol (2-ME). The homogenates were then centrifuged at 18,000 g for 45 min. The crude extract was precipitated sequentially with ammonium sulfate fractionation 40% and then 60% saturation. The precipitate was then suspended in a small volume of 20 mM Tris-HCl (pH 7.5) and dialyzed against the same buffer. Protein that precipitated during dialysis was removed by centrifugation.
The enzyme solution was applied to a DEAE Toyopearl 650S column (2.6 × 40 cm) (Tohso, Tokyo, Japan) equilibrated with 20 mM Tris-HCl (pH 7.5). The adsorbed materials were eluted with a linear gradient of NaCl concentration from 0 to 0.5 M in 500 ml of the buffer. The fractions having enzyme activity were collected and dialyzed against 20 mM Tris-HCl (pH 7.5) containing 0.5 M NaCl. The enzyme solution was loaded onto a Concanavalin A (Con A)-Sepharose column (1.6 × 40 cm) (Pharmacia Biotech, Uppsala, Sweden) equilibrated with dialysis buffer. After washing the column, the adsorbed enzyme was eluted with a linear gradient of D-(+)-mannose concentration gradient from 0 to 0.5 M in the equilibrated buffer. The fractions having enzyme activity were collected and dialyzed against 20 mM Tris-HCl (pH 7.5) containing 1.4 M ammonium sulfate. The dialyzed sample was applied to a Butyl Toyopearl 650S column (1.6 × 40 cm) (Tohso) equilibrated with the dialysis buffer. Bound protein was eluted with a linear gradient of ammonium sulfate concentration from 1.4 to 0 M in Tris-HCl buffer (pH 7.5). Active fractions were collected and concentrated to minimal volume using membrane filter YM1 or PM30 (Amicon, Beverly, MA).

PAGE and protein sequencing – Non-denaturating (native) PAGE was carried out at 4°C using a 5% polyacrylamide gel (pH 8.8). ACP activity staining was carried out by incubating the gels with acetate buffer (pH 5.0) containing 0.1 mg/ml Fast Black K salt (Sigma, St. Louis, MO) and 1 mg/ml α-naphthylphosphate in the dark at room temperature until colored bands appeared. SDS-PAGE was performed using a 10% polyacrylamide gel in the presence and absence of 2-ME. The separated protein bands were stained with Coomassie Brilliant Blue R-250 (CBB). The size markers were phosphorylase b (97 kDa), BSA (66 kDa), egg ovalbumin (45 kDa), carbonic anhydrase (30 kDa) and soybean trypsin inhibitor (20.1 kDa). Glycoprotein staining of the gel was
performed according to a periodic acid-Schiff (PAS) staining protocol.

For the N-terminal amino acid sequence, the proteins separated by SDS-PAGE were electroblotted onto a PVDF membrane using a semi-dry apparatus according to the method described previously (17). The peptide fragments were generated by limited proteolysis with \textit{Staphylococcal} V8 protease and \textit{Achromobacter lyticus} lysyl endopeptidase during electrophoresis. After the PVDF membranes were briefly stained with CBB, the stained bands were excised. The N-terminal amino acid sequences were determined using an automated protein sequence analyzer (Model 492HT, Applied Biosystems, Foster City, CA).

\textit{Analytical gel filtration} – A Superdex 200 HR 10/30 gel filtration column (1.0 \times 30 cm) (Pharmacia Biotech) was equilibrated with 20 mM Tris-HCl (pH 7.5) containing 0.5 M NaCl. The molecular mass of the purified enzyme was estimated by calibration with standard proteins, thyroglobulin (669 kDa), ferritin (440 kDa), aldolase (158 kDa), BSA (67 kDa) and ribonuclease A (13.7 kDa).

\textit{Deglycosylation of KeACP} – Deglycosylation of the purified KeACP was performed using \textit{N}-glycosidase F Deglycosylation Kit (Roche, Penzberg, Germany) according to the manufacturer’s instruction. The purified enzyme (50 µg) was denaturated in 10 µl of denaturation buffer (pH 8.6) containing 1% SDS at 95°C for 3 min, and was transferred into reaction buffer (pH 7.2) containing 0.5% Nonidet P-40. Reaction mixture was incubated with 12 units of peptide-\textit{N}-glycosidase F (PNGase) for 12 hrs at 37°C. Samples were loaded on SDS-PAGE, and the gels were stained by CBB and PAS reagent.

\textit{Assay for ACP activity} – The ACP activity of the enzyme was determined by following the hydrolysis of \textit{p}-nitrophenyl phosphate (\textit{p}-NPP) at 410 nm. The optimum
pH of the enzyme was around pH 6.0, and it showed activity in the range of pH 5 to 8 (data not shown). The routine assay was performed at 30°C by adding 50 µl of enzyme solution to 0.5 ml of reaction mixture consisting of 50 mM \( p \)-NPP and 200 mM Tris-maleate (pH 6.0). One enzyme unit was defined as 1 µmole of \( p \)-NPP hydrolyzed per 1 min.

