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FORMATION OF AROMATIC INTERMEDIATES IN THE BIOSYNTHESIS OF BITTER ACIDS IN *HUMULUS LUPULUS*

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Key Word Index—*Humulus lupulus*; Cannabaceae; hop; intermediates bitter acids; biosynthesis bitter acids; chalcone synthase.

Abstract—A new hypothesis concerning the formation of aromatic intermediates in the biosynthesis of hop bitter acids was tested. Two phloroglucinol derivatives, 2-(3-methylbutanoyl)-1,3,5-benzenetriol (phlorisovalerophenone = PIVP) and 2-(2-methylpropanoyl)-1,3,5-benzenetriol (phlorisobutyrophenone = PIBP), were detected in hop extracts. The formation of these compounds was shown to occur *in vitro* in hop protein extracts using the precursors malonyl-CoA plus isovaleryl-CoA or isobutyryl-CoA, respectively. We conclude that PIVP and PIBP are probably the first aromatic intermediates formed in the biosynthesis of humulone/lupulone and cohumulone/colupulone, respectively. We suggest that the reactions are catalysed by a chalcone synthase-like enzyme. The enzyme chalcone synthase and its activity were found to be present in hop protein extracts. The formation of naringenin, PIVP and PIBP in various hop protein extracts obtained during the development from flower bud to ripe cone was compared.

INTRODUCTION

The ripe cones of the hop plant, *Humulus lupulus* L. (Cannabaceae), contain up to 20% of bitter acids [1]. These compounds are prenylated derivatives of phloroglucinol and consist of α-acids, mainly humulone, cohumulone and adhumulone, and β-acids, mainly lupulone, colupulone and adlupulone (Fig. 1) [2]. During the brewing process the α-acids are converted into their isoforms, compounds which are important for the flavour and taste of beer. Therefore, it is interesting to gain knowledge of the biosynthesis of the various bitter acids in hop and its regulation.

Drawert and Beier [3] have studied the biosynthesis of the hop bitter acids with the use of labelled precursors and were able to detect labelled 2-(3-methylbutanoyl)-4-(3-methyl-2-butenyl)-1,3,5-benzenetriol (= compound X) and 2-(2-methylpropanoyl)-4-(3-methyl-2-butenyl)-1,3,5-benzenetriol (= compound co-X) [Fig. 1], suggested intermediates in the formation of lupulone and humulone, and colupulone and cohumulone, respectively. According to the hypothesis of Drawert and Beier, the compounds X and co-X should be the first aromatic intermediates in the biosynthesis, produced via prenylated long chain fatty acids. However, we have reasons to suggest that X and co-X are formed in a different way. A glycoside of 2-(2-methylpropanoyl)-1,3,5-benzenetriol (= phlorisobutyrophenone, abbreviated as PIBP) has been isolated from hop cones [4], and 2-(3-methylbutanoyl)-1,3,5-benzenetriol (= phlorisovalerophenone, abbreviated as PIVP), PIBP, X and co-X have been detected in low concentrations in methanol extracts from cones of several hop cultivars [5], which indicates that the formation of the aromatic ring could precede any of the prenylation steps and thus become the first step in the biosynthesis.

In the flavonoid pathway, the formation of the aromatic compound naringenin chalcone from one molecule of *p*-coumaroyl-CoA and three molecules of malonyl-CoA is catalysed by the key enzyme chalcone synthase (EC 2.3.1.74) [6]. From parsley chalcone synthase it is also known that the substrate *p*-coumaroyl-CoA can be replaced by certain aliphatic CoA esters, e.g. butyryl-CoA, leading to the formation of the respective chalcone analogues, like phlorobutyrophenone [7]. Flavonoids have also been detected in hop cones [8], indicating that chalcone synthase should be present. We therefore suggest that the first step in the biosynthesis of the main hop bitter acids could be the formation of the aromatic intermediates PIVP and PIBP from the reaction between the precursors malonyl-CoA with isovaleryl-CoA and isobutyryl-CoA, respectively, catalysed by chalcone synthase or a chalcone synthase-like enzyme. Subsequently, these aromatic intermediates could be prenylated to yield X and co-X, respectively. The hypothetical scheme of the biosynthesis of the hop bitter acids is given in Fig. 1.

