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HlPT-1, a membrane-bound prenyltransferase responsible for the biosynthesis of bitter acids in hops

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ABSTRACT

Female flowers of hop (*Humulus lupulus* L.) develop a large number of glandular trichomes called lupulin glands that contain a variety of prenylated compounds such as α- and β-acid (humulone and lupulone, respectively), as well as xanthohumol, a chalcone derivative. These prenylated compounds are biosynthesized by prenyltransferases catalyzing the transfer of dimethylallyl moiety to aromatic substances. In our previous work, we found HlPT-1 a candidate gene for such a prenyltransferase in a cDNA library constructed from lupulin-enriched flower tissues. In this study, we have characterized the enzymatic properties of HlPT-1 using a recombinant protein expressed in baculovirus-infected insect cells. HlPT-1 catalyzed the first transfer of dimethylallyl moiety to phloroglucinol derivatives, phlorisovalerophenone, phlorisobutyrophenone and phlormethylbutanophenone, leading to the formation of humulone and lupulone derivatives. HlPT-1 also recognized naringenin chalcone as a flavonoid substrate to yield xanthohumol, and this broad substrate specificity is a unique character of HlPT-1 that is not seen in other reported flavonoid prenyltransferases, all of which show strict specificity for their aromatic substrates. Moreover, unlike other aromatic substrate prenyltransferases, HlPT-1 revealed an exclusive requirement for Mg^{2+} as a divalent cation for its enzymatic activity and also showed exceptionally narrow optimum pH at around pH 7.0.

1. Introduction

Hop (*Humulus lupulus* L., Cannabinaceae) is a perennial and dioecious climbing plant and female plants of the species are cultivated world-wide for use as an essential ingredient of beer. Female flowers, also called hop cones, give the characteristic flavor and bitter taste to beer due to a variety of essential oils and aromatic compounds, which are biosynthesized and accumulated exclusively in yellow glandular trichomes, also designated lupulins, which develop at the basal part of hop cone bracts [1]. Among the secondary metabolites produced by the hop plant, prenylated acylphloroglucinols, conventionally called 'bitter acids', have received a large amount of attention because their characteristic bitter property is important for beer taste; moreover, their divergent biological activities, including radical scavenging activity, angiogenesis inhibition, and inducing effect for P450 enzyme, are beneficial for human health [2,3]. Hop cones also contain prenylated flavonoids, among which the major one is xanthohumol, a prenylated chalcone derivative, which has potential applications as a cancer chemopreventive agent [4].

The proposed biosynthetic pathway of bitter acids in hops, also called α- and β-acid (humulone and lupulone, respectively), are shown in Fig. 1, with the biosynthesis of xanthohumol illustrated in parallel. The aromatic core of humulone and lupulone is a phloroglucinol derivative formed by the condensation of an acyl-CoA and three molecules of malonyl-CoA via the function of valerophenone synthase [5]. There are three major phloroglucinol derivatives in hops according to the different sources of acyl-CoA, namely, phlorisovalerophenone (PIVP) biosynthesized from isovaleryl-CoA, which is the precursor for humulone and lupulone as representative bitter acids [5], phlorisobutyrophenone (PIBP) derived from isobutyryl-CoA leading to cohumulone and colupulone, and phlormethylbutanophenone (PMBP) given with 2-methylbutyryl-CoA, which is the precursor for adhumulone and adlupulone. After condensation with malonyl CoA, the resulting acylphloroglucinol derivatives undergo two or three prenylations with dimethylallyl diphosphate by an aromatic substrate prenyltransferase of hops. Mono-prenyl PIVP and di-prenyl PIVP (deoxyhumulone) are the key intermediates in the humulone and lupulone biosynthesis. Humulone is formed from deoxyhumulone