Substrate specificity of the KeACP was estimated by incubating the purified enzyme with various substrates (17 kinds). The assay was carried out at 30°C by adding 50 µl of the enzyme solution to a reaction mixture containing 10 mM target substrate and 50 mM Tris-maleate (pH 6.0), and phosphorus released during the 2 min incubation was measured according to a method described previously (18). In all assays one unit of enzyme activity was defined as 1 µmole of Pi released per 1 min.

Assays to examine the effects of metal ions and chemical agents (9 kinds) on ACP activity were carried out as above, by incubating \( p \)-NPP at 30°C for 30 min with native enzyme or enzyme that was dialyzed against buffer containing EDTA. All measurements were repeated five times and the results represent the arithmetic means.

Assay for chloroperoxidase (CPO) activity – Vanadate-substituted enzyme (V-CPO) was prepared by incubating the enzyme with various concentration of \( o \)-vanadate in 100 mM Tris-HCl (pH 7.5) at 30°C for 30 min. The purified enzyme was then dialyzed against 100 mM Tris-HCl (pH 7.5) containing 20 mM EDTA. The CPO activity was measured by following the increase in the absorbance at 290 nm due to the chlorination of monochlorodimedon (MCD) in the presence of hydrogen peroxide (11). The standard assay was carried out at 30°C in a reaction mixture containing 2 mM \( H_2O_2 \), 5 mM KCl and 100 mM citrate buffer (pH 5.0). One unit of CPO was defined as 1 µmole of MCD chlorinated per 1 min.
Cloning and nucleotide sequencing of the KeACP gene – RNA was isolated from kidney bean embryonic axes elongated through 48 hrs after germination. Embryonic axes (0.1 g) were ground in liquid nitrogen. Total RNA was extracted with RNeasy Plant Mini kit (QIAGEN, Hilden, Germany) according to “clean-up” recommendations of the manufacturer. The cDNA library was constructed using a SMART™ RACE cDNA amplification Kit (BD Biosciences Clontech, Palo Alto, CA) according to the manufacturer’s instruction. The primers used to amplify sequences were designed based on the partial nucleotide sequence of the KeACP gene and the A. thaliana putative ACP mRNA (GenBank Accession Number AY091415). The primers used were SAK01 for the 5’-RACE (5’-tgcacatcacgcaaggtcac-3’) and HAK01 for the 3’-RACE (5’-cctcaaggccttcaataacac-3’). Both 5’- and 3’-RACE PCR amplifications of cDNA were carried out using a Gene-Amp automated thermal cycler (Model 9700, Applied Biosystems) with appropriate cycle parameters. The purified 5’-RACE (600 bp) and 3’-RACE (1,100 bp) PCR products were subcloned into a sequencing plasmid using a TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA) according to the manufacturer’s instruction.

Total genomic DNA was extracted from fine meal of the kidney bean seed according to the plant DNA extraction method, as described previously (19). To isolate the KeACP gene from the total genomic DNA by PCR, primers were designed and synthesized based on the cDNA sequence of KeACP that was determined in this study, as listed in Table I. The PCR products were sequenced on both strands using an ABI BigDye-Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems). The dye-labeled extension products were analyzed with an automated DNA sequencer (Model 377, Applied Biosystems). Nucleotide sequences were analyzed using
MacVector sequence analysis software (Oxford Molecular, Ltd). The sequence data have been submitted to the GenBank database under accession numbers AB116719 for full-length cDNA and AB116720 for genomic DNA of KeACP.

*Northern blot analysis* – Total RNAs were extracted from 0.1 g of embryonic axes of the kidney bean at different periods of growth on the agar plate at 25°C in darkness until 72 hrs after the beginning of imbibition. RNAs were also extracted from 0.1 g of roots, leaf and stem after two weeks of growth. The total RNAs extracted (30 µg) using the RNeasy Plant Mini kit were separated by 1% formaldehyde agarose gel electrophoresis and transferred to a nylon membrane (Hybond N+, Pharmacia Biotech). For dot blot analysis, 60 µg of total RNAs from each tissue were transferred to a nylon membrane as well. A DNA probe (245 bp) encoding a partial region of KeACP cDNA was labeled using a PCR-based DIG-Labeling Mix (Roche) with primer pair SAK01 and HAK01 (Table I). Northern hybridization was performed by high stringency hybridization at 60°C overnight.

**RESULTS**

*Purification and molecular characteristics of KeACP from embryonic axes of kidney bean* – Table II presents a summary of the purification procedure parameters. The enzyme was isolated from embryonic axes that elongated during germination of the kidney bean seed. KeACP was purified 852-fold to near homogeneity by anion-exchange DEAE column chromatography followed by Con A affinity and hydrophobic column chromatography. The Con A purification step effectively served to remove a significant amount of contaminating proteins, indicating that KeACP may be a
glycoprotein. Through all column chromatography purification steps, a single chromatographic peak of KeACP activity was observed, and the final preparation was colorless. This suggests that neither purple color ACP, which has usually been found in dry seeds (20), nor multiple ACP forms are present in embryonic axes of kidney beans. The pH dependence of phosphatase activity was also investigated for KeACP, yielding a curve with a pH optimum at pH 6.0 (data not shown) that was almost identical to that of other ACPs.

