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Chalcone synthase homologues from *Humulus lupulus*: some enzymatic properties and expression

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Abstract

The enzymatic properties of four chalcone synthase homologues CHS_H1, VPS, CHS 2 and CHS 4 from *Humulus lupulus* L. were investigated after heterologous expression in *Escherichia coli*. It was found that both VPS and CHS_H1 can utilize isovaleryl-CoA and isobutyryl-CoA as substrates producing compounds with positions in thin layer chromatography characteristic for phloroisovalerophenone and phloroisobutyrophenone. These reactions are accompanied by the formation of associated byproducts. The formation of naringenin chalcone can be catalyzed primarily by CHS_H1. Comparatively the ability of VPS to perform chalcone synthase reaction is very limited. Since only CHS_H1 has true chalcone synthase activity, this enzyme can be considered a key enzyme in prenylflavonoid biosynthesis. Both CHS 2 and CHS 4 utilize isovaleryl-CoA and isobutyryl-CoA as substrates, but the reactions were prematurely terminated. In comparison with VPS and CHS_H1, the optimum pH of CHS 2 was shifted to lower value. High expression of chalcone synthase-like genes were found in maturating hop cones of cultivars with high bitter acid content (Agnus, Magnum, Target) by Northern and Western blotting using probes specific for *vps*, *chs*_H1, *chs* 4 and polyspecific serum risen against recombinant protein CHS4, respectively. It was also found that these cultivars maintained expression of CHS homologues for a longer period of time during cone development in contrast to time-limited expression of CHS homologues in cultivars with low bitter acids content.

Additional key words: bitter acids, hop, prenylflavonoids, valerophenone synthase.

Introduction

The cones of the hop plant are used in the brewing process. Their major contribution to beer is the characteristic bitterness that results from isomerization of the hop bitter acids. The biosynthetic pathway of α and β bitter acids was analyzed by Zuurbier *et al.* (1995). A condensation reaction catalyzed by a chalcone synthase-like enzyme was proposed for this pathway. The predicted enzyme, valerophenone synthase (VPS, E.C. 2.3.1.156), was purified from glandular tissue of hop cones by Paniego *et al.* (1999) and a corresponding gene *vps* was described by Okada and Ito (2001).

Another important group of secondary metabolites from hop cones are prenylflavonoids for their antiproliferative and anti-cancer effects (Stevens *et al.* 1997, Miranda *et al.* 1999). The presence of prenylflavonoids in hop cones suggests a key role for enzymes with chalcone synthase activity (E.C. 2.3.1.74). We have recently described in detail one such gene, *chs*-H1, and its predicted protein

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Abbreviations: CHS - chalcone synthase; VPS - valerophenone synthase.
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(Matoušek *et al.* 2002a,b).

Additionally, three other chalcone synthase-like genes in hop were identified: *chs* 4 (Novák *et al.* 2003), *chs* 2 (GenBank accession number AB061020) and *chs* 3 (AB061022). The enzymatic properties of the proteins derived from these genes were only partially determined. It was shown that VPS is able to catalyze the formation of phloroisovalerophenone and phloroisobutyrophenone, the precursors of bitter acids (Paniego *et al.* 1999). CHS_H1 can catalyze the formation of naringenin chalcone, suggesting true chalcone synthase. In addition, CHS_H1 catalyses formation of phloroisovalerophenone, but the reaction is accompanied by formation of a prematurely terminated byproduct (Matoušek *et al.* 2002a). Chalcone synthase and valerophenone synthase-type reactions proceed through the three-step decarboxylative condensation of three acetate residues from malonyl-CoA with other enzyme specific CoA ester. The final tetraketide undergoes the cyclization to an aromatic product (Jez *et al.* 2001). In the reactions, different CoA esters are used (*p*-coumaroyl CoA and isovaleryl-CoA or isobutyryl CoA) but the reaction type is the same for both chalcone and valerophenone synthase (Zuurbier *et al.* 1998).

Up to now, the enzymatic properties of the other homologous proteins have not been characterized. In this work, we tried to clarify the function of these homologues in hop by comparison of their enzymatic properties. As it has been previously reported that *vps*, *chs*_H1 and *chs* 4 are specifically expressed in hop cones (Okada and Ito 2001, Matoušek *et al.* 2002a, Novák *et al.* 2003), we were also interested in determining the expression levels of CHS homologues in hop cultivars with different bitter acids content.