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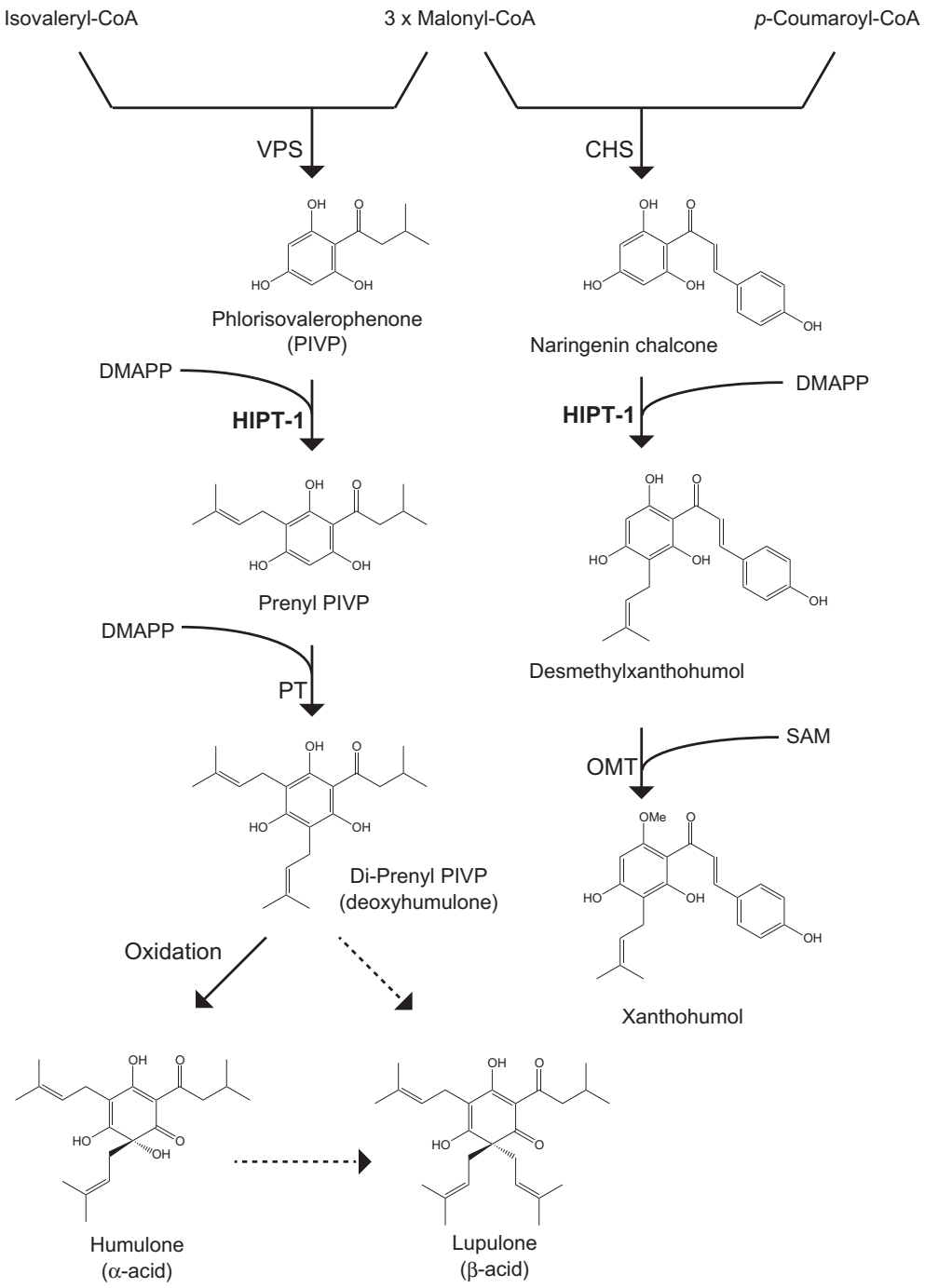


Fig. 1. Biosynthesis of bitter acids (humulone and lupulone) and xanthohumol in lupulin glands of hop. DMAPP, dimethylallyl diphosphate; VPS, valerophenone synthase; PT, prenyltransferase; CHS, chalcone synthase; OMT, *O*-methyltransferase; SAM, *S*-adenosylmethionine.