The apparent molecular mass of the purified enzyme was estimated to be around 96 kDa by analytical gel filtration on a Superdex 200 HR 10/30 column (Fig. 1). SDS-PAGE of the purified preparation both in the presence and in the absence of 2-ME revealed the presence of 56- and 45-kDa polypeptides with different staining intensities by CBB and PAS in a 3:1 ratio, respectively (Fig. 2A and B). This suggests that the native enzyme has a dimeric structure with no intersubunit disulfide linkages. Furthermore, native-PAGE of the purified enzyme preparation showed three bands of protein with both CBB staining (Fig. 2D) and enzyme activity staining (Fig. 2E), indicating that native KeACP presents three forms of subunit structure. Both bands on the gel were stained with a PAS reagent, and the enzyme retained on Con A column was eluted with D-(-)-mannose, strongly suggesting that they are glycosylated. Deglycosylation of the KeACP with N-glycosidase F under the denaturation condition was partially successful. After 12 hrs incubation, decreases in molecular mass of 2 and 1.4 kDa for dominant and minor subunits, respectively, on SDS-PAGE were observed (Fig. 2C). However, both bands were stained by PAS, indicating that oligosaccharide chain had still not been removed. The N-terminal amino acid sequences of both bands were determined to be NH₂-SEWPAVDIPLDHEAFAVPKG (Fig. 2B), implying that two
subunits have identical N-terminal sequences, but one probably has an excision of more than 100 amino acids in its C-terminal region, or/and has a different glycosylation pattern.

**Substrate specificity** – Because the physiological relevant substrate(s) for KeACP is still unknown, the purified enzyme was tested for its activity against a variety of phosphorylated substrates (Table III). The activity against p-NPP was taken to be 100%. Relatively high activity was observed with phosphoenolpyruvate and this was comparable to pyrophosphate, phosphorylated-Tyr, β-naphtylphosphate, glucose-1-phosphate and ADP, with more than 50% activity. Several other compounds were also dephosphorylated by KeACP at a much lower rate compared with p-NPP. Bis-p-NPP, phytate and 5’-AMP were apparently poor substrates. As a result, KeACP shows a wide variety of substrate specificity, but not a preference for other substrates over p-NPP. However, it is noteworthy that KeACP catalyzed dephosphorylation of phosphorylated-Ser, -Thr and -Tyr under the conditions employed.

**Effects of various chemicals on KeACP activity** – As shown in Table IV, like other ACPs, KeACP is inhibited completely by such typical inhibitors as vanadate (VO₄³⁻) and molybdate (Mo⁶⁺) at 100 µM, whereas the other metal ions (Zn²⁺, Fe³⁺ and Mn²⁺) at 10 µM concentration revealed no influence on KeACP activity. Metal chelating agents such tartarate, citrate, fluoride and EDTA at 100 µM concentration had no inhibitory effect, but rather caused a small increase in activity.

**Vanadate-substitute CPO activity** – After overnight dialysis against Tris-HCl buffer containing 20 mM EDTA to remove metals, chloroperoxidase activity was tested in a MCD-chlorination assay by the incubation of apo-KeACP with vanadate for 30 min at 30°C. In the presence of 100 µM vanadate, MCD was clearly chlorinated. In the
absence of vanadate, the apo-enzyme revealed phosphatase activity at the same level as the native enzyme, but no chloroperoxidase activity. On the other hand, vanadate-substituted purple color ACP prepared from kidney bean seed (20) revealed no chloroperoxidase activity under the same conditions, such as dialysis against EDTA buffer followed by incubation with vanadate. This means that the vanadate-substituted KeACP uniquely catalyzes the chloroperoxidation reaction. Figure 3A shows that more than 100 µM vanadate was required to obtain full chloroperoxidase activity, and that ACP activity disappeared at rates depending upon the concentration of vanadate added.

A modified Hill plot (21) with vanadate is shown in Fig. 3B. The interaction between enzyme and vanadate showed a linear relationship, and its Hill constant, calculated from the slope, was approximately 1.0. This may reflect the fact that the number of vanadate binding sites on the enzyme is one molecule binding to either subunit, or that two molecules bound to both subunits with equivalent affinity. The apparent dissociation constant, $K_d$, was determined to be 6.6 µM vanadate, as shown in Fig. 3B. The kinetic parameters, $K_m$ values for $H_2O_2$ and chloride (KCl), were determined as 0.79 mM and 7.6 mM, respectively, in the presence of 100 µM vanadate.