In this paper, we present a study on our hypothesis concerning the formation of phloroglucinol derivatives as intermediates in the biosynthesis of the hop bitter acids (Fig. 1). The presence of PIVP, PIBP and X and co-X was checked in hop extracts obtained during the development

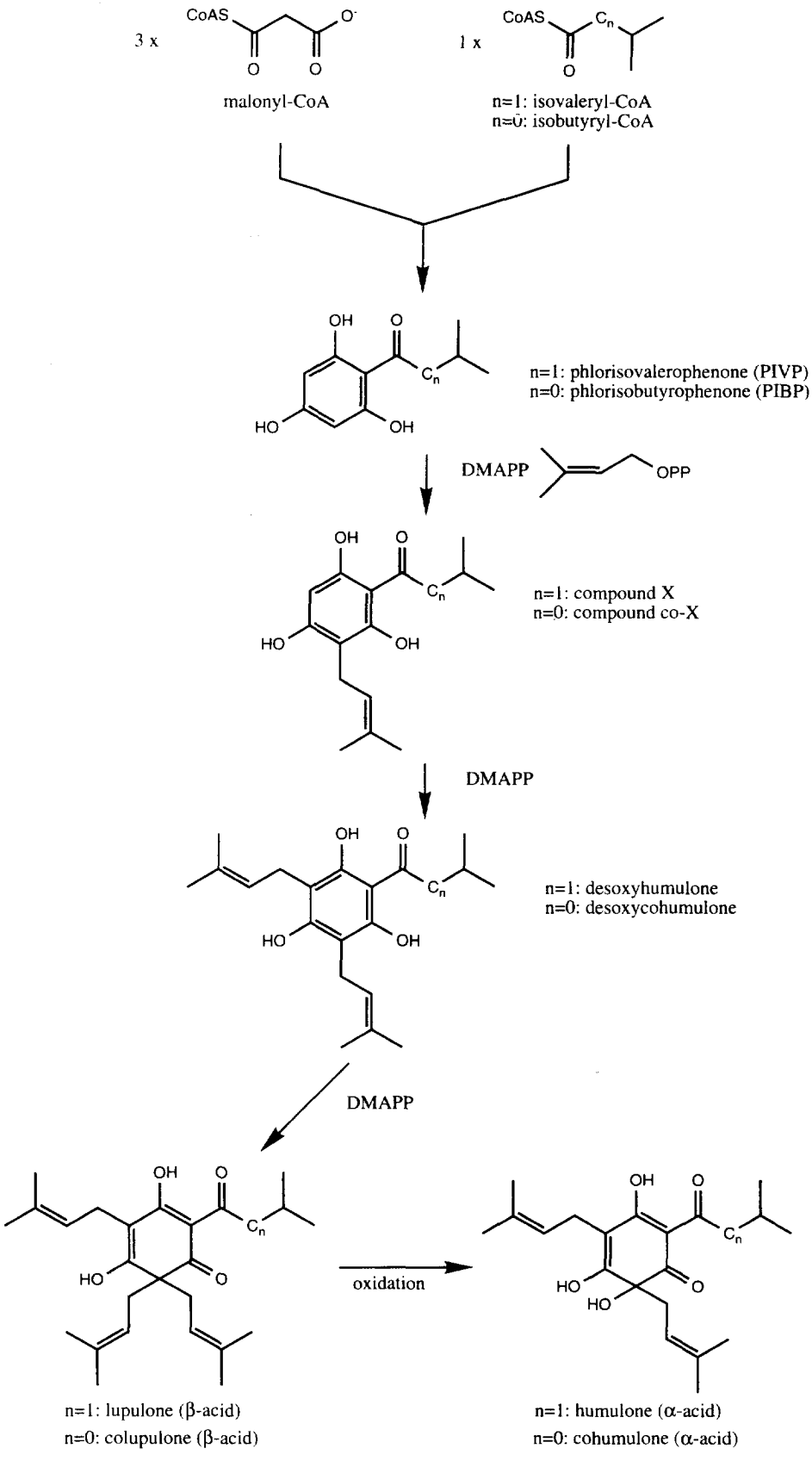

Fig. 1. Tentative scheme of the biosynthesis of the hop bitter acids lupulone and humulone. Colupulone and cohumulone are formed accordingly from the precursor isobutyryl-CoA instead of isovaleryl-CoA.

from flower buds into ripe cones. Hop protein extracts from the same development stages were used to determine the formation of PIVP and PIBP *in vitro*. Furthermore, the presence in hop of the enzyme chalcone synthase and its activity was investigated, and the involvement of chalcone synthase or a chalcone synthase-like enzyme in the first step of the biosynthesis of the hop bitter acids is discussed.