through oxidation [6], whereas lupulone, having three dimethylallyl moieties, is proposed to be synthesized either from deoxyhumulone or humulone by a further prenylation reaction [7]. In a manner similar to acylphloroglucinol derivatives, naringenin chalcone is biosynthesized via condensation of *p*-coumaroyl-CoA and three molecules of malonyl-CoA by chalcone synthase to yield the aromatic core of xanthohumol. *C*-prenylation of naringenin chalcone provides desmethylxanthohumol, which is then converted to xanthohumol by an *O*-methyltransferase in the presence of a methyl donor, *S*-adenosylmethionine [8]. In the biosynthesis of these secondary metabolites in hops, aromatic prenyltransferases play a crucial role for both phloroglucinol and flavonoid derivatives.

The plant prenyltransferases that recognize aromatic secondary metabolites are a new topic of research in plant molecular biology; the first flavonoid-specific prenyltransferase, naringenin 8-dimethyallyltransferase (SfN8DT), was identified in 2008, and thereafter a pterocarpan and isoflavonone-specific prenyltransferases, glycinol 4-dimethylallyltransferase (G4DT) and genistein 6-dimethylallyltransferase (SfG6DT), respectively, have been reported [9–11]. These enzymes are all divalent cation-requiring membrane-bound proteins, and those characterized to date have been localized in plastids. In our previous work on hops, we constructed a cDNA library from the lupulin gland-rich portion of female flower bracts, and randomly sequenced 11,233 EST clones, obtaining sequence information for 6613 non-redundant ESTs. Among them, a cDNA

designated *Humulus lupulus prenyltransferase-1* (HlPT-1) was a candidate for the gene coding for prenylation enzyme as it possessed three features of the plant aromatic prenyltransferase family, namely, a D-rich motif, multiple membrane-spanning domains, and a putative transit peptide sequence at the N-terminus [12]. Indeed, HlPT-1 was highly expressed in hop cones, especially in the lupulin glands. Moreover, a GFP fusion experiment showed that the transit peptide of HlPT-1 actually localized the GFP fusion protein to plastids in a manner similar to other flavonoid prenyltransferases in the legume plants [12]. In this study, we heterologously expressed HlPT-1 protein in insect cells and demonstrated its enzymatic function *in vitro* assays using phloroglucinol derivatives and various flavonoids as prenyl acceptor substrates in the presence of dimethylallyl diphosphate as a prenyl donor.

2. Materials and methods

2.1. Plant materials and reagents

We used HlPT-1 cDNA synthesized from hop plants (cv. Kirin II) as previously described [12]. The flavonoids used as substrate, naringenin, naringenin chalcone and 6′-*O*-methyl chalcone [helichrysetin, 1-(2,4-dihydroxy-6-methoxyphenyl)-3-(4-hydroxyphenyl)-2-propen-1-one], were purchased from Tokyo Chemical Industry (Japan), TransMIT (Germany), and Wako Pure Chemical Industries (Japan). Dimethylallyl diphosphate (DMAPP) was chemically synthesized as described elsewhere [13]. PIVP, PIBP, PMBP and those prenylated compounds were also chemically synthesized as previously reported [14].