Cloning and nucleotide sequence of KeACP cDNA – The full-length coding regions of selective fragments were obtained by 5’- and 3’-RACE. The KeACP cDNA was 1,641 bp long and contained a 1,375 bp open reading frame, a 46 bp 5’-flanking sequence and a 220 bp 3’-flanking sequence. A poly-adenylation signal was found in the 28 bp downstream region. The predicted KeACP protein consists of 457 amino acids and has a calculated molecular mass of 52,865 Da. To examine the partial amino acid sequences of the KeACP protein, the fragments derived by limited proteolysis with V8 protease and lysyl endopeptidase were analyzed for their N-terminal amino acid
sequences. The sequences determined derived from two major fragments, from Asp-237 to Phe-248, and from Arg-304 to Met-317. These were in complete agreement with those deduced from the nucleotide sequence of the KeACP cDNA (data not shown).

The deduced amino acid sequence of the enzyme was analyzed using the SignalP (Ver. 2) program (22), along with the iPSORT and TargetP algorithms. The results predict that the N-terminal region of the precursor protein has characteristics of a signal peptide, with a probable cleavage site between Ala-28 and Gly-29, as shown in Fig. 4. However, the N-terminal amino acid sequences of both subunits begin at Ser-37 but not Gly-29 (Fig. 4), indicating that the N-terminal region of KeACP undergoes further modification after processing by signal peptidase in the tissue. The calculated molecular weight of the 420-amino-acid mature protein was predicted to be 44,840 Da, which differed from the molecular weight of partially deglycosylated dominant subunit (54 kDa), as determined by SDS-PAGE.

Comparison with the sequence database using a ClustalW multiple sequence alignment analysis indicated that the known protein with the highest homology (92% identity) to KeACP protein was a purple ACP-like protein (GenBank Accession Number AAN85416) from soybean. The KeACP protein shares 87% identity to lupinus purple ACP (GenBank Accession Number AJ458943), 78% identity to A. thaliana putative ACP (GenBank Accession Number AY050812) and 77% identity to Oryza sativa ACP (GenBank Accession Number AF356352). Thus, amino acid sequence analysis indicated that the KeACP was a purple ACP-like protein. However, purple ACP (Swiss-Prot #P80366), which is found predominantly in kidney bean, shared only 54% identity to our KeACP.

Further computer-assisted motif analysis showed that KeACP contains several
putative domains that are conserved. The results from analyses using the InterPro algorithm suggest that the KeACP protein is predicted to contain a Ser/Thr specific protein phosphatase at the residue 162–197 segment, a metallo-phosphatase domain at the 161–359 segment, a purple acid phosphatase N-terminal domain at residues 42–153, and an ACP purple precursor hydrolase signal glycoprotein tartrate-resistant Fe phosphatase-like domain at residues 157–457.

The intron-containing region of the KeACP gene was also analyzed by direct sequencing of PCR products. These were amplified using primers designed from the cDNA sequence of KeACP, as determined in this study. A total of 3,228 nucleotides were sequenced, and the genomic DNA sequence encoding KeACP was found to consist of seven exons and six introns according to the GT-AG rule. The complete sequence showed that all intron sequences were removed from the fully processed cDNA.

Northern blot analysis – The accumulation level of the KeACP mRNA (1.6 kb) was analyzed by northern blots using total RNA extracted from elongated embryonic axes of germinated kidney bean at selected time intervals after the beginning of imbibition. Northern blots indicated that KeACP gene expression appeared soon after the imbibition and increased until 18 hrs imbibition, at which time the radicle germinates from the seed coats. Subsequently, the level of expression was constant (Fig. 5A). Dot blot analysis indicated the same tendency. On the other hand, for other tissues, such as roots, leaf and stem, the levels of mRNAs after two weeks of cultivation were very low compared with that of embryonic axes (72 hrs after imbibition), as shown in Fig. 5B. Accordingly, this suggests that the KeACP protein is specifically localized in embryonic axes tissue during elongation that occurs after the beginning of germination.
DISCUSSION

KeACP was purified to near homogeneity as a single activity peak from kidney bean embryonic axes by using ion-exchange, Con A-Sepharose and hydrophobic column chromatography. As shown in Table II, KeACP from the kidney bean embryonic axes was purified 852-fold to a final \( p \)-NPP specific activity of 383 units/mg protein, and with a recovery of 11%. Binding of the enzyme to a Con A column suggested that this KeACP is glycosylated. SDS-PAGE of purified KeACP in the presence or absence of reducing agent showed two bands, a dominant 56-kDa and minor 45-kDa polypeptides with a staining intensity ratio of 3:1 by CBB and PAS reagent. Deglycosylated enzyme through the use of \( N \)-glycosidase F showed that both subunits seem to have oligosaccharide chain containing at least 7–12 sugar residues (1.4–2 kDa). However, both subunits were still stained by PAS reagent, indicating that the partial fucosylation of some oligosaccharide core may make inaccessible to endoglycosidase employed. Using gel filtration chromatography, the molecular mass of purified KeACP was determined apparently to be around 96 kDa, demonstrating the dimeric structure of the enzyme. However, the molecular mass of the enzyme by gel filtration was somewhat lower than the expected 100–110 kDa by sum of combination of subunit masses, probably due to specific interactions between glycosyl residues and gel filtration resin.