Materials and methods

Chemicals: [2-^{14}C] malonyl CoA (185kBq) was obtained from *Amersham Pharmacia Biotech* (Freiburg, Germany), *p*-coumaroyl-CoA was obtained as a gift from Prof. Joachim Schröder (Universität Freiburg, Germany). The other CoA esters were purchased from *Sigma* (Prague, Czech Republic).

RNA isolation and cDNA preparation: RNA was isolated from the mature hop inflorescences collected from cultivar Osvald's72 using *RNeasy plant mini kit* (*Qiagen*, Hilden, Germany) with modification according to Gehrig *et al.* (2000). In RT PCR reactions, the following pairs of primers specific for *chs*_H1, chs2, *chs* 4 and *vps* were used:

chsH1Nde1 5' AGGACATATGGTTACCGTCGAGGAA 3'
chsH1BamH1 5' CTAGGATCCCACACTGTGAAGCAC 3'
chs2NdeI 5' CATATGACCTCCATGACTGTGGAC 3'
chs2BamH1 5' GGATCCGAATTTATTGGCCACGCTGTG 3'
chs4NdeI 5' CATATGGCATCTGTAACTATGGAGCAA 3'
chs4BamH1 5' GGATCCTTAAACCTTGTTTGCAACGC 3'
vpsNdeI 5' CCAACATATGGCGTCCGTAACTGTAGA 3'
vpsBamH1 5' TTAGGATCCTGTGGGCACGCTGTGCA 3'.

Restriction sites created in primers for cloning purposes are underlined.

Enzyme expression and purification: In all cases the protein coding region of the cDNAs was inserted *via* *Nde*I and *Bam*HI sites introduced into expression vector pET15-b (*Novagen*, Madison, USA) downstream to sequence coding histidine tag. Proteins were expressed in *E. coli* [BL21(DE3)] according to pET system manual (*Novagen*, Madison, USA). Expressed proteins were purified using affinity chromatography on Ni-Agarose column according manufacturer's protocol (*Qiagen*) and purity was visually checked in SDS gels stained with Coomassie blue.

Enzyme assays: The reactions were performed according Zuurbier *et al.* (1998). In a standard experiment, the reaction was performed in 0.1 cm^3 of 0.1 M phosphate buffer, 10 μM CoA ester (*p*-coumaroyl, isovaleryl or isobutyryl), 15 μM [2-^{14}C] malonyl-CoA (4.20 MBq per assay). The reactions were started by addition 2 μg of protein then the samples were incubated for 30 min at 37 °C. When *p*-coumaroyl-CoA was used as substrate, incubation was followed by the addition of 1 M Tris/HCl buffer pH 9 to convert the chalcone non-enzymatically to naringenin (Lanz *et al.* 1991). This step was omitted in the reactions with isovaleryl CoA and isobutyryl CoA. The reaction products were then extracted two times into 0.05 cm^3 of ethyl acetate. The solvent was evaporated and the remaining pellet was dissolved in methanol. Samples were analyzed by TLC on a cellulose plate (*Merck*, Darmstadt, Germany) with 20 % acetic acid used as the solvent (Lanz *et al.* 1991). TLC plates were scanned and radioactivity signals quantified using a *STORM* device and *ImageQuaNT* software (*Molecular Dynamics*, Sunnyvale, USA). For kinetic studies, analyses were performed with varying concentrations of isovaleryl-CoA (0.25 - 50 μM), isobutyryl-CoA (0.25 - 50 μM) or *p*-coumaroyl-CoA (0.25 - 400 μM) in the presence of 30 μM malonyl-CoA. The apparent enzyme kinetic constants were calculated by non-linear regression according to Hernandez and Ruiz (1998). The optimum pH was measured as described, but different buffers were used for each particular pH (0.1 M Tris/HCl, pH 9 and 8.4; 0.1 M Mes pH 5.7 and 5; 0.1 M phosphate pH 6.5, 7, and 7.5).