2.2. Expression of recombinant HlPT-1 in insect cells

PCR primers were designed to subclone the coding sequence of HlPT-1 into a baculovirus vector (Fw-primer, 5′-tcgcgaatggagctc tcttcagtttctag-3′ and Rv-primer, 5′-ggtaccctaaatgaacagatatacaacgta-3′), to which *Nru*I and *Kpn*I restriction sites (underlined) were added to the 5′- and 3′-ends of HlPT-1, respectively. PCRs were performed with *TaKaRa Ex Taq* DNA polymerase and HlPT-1 cDNA (GenBank ID: AB543053) as the template [12]. TA coning of the PCR product was carried out with TOPO TA Cloning® Kit for sequencing (Invitrogen). The PCR product was subcloned into a baculovirus transfer plasmid pPSC8 (Protein Science Corporation) by restriction enzyme digestion followed by ligation to yield pPSC8-HlPT-1. Co-transfection of insect cells (Sf9) was carried out with the transfer vectors and *Autographa californica* nuclear polyhedrosis virus (AcNPV) DNA. Plaque assay was performed to purify a clonal population of recombinant virus, and Sf9 cells were transfected again with the purified virus to amplify the virus titer. To produce the recombinant HlPT-1 protein, *Expres*SF$^{+®}$ cells (Protein Science Corporation) were transfected with the amplified virus fluids prepared above. Co-transfection, purification and amplification of virus, and the heterologous expression in *Expres*SF$^{+®}$ cells were performed according to the protocol of Wako Pure Chemical Industries, Ltd. (Japan).

2.3. Enzyme assay

Microsomal fraction of *Expres*SF$^{+®}$ cells expressing HlPT-1 protein were suspended with 100 mM Tris–HCl buffer (pH 7.5) to form the enzyme solution for the prenyltransferase assay. A typical incubation mixture contained 1 mM aromatic substrate, 1 mM DMAPP, 10 mM $MgCl_2$ and enzyme solution (ca. 0.5 mg protein) in a total volume of 100 μl, which was incubated at 30 °C for 1 h. The reaction mixture was extracted with 1 ml of ethyl acetate, and the organic phase was separated and evaporated. The residue of organic phase was dissolved in methanol, and after centrifugation the supernatant was analyzed by HPLC for routine assay. LCMS-IT-TOF (liquid chromatography ion trap time of flight mass spectrometry) was employed for identification of enzyme reaction products.

2.4. Analysis of enzymatic reaction products by HPLC and LCMS-IT-TOF

The enzymatic reaction products, namely, mono-prenylated PIVP, PIBP and PMBP, were quantitatively analyzed with the peak area at 291 nm in HPLC, which was conducted with a LaChrom Elite System equipped with L-2455 photo diode array detector (HITACHI, Tokyo, Japan). The chromatography was carried out using C18-column (Tosho, TSKgel ODS-80Ts, 2.0 mm ID × 25 cm) with a gradient program of solvent A (0.3% formic acid) and solvent B (100% acetonitrile), ranging from 40% to 80% B for 20 min, and isocratic elution at 80% B for 10 min at 40 °C. In LCMS-IT-TOF analysis, the same reversed phase chromatography was done, and separated samples were ionized by both positive- and negative-ion mode with detection range from *m*/*z* 200 to 500 for mass spectrometry.