The N-terminal sequences (20 residues) of both polypeptides were identical, suggesting that the 45-kDa polypeptide is derived from deletion of about 100 amino acids from C-terminal of the 56-kDa polypeptide. Both CBB and enzyme activity staining on native-PAGE showed three bands corresponding to each form. Accordingly, the dimer of KeACP is composed of two non-covalently bound subunits, and seems to
exist as a mixture of three dimeric forms, a homodimer of the 56-kDa subunit, or the 45-kDa subunit and a heterodimer of each subunit. Red kidney bean ACP (7) is well characterized with respect to its three-dimensional structure and metal binding site, which indicates that it is a dimeric glycoprotein (110 kDa) with an intersubunit disulfide linkage. The plant ACPs are mostly dimeric glycoproteins, about 110 kDa per homodimer (14, 23) or heterodimer. Similarly, heterogeneity in the molecular masses of the subunits of plant ACPs has also been reported for yellow lupin (24), sunflower seed (25), sycamore seeds (26) and tomato (27), reflecting partial proteolysis of a polypeptide chain or a change in the glycosylation pattern.

We have cloned a full-length cDNA for the \textit{KeACP} gene from embryonic axes of the kidney bean. The sequence is composed of 1,641 nucleotides, including a single open reading frame of 1,375 nucleotides (GenBank Accession Number AB116719) that encodes 457 amino acids and contains putative signal peptide of 28 amino acids, as predicted by the SignalP program supported by the iPSORT prediction and TargetP prediction programs. In plants, ACPs (or ACP activities) have been localized to many cellular compartments including vacuoles, chloroplasts, cell walls, membranes, the Golgi complex, and the cytoplasm (1, 28). These analyses provide information about possible cellular location of the \textit{KeACP} protein, and the results clearly indicate that the \textit{KeACP} protein is targeted to the secretory pathway via the endoplasmic reticulum.

The precursor protein has characteristics of a signal peptide at its N-terminal region, with a probable cleavage site between Ala-28 and Gly-29, as shown in Fig. 4. However, this prediction differs from the sequence of the purified enzyme, in which the N-terminal amino acid sequences of both subunits begin at Ser-37, not Gly-29. It is likely that the N-terminus of purified \textit{KeACP} results from further modification after
processing by signal peptidase in the embryonic axes tissue, or as a result of certain
protease(s) in the crude homogenate, which are probably trypsin-like, because Arg-36 is
at the amino side of Ser-37. The molecular weight predicted for the mature protein (420
amino acids, 44,840 Da) appeared to match very well with that determined by
SDS-PAGE (45–56 kDa), considering that both subunits of the purified enzyme are
glycosylated.

Comparative analysis of the structures of purple ACPs from higher plants has
allowed the identification of conserved sequence and structural motifs in this type of
enzyme from many eukaryotic species (2, 9). Generally, plant purple ACPs belong to
the metallo-phosphoesterase family of proteins, indicating that the composition of
dinuclear center is Fe$^{3+}$-Zn$^{2+}$ in red kidney bean (7) and soybean (6), or Fe$^{3+}$-Mn$^{2+}$ in
sweet potato (29). They can be distinguished from other ACPs by their purple color,
which is due to a charge transfer complex with Fe$^{3+}$ and a nearby tyrosine residue. The
KeACP in concentrated solution did not reveal a purple color, despite the fact that motif
analysis using the InterPro algorism predicted a metallo-phosphoesterase at domain at
residues 161–359. Moreover, addition of Fe$^{3+}$ and Zn$^{2+}$ to the KeACP protein did not
lead to development of purple color when it was added after dialysis against buffer
containing EDTA, and various metal chelating agents such as tartrate, citrate and
fluoride did not inhibit native KeACP activity. Evidently, the KeACP enzyme is distinct
from other plant purple ACPs, even though it resembles the purple ACP family in
several of its biochemical properties.

In this study we showed that KeACP converted to CPO with loss of phosphatase
activity when ortho-vanadate (VO$_4^{3-}$) was added to the enzyme after dialysis against
EDTA-containing buffer. To our knowledge, KeACP is the first V-substituted CPO in
plants to be characterized. CPOs are members of the enzyme group called haloperoxidases. These non-heme vanadate-containing peroxidases were first isolated from seaweed brown macro-algae (30), and have also been found in fungi (31) and lichen (32). Recently, Littlechild et al. (33) reviewed structural and functional comparisons between vanadium haloperoxidases and ACPs, finding that they belong to a completely different group of enzymes. The ACPs are classified into several different groups, and membrane-associated phosphatases, including type 2 phosphatidic ACPs, bacterial ACPs and mammalian glucose-6-phosphatase shared an active site sequence motif (GSYP SGHT) with the V-dependent CPOs. However, this type of motif was not found in KeACP, which is classified as a soluble phosphatase.