RESULTS AND DISCUSSION

In the present study three hop cultivars were used, i.e. Hallertau Mittelfrüh, Olympic and Wye Northdown. Since only relatively small amounts of the plant material were available, it was not possible to perform the experiments described with one and the same cultivar.

The accumulation of bitter acids starts during the development of hop flowers into cones [2]. For 'Olympic', HPLC and GC analyses showed that traces of PIVP and PIBP ($< 2.2\ \mu g\ g^{-1}$ fresh weight) were present in the methanol extracts of various stages of flowers and cones (Fig. 2A). The low concentration of PIVP and PIBP in all development stages is in accordance with our hypothesis that they are intermediate products in the biosynthesis of the main hop bitter acids. Co-X and X also occurred in these extracts; the concentration of X in cones was significantly higher than that of the three other intermediates. Figure 2B demonstrates the accumulation of the main α and β hop bitter acids, which started at the flower stage and continued until the stage of the ripe cones. In the HPLC system used, no distinction was possible between the individual compounds of the two isomeric pairs humulone/adhumulone and lupulone/adlupulone, but humulone has been reported to be the main bitter acid in hop [2]. The relatively high concentration of the intermediate X compared with that of co-X does not correlate with the ratio found for the end products lupulone plus humulone and colupulone plus cohumulone, respectively. This result indicates that the rates of formation and/or conversion of X could be different compared with that of co-X.

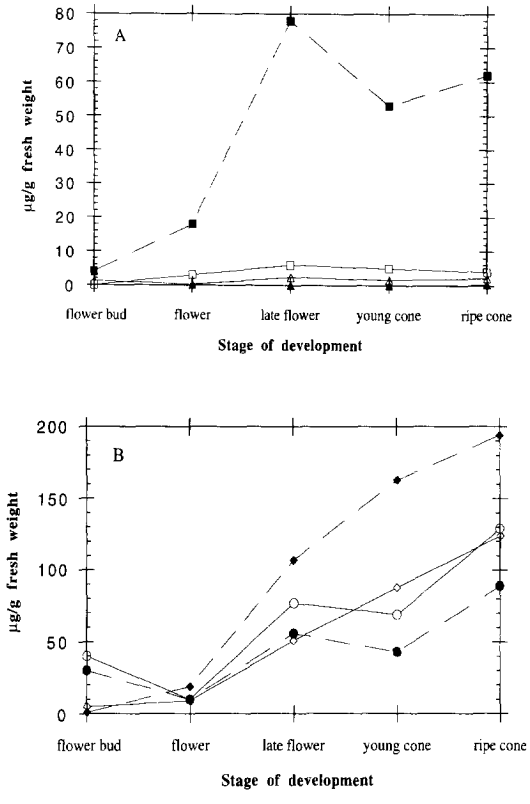

Fig. 2. Concentrations of the aromatic intermediates (A) and the main bitter acids (B) during the development of cones in the hop cultivar Olympic. PIVP (—▲—), PIBP (—△—), X (--■--), co-X (—□—), lupulone + adlupulone (—●—), humulone + adhumulone (—◆—), culupulone (—○—) and cohumulone (—◇—) were detected.

A protein extract from hop flowers of the cultivar Hallertau Mittelfrüh was used to investigate whether chalcone synthase activity was present in hop, which catalysed the formation of naringenin from malonyl-CoA and *p*-coumaroyl-CoA. Furthermore, experiments were conducted to establish the production of PIVP and PIBP by the protein extract, when *p*-coumaroyl-CoA was replaced, respectively, by isovaleryl-CoA and isobutyryl-CoA. The recently developed HPLC assay for chalcone synthase determination [9] was used to measure the enzyme products naringenin, PIVP and PIBP. In Fig. 3 the HPLC elution profiles obtained from the three assays are given, which show signals at the expected positions for naringenin, PIVP and PIBP. In addition to naringenin, *p*-coumaric acid (elution time of 2.7 min) could be detected, as is also described in ref. [9]. The compounds were identified by photodiode array detection. In order to confirm the results, the radioassay for chalcone synthase activity [10] using [2-^{14}C]malonyl-CoA was used. In Fig. 4 the radio thin layer chromatogram is shown. It is clear from this figure that labelled naringenin and PIVP were formed from [2-^{14}C]malonyl-CoA plus *p*-coumaroyl-CoA or isovaleryl-CoA, respectively.