3. Results and discussion

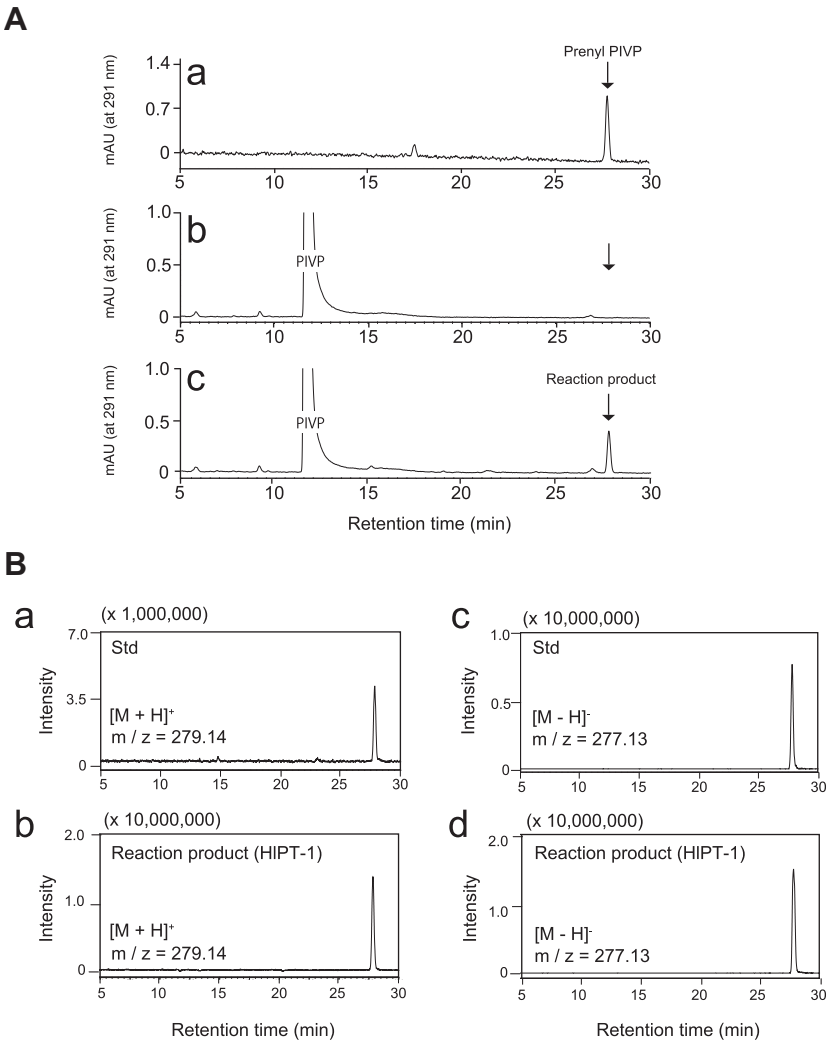
3.1. Enzymatic function of recombinant HlPT-1

HlPT-1 cDNA was expressed in insect cells (*Expres*SF$^{+®}$) by a baculovirus system. Enzyme assay was performed using the microsomal fraction of insect cells expressing the HlPT-1 protein. *Expres*SF$^{+®}$ cells transfected with empty vector (pPSC8) was used as the negative control. As representative substrates, PIVP and DMAPP were employed as a prenyl acceptor and a prenyl donor, respectively. The enzymatic reaction product showed both identical retention time and identical molecular mass with a standard specimen of mono-prenyl PIVP in HPLC and LCMS-IT-TOF analysis, i.e., the retention time at 27.8 min, the parent ion *m*/*z* 279.143 and a daughter ion *m*/*z* 223.084 (Fig. 2A, B). No detectable mono-prenyl PIVP was seen in the negative control. The identity of prenylated PIVP was confirmed also in negative mode, where the value of $[M-H]^-$ 277.129 and the fragment size of *m*/*z* 234.150 indicated that HlPT-1 protein catalyzed the dimethylallylation of PIVP (Fig. 2C).

In the biosynthesis of humulone and lupulone, multiple prenylation steps are involved after the formation of PIVP via condensation of isovaleryl-CoA and malonyl-CoA (Fig. 1). It could thus be expected that the second and the third prenylation can be also catalyzed by HlPT-1, but neither di-prenyl PIVP (deoxyhumulone), humulone nor lupulone were detected in the enzyme reaction. These results suggested that HlPT-1 is responsible for the first aromatic prenylation in the biosynthesis of humulone and lupulone, and the second and/or the third aromatic prenylations may be managed by another prenyltransferase. In any case, the successive prenylation was not observed under our assay conditions.

3.2. Substrate specificity of HlTP-1

Bitter acids in lupulin glands of hops are composed of α- and β-acids, which are respectively a mixture of n-, co- and ad-humulone; and a mixture of n-, co-, and ad-lupulone. The chemical difference between these acylphloroglucinol derivatives lies in their branched side chains, which are derived from leucine, valine, and isoleucine, respectively [15]. The intermediates of humulone and lupulone derivatives, namely, PIVP, PIBP and PMBP are the precursors of n-, co- and ad-compounds, respectively (Fig. 3A). The substrate specificity of HlPT-1 was studied with these phloroglucinol