The amount of vanadate required for full CPO activity seems relatively high, 100 µM, and an apparent dissociation constant of 6.6 µM for vanadate was calculated. Moreover, KeACP displayed relatively high $K_m$ values of 0.72 mM for $H_2O_2$ and 7.4 mM for $Cl^-$ at pH 5.0, compared with $K_m$ values of 4–27 µM for $H_2O_2$ and 0.18–19.6 mM for $Cl^-$, depending upon pH, reported for a representative CPO from the fungus Curvularia inaequalis (34). It is well known that soils contain approximately 2 mM vanadate (31), which is likely enough to obtain a fully activated CPO in the plant in vivo. However, the fact that CPO activity has not been detected earlier in kidney bean homogenates may be related to the fact that addition of vanadate to the apo-form of the enzyme is necessary for CPO activity. Indeed, a general property of V-bromoperoxidase is that vanadate can be removed by dialysis against phosphate-containing buffers, and the CPO activity may be restored only by addition of vanadate (35).

The CPOs are defined by their ability to oxidize electrophilic halide species, $Cl^-$, $Br^-$ and $I^-$, to the corresponding hypohalous species in the presence of $H_2O_2$. However,
V-CPO derived from KeACP specifically oxidized Cl\textsuperscript{−} to hypochlorite but was inactive against other halides. This property of vanadate-KeACP is distinct from other V-CPOs from various organisms. Assuming that V-CPO carries out its function \textit{in vivo}, the physiological role of this enzyme has been unclear, as it produces a strong oxidizing agent, hypochlorite, at an early stage of elongation of the embryonic axis during germination. There is increasing evidence suggesting that the H\textsubscript{2}O\textsubscript{2} producing-reaction locates in photosynthetic or respiratory electron transport systems in plants, and a few reports using soybeans suggested that seed germination is accompanied by a generation of reactive oxygen species containing H\textsubscript{2}O\textsubscript{2} in the embryonic axis (36, 37). Since seed germination represents the developmental period that is most sensitive for pathogen infection (38), it is more likely that hypochlorite produced in this stage plays an important role in protecting the embryo against attack by pathogens or parasitic organisms. Another possible explanation is that hypochlorite produced by V-CPO may promote seed germination by decomposing antioxidants, which are derivatives of well-known germination inhibitors present in plant seeds (39, 40).

The full-length \textit{KeACP} gene was most closely related to the soybean purple ACP-like gene \textit{GmPAP3} (41) with 92\% identity, lupin ACP (42) with 87\% identity and red kidney purple ACP gene (9) with 54\% identity. Motif analysis predicts that the KeACP protein possesses a metallo-phosphatase domain, a purple ACP N-terminal domain and an ACP precursor hydrolase signal glycoprotein tartrate-resistant Fe phosphatase-like domain, and that these are conserved in all purple ACPs (6, 9). These results suggest that KeACP belongs to the classical purple ACP family. Nevertheless, KeACP is a novel ACP that differs from the previously identified purple ACPs in plant. Unlike most purple ACPs that contain a binuclear Fe-Zn(Fe) center on their active sites
(6), KeACP develops no purple color or CPO activity after the addition of vanadate. Furthermore, northern blot data presented here demonstrated that the KeACP gene transcript specifically accumulated during rapid elongation of embryonic axes of the kidney bean, but not other tissues (Fig. 5). However, it is interesting that the soybean purple ACP-like GmPAP3 gene, which has a sequence similarity to KeACP, was induced by salt stress through its involvement in reactive oxygen species (41). Further studies are needed to demonstrate the subcellular localization of KeACP in embryonic axes, and how induction of the KeACP gene expression responds to various stresses, including oxidative stresses and pathogens (43), at the germination stage.

The data presented here demonstrate that KeACP is a novel ACP that is clearly distinct from previously reported ACPs in plants. However, it might be premature to speculate on possible functions of KeACP. Further characterization of its precise structure and physiological substrates will be required to clarify its possible roles in kidney bean embryonic axes cells.

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FOOTNOTES

1The abbreviations used are: ACP, acid phosphatase; CPO, chloroperoxidase; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PAS, periodic acid-Schiff; p-NPP, *p*-nitrophenyl phosphate; 2-ME, 2-mercaptoethanol; MCD,
monochlorodimedon.

FIGURE LEGENDS

Fig. 1. Molecular size estimation of KeACP by an analytical gel filtration Superdex 200 HR 10/30 column. Purified KeACP (40 µg) was applied to a column equilibrated with 20 mM Tris-HCl (pH 7.5) containing 0.5 M NaCl. Molecular size of the target protein was calculated by a line fitting the calibration curve to the data with standard size marker proteins. Measurements were repeated three times, and the results represent the arithmetic means.