In order to compare the above described three synthase activities at various developmental stages of hop cones, crude protein extracts from flower buds, flowers, late-stage flowers, young cones and ripe cones from the cultivar Wye Northdown were analysed. In Fig. 5 the enzyme activities are plotted against the various developmental stages. It was shown that naringenin formation *in vitro* increased up to the late flower stage, and then declined. The activity for PIVP formation was greater than that for naringenin at each developmental stage, was almost four times higher in ripe cones, and showed an optimum in young cones. Compared with PIVP, the PIBP formation was lower, but increased continuously from zero level in flower buds to the same value as that of PIVP in ripe cones. Except for the value measured in ripe cones, it showed a developmental pattern similar to that of PIVP. Under the conditions used, the activity of the PIVP and PIBP forming enzyme(s) rose during the development from flower bud to ripe cone, whereas that of naringenin decreased after the late flower stage. Similar

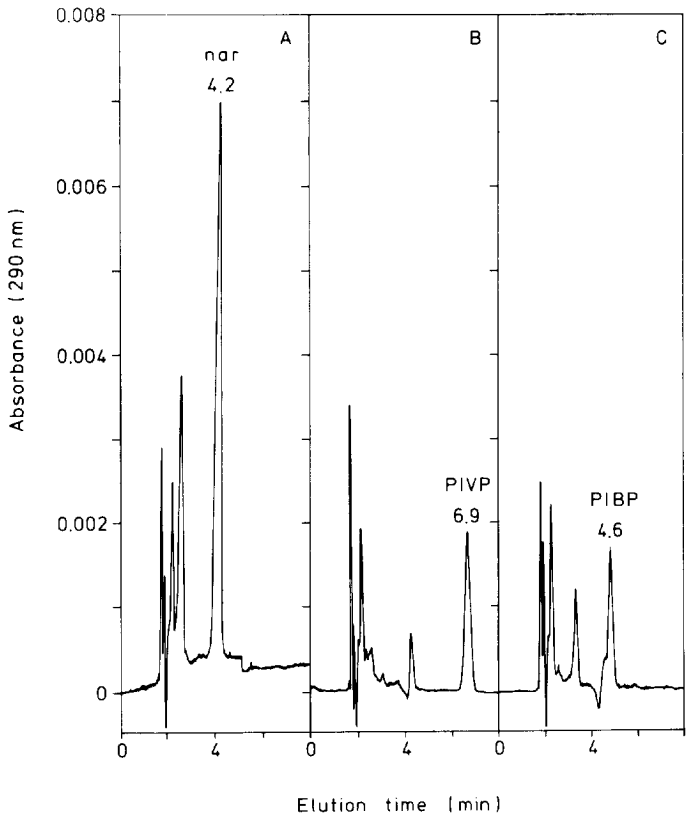

Fig. 3. HPLC elution profiles obtained for assays of chalcone synthase activity in a protein extract of hop flowers (cv Hallertau Mittelfrüh) using various substrates. The assay was performed in the presence of malonyl-CoA plus *p*-coumaroyl-CoA (A), isovaleryl-CoA (B) or isobutyryl-CoA (C). For assay and HPLC conditions, see Experimental.

results were obtained with the cultivar Olympic. These results on enzyme activities *in vitro* are in agreement with the observation that the hop bitter acids, which are possibly formed via PIVP and PIBP, accumulate in the cone throughout its development (Fig. 2B). The results furthermore suggest that the formation of the hop bitter acid precursors is catalysed by an enzyme similar to but different from chalcone synthase, and that PIVP and PIBP may even be formed by two different enzyme species.

SDS–PAGE in combination with immunoblotting was used to demonstrate the presence of chalcone synthase protein or isoforms of it in the above described protein extracts from flower buds, flowers, late-stage flowers, young cones and ripe cones of 'Wye Northdown'. The immunoblot in Fig. 6 shows that each of the protein extracts contained one protein band of M_r *ca* 45×10^3 which was recognized by antibodies raised against parsley chalcone synthase. This M_r is in agreement with the literature values of chalcone synthase determined using SDS–PAGE [11–13]. These results, however, do not exclude the possibility that the new synthase enzyme(s) which may have a M_r close to that of chalcone synthase, is (are) also recognized by the parsley antibodies. The intensity of the immunostained band, related to the amount of protein which was loaded, was greatest in young cones and very low in flower buds.