Plants, protein extraction and immunoblotting: Hop (*Humulus lupulus* L.) plants were maintained in a breeding hop garden at the Hop Research Institute, Žatec. Cultivars Agnus, Sládek, Osvald's72, Galena, Taurus, Target, Magnum, Fuggle and Nugget were used in our analyses. Proteins for immunoblotting were extracted from hop flowers, young cones and mature cones. The frozen plant material was ground using a pestle and mortal in the presence of sea sand and 10 % (m/m) polyvinyl-polypyrrolidone (*Sigma*, Prague, Czech Republic). Frozen powder was mixed with extraction buffer containing 0.5 M potassium phosphate pH 8, 1.5 % polyethylenglycol (M_r 6000), 400 mM sucrose, 1 mM $CaCl_2$, 200 mM ascorbic acid, 50 mM EDTA, 0.2 mM phenylmethanesulfonyl fluorid and 100 mg.dm^{-3} *Nonidet P-40*. After thawing, the homogenate was centrifuged at 18 000 *g* for 20 min and stored at -80 °C. For SDS PAGE electrophoresis, total protein extracts were desalted using a PD 10 column (*Amersham Pharmacia Biotech*, Freiburg, Germany) and 50 mM Tris/HCl pH 6.8 according to the manufacturer's protocol. SDS-PAGE and immunoblotting were performed according to Harlow and Lane (1988). Mouse antibodies for the detection of CHS homologues were prepared by immunization with purified CHS 4 expressed from *E. coli*. The specific reaction of the antibody with CHS 4 and cross-reactions with VPS, CHS 2 and CHS_H1 were determined (not shown). Anti-mouse IgG alkaline phosphatase conjugate was used as the detection system.

Northern blot analyses: Total RNA samples of 35 μg each were separated on formaldehyde-denaturating agarose gel and blotted onto *Biodyne A* transfer membrane (*Pall*, Hampshire, England). Prehybridization and hybridization were carried out according to Ausubel *et al*. (1987) using formamide-based (pre)hybridization buffer at 42 °C. The final washing was performed in 0.25×SSC plus 0.1 SDS at 50 °C for 20 min. The probes for the detection of chs 4, chs_H1 and vps were prepared from PCR fragments amplified using specific primers (chs4NdeI, chs4BamH1, chsH1NdeI, chsH1BamH1, vpsNdeI, vpsBamH1) and using *Redivue*™ [α-^{32}P] dCTP 110 TBq mmol^{-1} *Rediprime*™ II random prime labeling system (*Amersham Pharmacia Biotech*, Freiburg, Germany). The autoradiograms were scanned using a *STORM* device and *ImageQuaNT* software (*Molecular Dynamics*, Sunnyvale, USA).

Quantification of lupulin compouds: Hop resins and xanthohumols were estimated according to the EBC 7.7 procedure (1997) on an HPLC column nucleosil RP C_{18} (*Machererey Nagel*, Germany, 5 mm, 250 × 4.6 mm) using chromatograph *Shimadzu LC-10A* (Tokyo, Japan). The quantification was performed using the external standard ICE 2. For chemical analyses, the same plants were used as in the case of the RNA and protein isolations.

Results

Enzyme activities of CHS homologues: The ability of CHS homologues to catalyze the reaction with the different substrates was tested. VPS and CHS H1 catalyzed the formation of compounds corresponding to phloroisobutyrophenone and phloroisovalerophene markers, but both enzymes also formed a considerable portion of prematurely terminated byproducts (peaks with higher mobility on Fig. 1*A*,*B*). The most abundant byproducts were identified as prenylated pyrons (LC/MS results - not shown). In the reactions catalyzed by CHS 2 and CHS 4, only the formation of byproducts was observed. The reactions with *p*-coumaroyl-CoA, a physiological substrate of chalcone synthase, were catalyzed by CHS_H1 and VPS (Fig. 1*C*). Both enzymes catalyze formation of naringenine chalcone (confirmed by LC/MS – data not shown) without formation of a notable amount of byproduct, even though CHS_H1 performs the reaction at a much higher rate than VPS. In the same experiment with CHS 2 and CHS 4, no products were observed (data not shown).

To characterize distinctions in the substrate specificities of VPS and CHS_H1, we determined the kinetic constants for each substrate (Table 1). Significant differences in enzyme activity were found primarily for reactions using *p*-coumaroyl-CoA. The V_{lim} value for CHS_H1 was forty-four times higher than value for VPS, this being the main distinction between these chalcone synthase homologues.