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Peak	R.T. (min)	[M + H]+	Fragment (ms/ms)	[M - H]-	Fragment (ms/ms)
Prenyl PIVP (Standard)	27.8	279.141	223.081	277.129	234.147
HlPT-1 product	27.8	279.143	223.084	277.129	234.150

Fig. 2. LCMS-IT-TOF analysis of enzymatic reaction product. (A) HPLC chromatograms at UV 291 nm; (a) standard specimen of mono prenyl PIVP, (b) negative control (empty vector) of enzyme assay with PIVP and DMAPP as substrates, (c) enzymatic reaction product of HlPT-1 incubated with PIVP and DMAPP. Arrows indicate the retention time of mono prenyl PIVP. (B) MS chromatograms of positive ion mode (a, b) and negative ion mode (c, d) in LCMS-IT-TOF analysis. Data of standard specimen of mono-prenyl PIVP are shown in (a, c) and those of enzymatic reaction product of HlPT-1 are shown in (b, d). (C) Summary of parent ions and representative fragment ions of HlPT-1 reaction product monitored both in positive and negative ion modes. The data were directly compared with the standard specimen.

derivatives as prenyl acceptors with DMAPP being used as a prenyl donor. In parallel, various flavonoids were tested as other prenyl accepter substrates, because prenylated flavonoids, like xanthohumol, also exist in lupulins in large quantities (Fig. 3B).

The most preferable aromatic substrate for HlPT-1 was found to be PIVP [688 ± 29.7 pmol mg protein^{-1} h^{-1} (n = 3), 100%], followed by PMBP and PIBP [61.9% and 29.5% relative activities, respectively (n = 3)]. The apparent *Km* value for PIVP was calculated to be 59.5 ± 10.3 μM (n = 3). We attempted to detect di-prenylated products in assays where PIBP and PMBP were used as substrates, but as was the case when PIVP was used as the substrate, neither di-prenyl PIBP (deoxycohumulone), nor di-prenyl PMBP (deoxyadhumulone) were detected. Also, further prenylated compounds like colupulone or adlupulone were not detectable, either. We considered the possibility that the successive prenylation did not occur because of the low amount of mono-prenylated PIVP in the reaction mixture of the first prenylation. Thus, we also carried out an *in vitro* assay using mono- and di-prenyl PIVP, PIBP and PMBP as aromatic substrates individually, but in fact no enzymatic reaction product was obtained with HlPT-1 (Fig. 3B), supporting the hypothesis that the second and the third prenylation is catalyzed by (an) other prenyltransferase(s).

Incubation of HlPT-1 with naringenin chalcone and DMAPP gave a prenylated chalcone. This reaction product showed the molecular mass representing $[M+H]^+ = m/z$ 341.10 and $[M-H]^- = m/z$ 339.10 in positive and negative ion mode of LCMS-IT-TOF analysis, respectively. These figures are identical to those obtained with those of desmethylxanthohumol, a penultimate intermediate of xanthohu-