Fig. 2. SDS- and native-PAGE analyses in the presence and absence of 2-mercaptoethanol (2-ME), and N-terminal amino acid sequence of KeACP. (A) SDS-PAGE of the purified KeACP (2 µg) in the presence of 2-ME (lane 1, indicated by 2-ME(+)) and absence of 2-ME (lane 2, indicated by 2-ME(−)) using a 10% separating gel. Protein bands in both lanes were stained by CBB. The N-terminal amino acid sequences of the bands are shown. (B) Glycoprotein staining of the purified KeACP (2 µg) separated from SDS-PAGE. Protein bands were stained by PAS. (C) Deglycosylation of the KeACP (2 µg) with N-glycosidase F separated from SDS-PAGE. Protein bands were stained by CBB and PAS. (D) Native-PAGE of KeACP (1 µg) using a 5% separating gel stained by CBB. (E) Detection of ACP activity in the gel after native-PAGE using Fast Black K salt/α-naphthylphosphate as substrate. Bands stained are indicated by arrows.

Fig. 3. ACP and CPO activities of the vanadate-substituted enzyme, and modified
Hill plot. (A) After the enzyme was dialyzed against buffer containing EDTA, the enzyme was incubated with various concentration of vanadate for 30 min at 30˚C. The activities for ACP (Δ) using p-NPP and for V-CPO (O) using chlorination of MCD in the presence of H₂O₂ as substrates were taken to be 100%, respectively. (B) Modified Hill plots of CPO activity versus vanadate concentration. The Hill constant was calculated from the linear slope, and the apparent dissociation constant Kd was obtained from the intercept on the horizontal axis at half activity, as indicated by the arrow. V₀ and V represent maximum velocity and velocities of CPO reaction at each concentration of vanadate, respectively.

Fig. 4. Schematic representation to compare N-terminal amino acid sequences of the precursor form, the predicted signal peptide, and the purified KeACP protein. N-terminal amino acid sequence of the precursor protein was deduced from full-length cDNA. Predicted cleavage site by signal peptidase was predicted by the SignalP program. The N-terminal amino acids of the purified enzymes (see Fig. 2B) are marked with arrows.

Fig. 5. Northern blot analysis of total RNA isolated from each tissue of the kidney bean after germination using a DIG-labeled nucleotide probe specific for the KeACP gene. Kidney bean seeds were germinated and grown as described in Experimental Procedures. (A) Accumulation of KeACP gene transcripts in embryonic axes at different periods of growth during the 72 hrs following imbibition. Total RNA in each lane was separated on an agarose gel, and sampling periods are indicated. (B) Northern dot blot analysis of total RNAs from stem, leaf and root tissues of the kidney
bean (two weeks growth) and embryonic axes (72 hrs growth) following the beginning of imbibition. In the top gels, total RNA (30 µg) in each lane was separated on the agarose gel, and sampling periods (A) and in tissues tested (B) are indicated. Middle gels in both A and B were stained with ethidium bromide. Bottom gels in both A and B show dot blot analysis of total RNA (60 µg) that was directly transferred to the membranes.
| primer | Forward primer         | Reverse primer         |
|--------|------------------------|------------------------|
|        | sequence                | sequence                |
| T01F   | 5’-TCC AGC TAT TCC GCC ATT TG-3’ | 5’-CTC CAT TAC TGT TGT AGA GTG-3’ |
| T02F   | 5’-TAA CAG GTA TCC CTT ACC TG-3’ | 5’-TGT CAG GTA AGG GAT ACC TG-3’ |
| T03F   | 5’-CGG TGA TTC TCC TCG AGA G-3’ | 5’-GCA CGC CTC ACT GCA TAC-3’ |
| T04F   | 5’-GCA TAA CCA GTA TTG GTA GG-3’ | 5’-CAT CAT CAT TGC GGT TCC AG-3’ |
| T05F   | 5’-GGT TAT GAC TCT GTA GCC AG-3’ | 5’-GTC TCG CCA TTT GTT CAA ATG-3’ |
| T07F   | 5’-ACA GGT ATC CCT TAC CTG AC-3’ | 5’-ACG TGC ATG AGC ACA ATG AG-3’ |
| S01F   | 5’-ACT ACT CCC TAT TTG GCC TC-3’ | 5’-CTA AGC CAT AAC GAT ATT AAA CTT CTA CC-3’ |
| S02F   | 5’-TCA TTG TGC TCA TGC ACG TG-3’ | 5’-CCT CAA GGC CTT CAA TAA CAC -3’ |
| S03F   | 5’-CAA GCT ATG GAC ACT CTA CC-3’ | 5’-TTC CAT CGT AGT CAC CTT GC-3’ |
| S04F   | 5’-CTT GCT GTT GTG AGG AAT TG-3’ | 5’-AAG CAT CCG GGT CAA CTT TG-3’ |
| SAK01  | 5’-TGC ACA TCA CGC AAG GTC AC-3’ | 229-249                |
| Eri02  | 5’-GGC CGA TTT GTT GAA AGG AG-3’ | 705-725                |
| Purification stage      | Activity (U) | Total Protein (mg) | Specific activity (U/mg) | Purification-fold | Yield (%) |
|-------------------------|--------------|--------------------|--------------------------|-------------------|-----------|
| Crude extract           | 3371         | 7400               | 0.45                     | 1                 | 100       |
| (NH₄)₂SO₄ (40-60 %)     | 1530         | 1480               | 1.03                     | 2                 | 45        |
| DEAE Toyopearl 650S     | 990          | 338                | 2.29                     | 5                 | 29        |
| Con A-Sepharose         | 645          | 10                 | 64.54                    | 143               | 19        |
| Butyl Toyopearl 650S    | 368          | 0.96               | 383.33                   | 852               | 11        |
| Substrate                     | Relative activity (%) |
|-------------------------------|-----------------------|
| $p$-NPP                       | 100                   |
| ADP                           | 54                    |
| $5'$-AMP                      | 13                    |
| ATP                           | 40                    |
| $\alpha$-naphtylphosphate     | 32                    |
| $\beta$-naphtylphosphate      | 59                    |
| bis-$p$-NPP                   | ND\textsuperscript{1} |
| glucose-1-phosphatase         | 58                    |
| glucose-6-phosphatase         | 46                    |
| pyrophosphate                 | 65                    |
| 2-phosphoglycerate            | 32                    |
| 3-phosphoglycerate            | 34                    |
| phosphoenolpyruvate           | 67                    |
| phytate                       | 16                    |
| phosphorylated-Thr            | 42                    |
| phosphorylated-Ser            | 28                    |
| phosphorylated-Tyr            | 63                    |