Table 1. The apparent kinetic constant of VPS and CHS H1 with different substrates. In reactions with isobutyryl-CoA and isovaleryl-CoA, where formation of byproducts was observed, only amount of phloroisobutyrophenone and phloroisovalerophenone was considered in calculation. Confidence intervals are given at $\alpha = 0.05$.

Substrate		VPS	CHS H1
Isovaleryl CoA	K_M [μM]	5.0 ± 0.2	8.0 ± 0.2
	V_{lim} [pKat μg^{-1}]	65.2 ± 6.6	196.8 ± 20.0
Isobutyryl CoA	K_M [μM]	14.6 ± 0.1	14.9 ± 0.2
	V_{lim} [pKat μg^{-1}]	57.3 ± 4.4	106.5 ± 10.3
p-Coumaroyl CoA	K_M [μM]	29.0 ± 0.2	40.9 ± 0.1
	V_{lim} [pKat μg^{-1}]	5.8 ± 1.0	256.5 ± 28.1

As the homologues differ in amino acid residues forming active site, we were interested in the impact of these changes to the optimum pH of individual enzymes. Because CHS 4 and CHS 2 are able to form only the prematurely terminated product, the byproduct formation was quantified for optimum pH determination (Fig. 2) In experiments with isovaleryl-CoA as substrate, we got similar results for CHS 2 and CHS 4 (data not shown). The highest activity of CHS_H1 was found at pH 7 in contrast to the optimum of CHS 2 which was shifted to pH 5.7. Meanwhile, altering pH had very little effect on the observed activity of CHS 4.

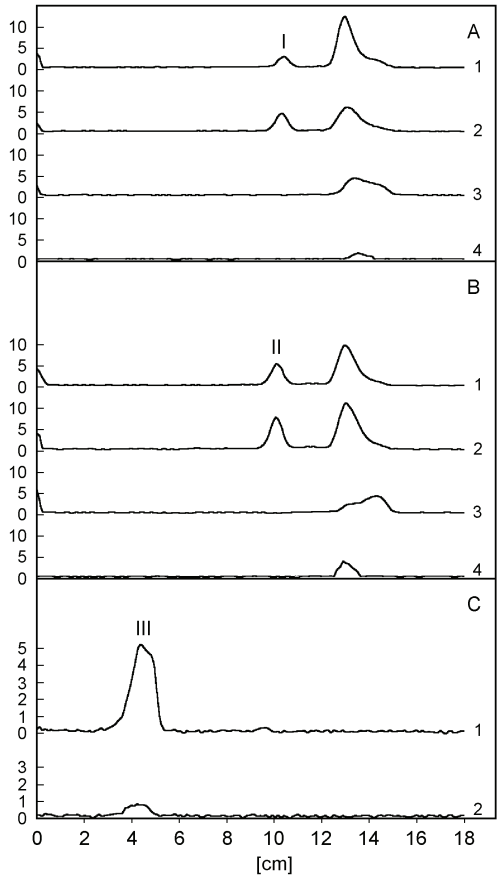

Fig. 1. TLC-scan of the reactions with different substrates: *A* - isobutyryl-CoA, *B* - isovaleryl-CoA, *C* - *p*-coumaroyl-CoA; Line 1 - CHS_H1, line 2 - VPS, line 3 - CHS 2, line 4 - CHS 4. Peaks I, II and III represent phloroisobutyrophenone, phloroisovalero-phenone and naringenin, respectively. The numbers at the ordinate indicate radioactivity signal, estimated using STORM device and ImageQuaNT software in pixel intensities × 10^{-4}. All reactions were performed in phosphate buffer pH 7.

A comparative analysis of expression of CHS homological genes: It is known that the accumulation of bitter acids in the hop starts at the late flower stage, continues during the development of hop cones and the highest content of bitter acids is in the ripe cones. To elucidate the role of CHS homologues, their contents were analyzed during these three stages of development using immunoblotting (Fig. 3). Because anti-CHS 4 antibody cross-reacted with all tested CHS homological proteins (see materials and methods), the immunoblot

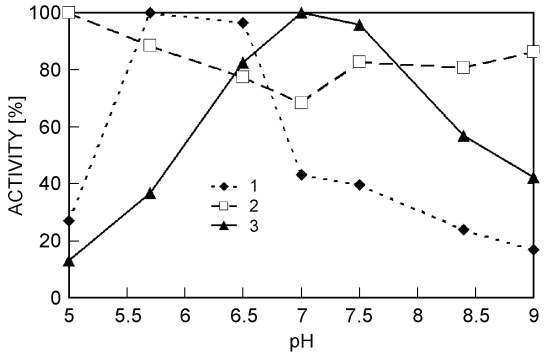

Fig. 2. pH - activity dependence. 1 - CHS 2 with isobutyryl-CoA, 2 - CHS 4 with isobutyryl-CoA, 3 - CHS H1 with *p*-coumaroyl-CoA.