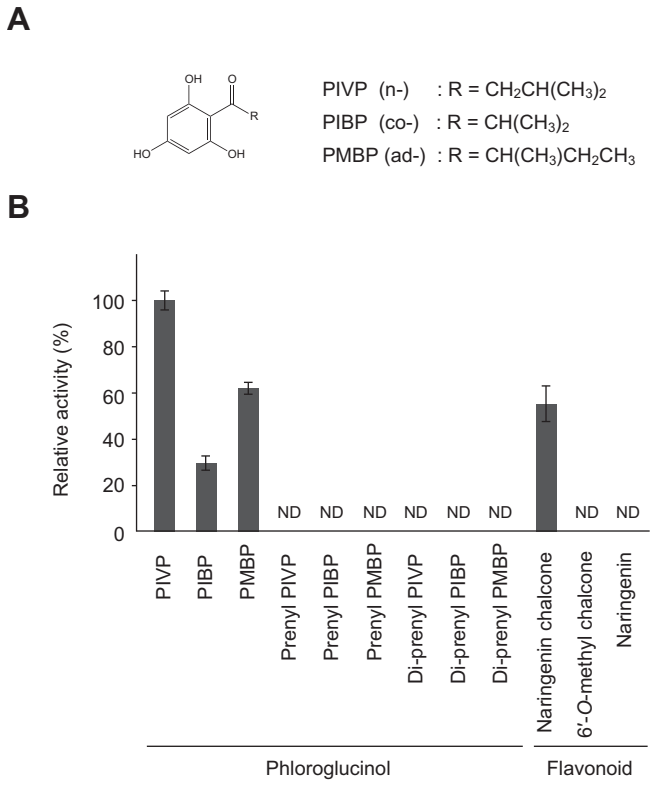

Fig. 3. Substrate specificity of recombinant HlTP-1 expressed in insect cells. (A) Prenyltransferase assays were performed with 0.5 mM aromatic substrate, 0.5 mM DMAPP, 10 mM $MgCl_2$ and enzyme solution (ca. 0.5 mg protein) in a total volume of 100 µl, which was incubated at 30 °C for 15 min. ND, not detected. Enzyme activity of 100% represents 688 ± 29.7 pmol mg protein^{-1} h^{-1}. Amount of desmethylxanthohumol was calculated as naringenin chalcone. (B) Structures of three types of acylphloroglucinol substrates for the biosynthesis of humulone and lupulone derivatives. PIVP, phlorisovalerophenone (n-humulone type); PIBP, phlorisobutyrophenone (co-humulone type); PMBP, phlormethylbutanophenone (ad-humulone type).

mol. We also employed other related flavonoids in the enzyme assay, but 6′-*O*-methylated chalcone and naringenin were not accepted as substrates of HlPT-1 (Fig. 3B). Plant prenyltransferases for polyphenols cloned thus far show strict specificity to the aromatic substrates [16,17]. However, our data in this study suggested that HlPT-1 is a prenyltransferase capable of catalyzing the addition of dimethylallyl moiety to not only acylphloroglucinol derivatives, but also naringenin chalcone (and thereby producing desmethylxanthohumol). Once naringenin chalcone had been methylated, HlPT-1 was unable to recognize the 6′-*O*-methyl chalcone as a substrate for prenylation, indicating that the prenylation of naringenin chalcone first yields desmethylxanthohumol, which is then methylated to form the final product xanthohumol in lupulin glands. These results are in conformity with the report of another group, which found that *O*-methyltransferase (OMT1) of hops preferentially catalyzed the methylation of desmethylxanthohumol compared to naringenin chalcone [8].

The broad substrate specificity of HlPT-1 to prenyl acceptor stands in clear contrast to the flavonoid-specific prenyltransferases SfN8DT-1 and SfG6DT identified in *Sophora flavescens*, which catalyze the transfer of a prenyl residue to naringenin and genistein, respectively [9,11]. A soybean enzyme G4DT, pterocapan-specific prenyltransferase, also shows very narrow specificity for its aromatic substrate [10]. It is to be noted that lupuline glands contain 8- and 6-dimethylallyl naringenin [4], while HlPT-1 does not prenylate naringenin, suggesting the existence of other prenyltransferases expressed in lupulins despite the fact such candidates were not found in our 11,233 EST data. Increase in the EST sequence will help to find new prenyltransferases. Besides, the possibility that desmethylxanthohumol is converted prenylated naringenin by an endogenous chalcone isomerase (CHI) of hops cannot be denied.

3.3. Other enzymatic properties of HlPT-1

HlPT-1 polypeptide possesses asparate-rich motifs (NQxxDxxxD and KDxxDxxGD) conserved among the Mg^{2+}-dependent plant aromatic prenyltransferase family [9–11]. We examined the divalent cation preference of HlPT-1, to demonstrate that this enzyme exclusively required Mg^{2+} for its activity and that while in the presence of EDTA the prenyltransferase activity of HlPT-1 was completely abolished (Fig. 4A). It is noteworthy that other divalent cations such as Mn^{2+} and Zn^{2+} could not be substituted for Mg^{2+}, which is another unique property of HlPT-1 that distinguishes it from other plant prenyltransferases [9–11]. In prokaryotes, prenyltransferases requiring no divalent cation have been reported [16], but homologs are not found in plants at least to our knowledge [17].