\textsuperscript{1}ND, not detected.
**TABLE IV**

*Effect of metal ions and chemical agents on the KeACP activity*

| Addition | Relative activity (% of control) $^\ddagger$ | 10 µM | 100 µM |
|----------|---------------------------------------------|-------|--------|
| Zn$^{2+}$ |                                             | 101.0 | 70.3   |
| Fe$^{3+}$ |                                             | 108.4 | 102.1  |
| Mn$^{2+}$ |                                             | 105.5 | 94.8   |
| VO$_4^{3-}$ |                                         | 5.3   | 9.5    |
| Mo$^{6+}$  |                                             | 37.6  | 12.6   |
| Tartrate  |                                             | 97.6  | 110.3  |
| Citrate   |                                             | 99.1  | 99.3   |
| NaF       |                                             | 114.2 | 107.4  |
| EDTA      |                                             | 96.8  | 103.7  |

$^\ddagger$Data represent the arithmetic means of five experiments
FIG. 1

Molecular mass (kDa)

Elution volume (ml)

- thyroglobulin
- ferritin
- aldolase
- 96 kDa
- BSA
- ribonuclease A
FIG. 2

A

B

C

D

E

56 kDa (SEWPAVDIPLDHEAFAVPKG)

45 kDa (SEWPAVDIPLDHEAFAVPKG)

54 kDa

43.6 kDa

2-ME(−)

2-ME(+)

2-ME(−)

CBB

PAS

CBB

PAS

CBB

ACP activity

M

M

M

M

M

(kDa)

(kDa)

(kDa)
FIG. 3

A

CPO activity (µM/min)

ACP activity (%)

Vanadate [µM]

0 5 10 15 20 25

0 5 10 15 20 25

0

0

5

10

15

20

25

0

20

40

60

80

100

120

CPO activity

ACP activity

B

Log(V_0/V_0 - V)

Slope = 0.95

Kd = 6.64 x 10^-6 (M)

Log [V]

-6

-5

-4

-3

-2

-1

0

1

2

1
Predicted signal peptide cleavage site

Precursor protein

Predicted signal peptide

Purified KeACP

N-terminus of purified KeACP

FIG. 4
FIG. 5

A  Time course (hrs)

|   | 6   | 12  | 18  | 24  | 36  | 48  | 60  | 72  |
|---|-----|-----|-----|-----|-----|-----|-----|-----|
|   | ![Image](image1.png) | ![Image](image2.png) |

Northern blot analysis

Total RNA

Dot blot analysis

B  Tissue

| Embryonic axis | Stem | Leaf | Root |
|---------------|------|------|------|
| ![Image](image3.png) | ![Image](image4.png) |
| ![Image](image5.png) | ![Image](image6.png) |
| ![Image](image7.png) | ![Image](image8.png) |
Characterization of a novel acid phosphatase from embryonic axes of kidney bean exhibiting vanadate-dependent chloroperoxidase activity
Tohru Yoneyama, Masae Shiozawa, Masao Nakamura, Tomonori Suzuki, Yoshimasa Sagane, Yoshinobu Katoh, Toshihiro Watanabe and Tohru Ohyama

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