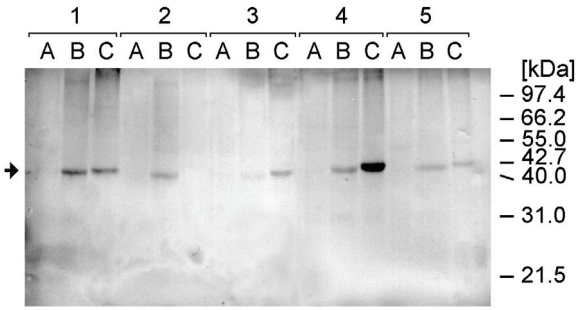

Fig. 3. Immunoblot of SDS-PAGE of hop protein extracts and immunostained using antibodies raised against hop CHS 4. 20 μg of total protein was loaded in each line. Proteins were extracted from cultivars Agnus (1), Osvald's72 (2), Sládek (3), Magnum (4) and Target (5) at different stages of development: flowers (A), young cones (B), mature cones (C). Position of chs-specific bands is indicated by the *arrow* on the side.

reflects the total level of CHS homologues. No CHS homologues were detected in flowers. In protein extracts from young cones and mature cones we got single bands of M_r approximately 43 kDa. Since the antibody used did not allow us to discriminate between particular CHS homologues, the mRNA levels of the three individual homologues, *vps*, *chs* 4 and *chs*_H1, were detected in mature cones by Northern blotting (Fig. 4). Signals with different intensities from the individual cultivars were detected at the position of about 1.5 kb for all three probes used (Fig. 5*C*). The highest expression of *vps* was found in cultivars Magnum and Sládek. Additionaly, the highest levels of *chs* 4 mRNA was detected in cultivars Agnus, Sládek, Galena, Taurus, Target and Magnum and the highest expression of *chs*_H1 was found in cultivar Magnum. Little or no expression of CHS homologues

were detected in "non-comprehensive" cultivars Osvald's72 and Fuggle. Taking these results together with the chemical analyses of lupulin in the tested cultivars (Fig. 5*A*), some correlation can be seen between total bitter acids content and *vps* and *chs* 4 expression, but the correlation is not absolute. For example, in RNA isolated from hybrid cultivar Sládek, high levels of mRNA of *vps* and *chs* 4 were observed in mature cones in contrast to a low final concentration of bitter acids. Simlar expression results were obseved in the protein levels, if one compares the immunoblot signals from different stages of cone development (Fig. 3).

As CHS_H1 was proposed to be responsible for the synthesis of hop flavonoids, especially prenylated flavonoid, we expected a strong correlation between prenylflavonoid content and the level of *chs*_H1 mRNA. The content of xanthohumol, major component of hop prenylflavonoids, was estimated and compared with the level of *chs*_H1 mRNA but no dependence was observed (compare Fig. 4*C* and 5*B*,*C*) suggesting that there are other factor(s) affecting the content of prenylflavonoids. On the other hand, it seems that the level of *chs*_H1 mRNA follows more less the levels of *vps* and *chs* 4 mRNAs in mature cones.