The optimum pH of HlPT-1 was observed at pH 7.0 in the range 5.0–8.0, showing relatively sharp preference (Fig. 4B). This also provides a strong indicator of how HlPT-1 different from other aromatic substrate prenyltransferases in its biochemical properties, since other polyphenol prenyltransferases commonly show broad optimum pH in the alkaline range of 8.0–10.0 [9,11].

In summary, the HlPT-1 identified in hops catalyzes the first step in prenylation of aromatic substances, adding dimethylallyl moiety to phloroglucinol derivatives and leading to the formation

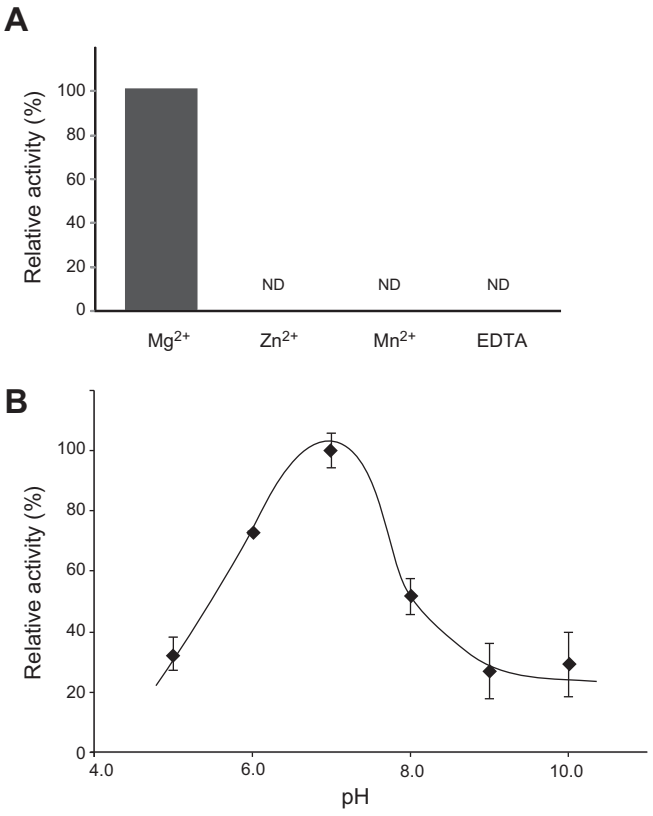

Fig. 4. Enzymatic properties of HlPT-1. (A) Divalent cation requirement of HlPT-1. ND, not detected. (B) pH dependency of HlPT-1. Microsomal fraction expressing recombinant HlPT-1 was suspended with various buffers and used as enzyme solution; 100 mM Citrate–NaOH buffer (pH 5.0), 100 mM MES–NaOH buffer (pH 6.0), 100 mM K–Pi buffer (pH 7.0), 100 mM Tris–HCl buffer (pH 8.0) and 100 mM CHES–NaOH buffer (pH 9.0, 10.0).

of humulone and lupulone derivatives. HlPT-1 is also responsible for the formation of xanthohumol by transferring prenyl residue to chalcone. Here, we found that HlPT-1 has many characteristic features as an aromatic prenyltransferase that set it apart from other reported members; namely, narrow optimum pH at around neutral pH, sharp preference for Mg^{2+} as a divalent cation, and broad substrate specificity. It is likely that, HlPT-1 recognizes a phloroglucinol portion as the prenyl acceptor, which is a common structure for the A-ring of naringenin chalcone. There should be other prenyltransferase members showing different substrate specificity including soluble-type enzyme expressed in lupulin glands [7]. Screening of other prenyltransferase mediating the second and third prenylation step to convert mono prenylated phloroglucinol derivative into humulone and lupulone is in progress.

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