Summarizing, PIVP and PIBP were found in hop plant extracts and were formed *in vitro* by hop protein extracts from malonyl-CoA plus isovaleryl-CoA or isobutyryl-CoA, respectively. We suggest that PIVP and PIBP are the first aromatic intermediates formed in the biosynthesis of the hop bitter acids lupulone/humulone and colupulone/cohumulone, respectively. The chalcone synthase protein as well as its enzymic activity were found in hop protein extracts. Attempts to purify any one of the synthases described here have not been successful so far, owing to their obviously low stability under the conditions used. The observation that the pattern of PIVP/PIBP formation during cone development was different compared with that of naringenin indicates that PIVP and PIBP are not produced by the chalcone synthase protein, but by another enzyme activity closely related to that of chalcone synthase. Other examples of such enzymes are stilbene synthases, detected for example in groundnut, grapevine and Scots pine [14]. These enzymes catalyse the reaction of (hydroxy)cinnamoyl CoA esters and malonyl-CoA to stilbenes. Stilbene synthases and chalcone synthase are closely related in function and structure, even cross-reactivity of stilbene synthase from

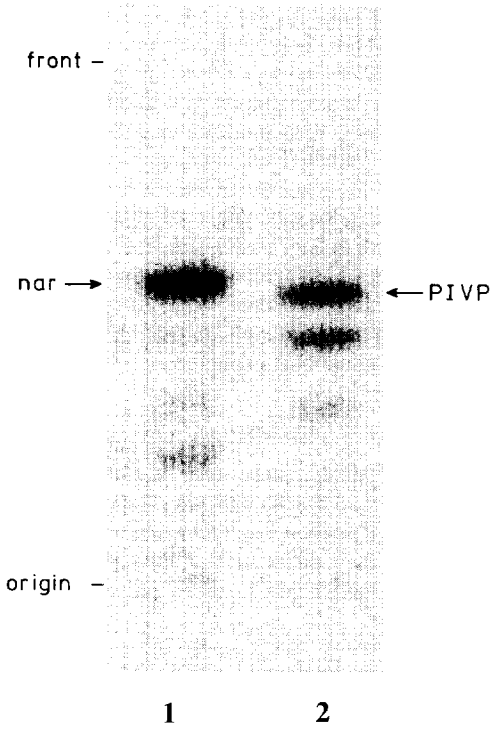

Fig. 4. Betascope of a TLC plate after separation of EtOAc extracts from chalcone synthase assays using a protein extract of hop flowers (cv Hallertau Mittelfrüh) and various substrates. The assay is described in the Experimental. The spots in the chromatogram represent ^{14}C radioactivity. The assay was performed in the presence of [2-^{14}C] malonyl-CoA plus *p*-coumaroyl-CoA (lane 1) or isovaleryl-CoA (lane 2). The arrows represent the positions of the unlabelled reference compounds naringenin and PIVP as detected by UV light.

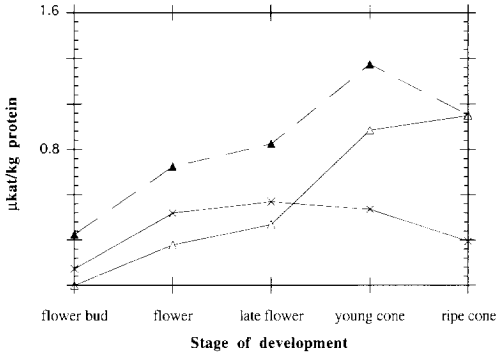

Fig. 5. Relative synthase activities using various substrates and hop protein extracts prepared for different stages of female flower development from flower bud to ripe cone (cv Wye Northdown). The substrates malonyl-CoA plus *p*-coumaroyl-CoA, isovaleryl-CoA or isobutyryl-CoA were used and the corresponding products naringenin (—X—), PIVP (—▲—) and PIBP (—△—) were detected. Average values from two measurements are shown.

Scots pine has been found using antibodies raised against parsley chalcone synthase [15]. Further studies will be necessary in order to confirm the identity of the enzyme(s) involved in the formation of PIVP and PIBP.