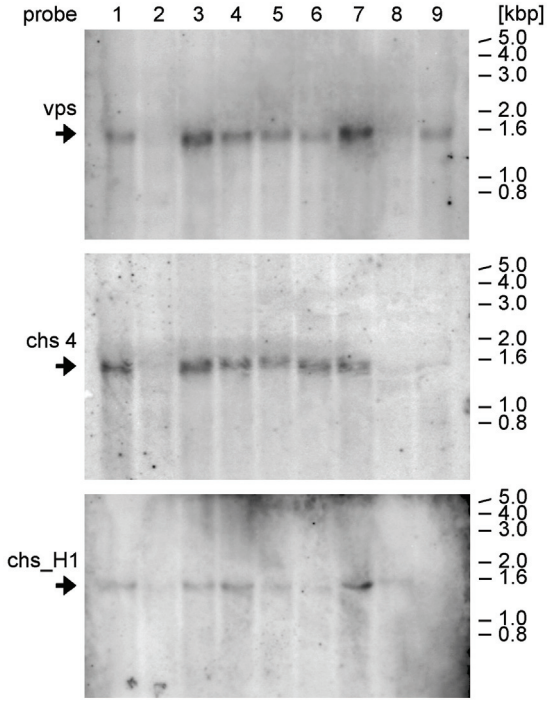

Fig. 4. Northern blot analyses of total RNA isolated from mature cones of hop cultivars Agnus (1), Osvald's72 (2), Sládek (3), Galena (4), Taurus (5), Target (6), Magnum (7), Fuggle (8), Nugget (9). Probes derived from cDNAs of vps, chs 4 and chs_H1 were used for hybridization. Positions of chs-specific bands are indiated by the *arrows* on the side.

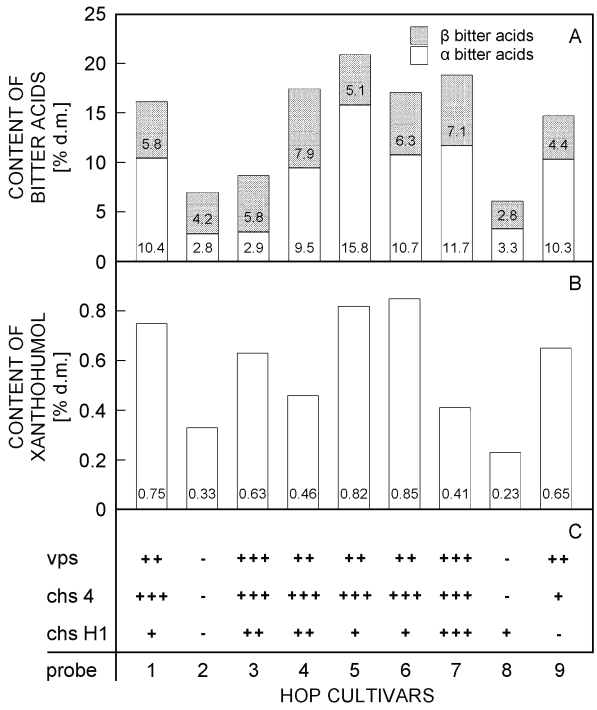

Fig. 5. Quantification of lupulin components in selected hop cultivars: Agnus (1), Osvald's72 (2), Sladek (3), Galena (4), Taurus (5), Target (6), Magnum (7), Fuggle (8), Nugget (9). Bitter acids (*A*) and prenylflavonoid xanthohumol (*B*) were estimated as percentage of dry mass of hop cones. Expressions of individual homologues detected by Northern blot are summarized (*C*). Measurements were performed once and expanded uncertainty was used to calculate accuracy of estimation at level a = 0.05. Uncertainties for measurement of xanthohumol, alfa acids, beta acids were 3.5, 4.6, and 3.9 %, respectively.

Discussion

The identities between the four tested homologues are in the 67 - 77 % range for amino acid similarity (Novák *et al.* 2003). Important differences were found especially in amino acids forming the active site. CHS_H1 retains all the amino acids important for function of chalcone synthase unchanged (Matoušek *et al.* 2002a). On the other hand, a number of amino acids forming catalytic site in CHS 2, CHS 4 and VPS are different. Two mutations were found in the active site of VPS, seven mutations in CHS 2 and two mutations in CHS 4, when compared with true chalcone synthase from alfalfa (Ferrer *et al.* 1999). As expected from previous molecular genetic studies, some differences in the enzymatic properties of analyzed CHS homologues were found. In this study the competent

substrate for CHS 2 and CHS 4 was not found. These enzymes utilize the substrates isobutyryl-CoA and isovaleryl-CoA, but the reactions are prematurely terminated. The core compound of bitter acids is formed by phoroglucinol and the individual compounds differ only by the nature of their acyl side chains. The most abundant are humulone, cohumulone, lupulone and adlupulone with isovaleryl or isobutyryl acyl chain. Other less abundant bitter acids possesses 2-methylbutyryl, propionyl, 4-methylpenthanoyl or 4-methylhaxanoyl acyl chain (Hornsey 2000). Even though CHS 2 and CHS 4 are not specific for isovaleryl-CoA or isobutyryl-CoA (Fig. 1*A*,*B*), there is some possibility that these homologues can catalyze formation of other precursor(s) of bitter acids using different CoA esters. This is supported by the fact that amino acids forming the active site cavity of CHS 4 and CHS 2 are different from corresponding amino acids in VPS and CHS_H1 (Novák *et al.* 2003). Besides, it was experimentally confirmed that the shape and the volume of an active site cavity determine the specificity and the number of condensation reactions catalyzed by chalcone synthase like enzymes (Jez *et al.* 2000, 2001).

Another possibility is that CHS 2 and CHS 4 expressed and isolated from a bacterial system do not fulfill the right catalytic function *in vitro.* Likewise, VPS does not perfectly catalyze the formation of phloro-isovalerophenone or phloroisobutyrophenone *in vitro* (Fig. 1*A*,*B*). Okada and Ito (2001) and Yamazaki *et al*. (2001) also observed the formation of byproduct by VPS expressed in the heterologous bacterial system. The main catalytic difference found between VPS and CHS_H1 is the very low capability of VPS to act as a chalcone synthase in comparison with CHS_H1 (Table 1). On the other hand, from the estimated kinetic constants for isovaleryl-CoA and isobutyryl CoA it seems that CHS_H1 can fulfill the function of a valerophenone synthase.

It was previously reported that the optimum pH of VPS is about 7 (Paniego *et al.* 1999). This value is the same as the estimated optimum pH for CHS_H1. Changing of pH has only a little effect on the activity of CHS 4. The optimum pH of CHS 2 was shown to be shifted to lower values in comparison with VPS and CHS H1. This can be explained by the differences in the active sites of the individual homologues. Three amino acid residues E192, T194 and T197 create the coumaroyl-binding pocket in CHS_H1 (Matoušek *et al*. 2002a). The corresponding amino acids in VPS are E194, T196 and I199 and equivalent residues in CHS 2 are D194, M196 and H199 (Ferrer *et al*. 1999, Novák *et al*. 2003). The presence of a histidine residue at position 199 in CHS 2 is the probable reason for the shifted in its optimum pH, in contrast to the neutral amino acid residues found in corresponding position in both VPS and CHS_H1. A similar phenomenon was observed in the other chalcone synthase homologue - stilbene synthase (Raiber *et al.* 1995).

In contrast to the differences in the enzymatic activities of the CHS homologues, the expression patterns of *vps*, *chs*_H1 and *chs* 4 were very similar in hop mature cones. The high expression of the tested homologues was observed in cultivars with a high bitter acids content. In addition, the expression of CHS homologues in "comprehensive" hops obviously proceeded for a longer period in comparison to "non-comrehensive" hops. The similar expression pattern of individual homologues suggests the existence of some common *cis*-regulatory elements, which were also predicted from genomic comparisons in our previous work (Matoušek *et al.* 2002b). Moreover, the nearly identical expression of *chs* 4 and *vps* is in agreement with previous findings showing that the *vps* and *chs* 4 genes are organized in a cluster (Novák *et al*. 2003). Likewise, for example, a similar expression pattern was previously reported in closely related CHS genes in the pea multigene family (Ito *et al* 1997). Although the expression analyses of CHS homologues by Northern blot were performed at stringent conditions, we cannot exclude the posibility that other closely related sequences were also detected. In previous papers we have shown by Southern blot that there are at least six sequences in the hop genome which hybridize to our chs_H1 probe (Matoušek *et al.* 2002a) and another six sequences which hybridize to our chs 4 probe (Novák *et al.* 2003). These results suggest that the expression spectrum of CHS gene homologues and the contributions of individual homological enzymes affect the final levels of bitter acids. According to the estimated enzymatic properties, VPS can only contribute to the biosynthesis of bitter acids but, CHS_H1 can probably contribute to both, bitter acid synthesis and prenylflavonoids synthesis.

Although we predicted a correlation between *chs*_H1 level and the level of xanthohumol, we did not find any such correlation. As hop flavonoids comprise flavonol glycosides, condensed tannins and prenylflavonoids (Stevens *et al*. 1998), we can assume that the content of prenylflavonoids is dependent not only on chalcone synthase activity but also on the activities of other enzymes which act in subsequent steps of flavonoid biosynthetic pathways.

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