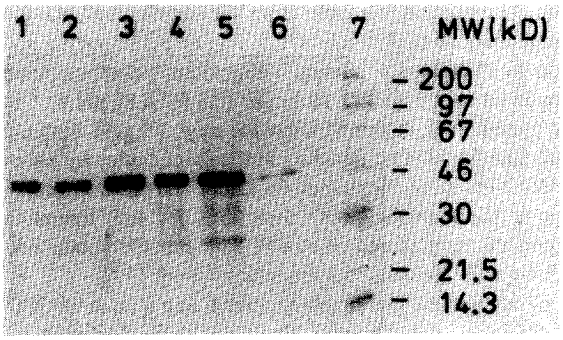

Fig. 6. Immunoblot of SDS–PAGE applied with hop protein extracts and immunostained using antibodies raised against parsley chalcone synthase. Protein extract of cones from cv Olympic (1; 12 μg protein) and of cones (2; 11 μg protein), young cones (3; 12 μg protein), late-stage flowers (4; 18 μg protein), flowers (5; 23 μg protein) and flower buds (6; 11 μg protein) from cv Wye Northdown. In lane 7, the M_r markers are shown.

EXPERIMENTAL

Plant material. Humulus lupulus L. cv Hallertau Mittelfrüh, Wye Northdown and Olympic were grown in our garden (Division of Pharmacognosy, Leiden). Flower buds, flowers, late-stage flowers, young cones and ripe cones were collected, frozen in liquid N_2 and stored at −80°.

Chemicals. [2-^{14}C]Malonyl-CoA (925 kBq, 1.89 GBq mmol^{-1}) was obtained from Amersham U.K., unlabelled malonyl-CoA, isovaleryl-CoA, isobutyryl-CoA and naringenin were from Sigma. The ref. compounds PIVP, PIBP, X and co-X were prepd according to ref. [5]. *p*-Coumaroyl-CoA was a gift from Dr R. Welle (Freiburg, Germany). The antibodies raised against parsley chalcone synthase were a gift from Dr E. Schmelzer (Max-Planck-Institut für Züchtungsforchung, Cologne, Germany). The anti-rabbit IgG alkaline phosphatase conjugate was from Sigma Immuno Chemicals.

Analysis of bitter acids and their aromatic intermediates. The extraction and analysis of metabolites by HPLC and GC-MS were performed according to ref. [5]. A supercritical CO_2 extract from hop cones which contained 45.4% α-acids and 21.2% β-acids was used as a ref. mixt. (sample provided by L. C. Verhagen, Heineken Technical Services, Zoeterwoude, The Netherlands).

Protein extraction of hop flowers, cv Hallertau Mittelfrüh. All steps were carried out at 0–4°. Frozen plant material (25 g) was ground using a pestle and mortar in the presence of sea sand and 10% (w/w) polyvinylpolypyrrolidone. The frozen powder was mixed with extraction buffer according to ref. [16] and flushed with N_2 before use. The mixt. contained 0.5 M KPi buffer pH 8, 1.5% (w/v) polyethyleneglycol 6000, 400 mM sucrose, 1 mM $CaCl_2$, 0.1% (w/v) bovine serum albumin, 200 mM ascorbic acid, 50 mM EDTA, 50 mM cysteine, 10% (w/v) Dowex 1 × 2. After thawing, the homogenate was filtered using a nylon filter and then centrifuged at 13 000 *g* for 20

min. The protein was pptd from the supernatant using a range from 30 to 75% of $(NH_4)_2SO_4$ satn and centrifugation at 13000 *g* for 20 min. The protein pellet was then dissolved in 2.5 ml PD 10 buffer (0.5 M KPi pH 8, 5% (w/v) trehalose, 200 mM ascorbic acid, 50 mM cysteine and 50 mM EDTA), desalted in the same buffer by use of a PD 10 column (Sephadex G-25M, Pharmacia) according to the manufacturer's directions for use, frozen in liquid N_2 and stored at $-80°$. The protein concn was 3.5 mg ml^{-1} determined according to ref. [17].

Crude protein extraction of several hop organs, cv Wye Northdown and Olympic. The first step of the above described protein extraction method was used. Amounts of frozen material from cv Wye Northdown and Olympic, respectively, were 0.8 and 1.3 g flowerbuds, 4.9 and 7.0 g flowers, 3.7 and 5.0 g late-stage flowers, 6.7 and 5.3 g young cones, and 5.3 and 5.8 g ripe cones. After centrifugation of the nylon-filtered extracts, the supernatants were used for the HPLC chalcone synthase assay immediately after prepn, or after freezing in liquid N_2 and storage at $-80°$. The protein concns were determined according to ref. [17].

Chalcone synthase assay using HPLC. Chalcone synthase activity was determined as described earlier [9]. Apart from *p*-coumaroyl-CoA (5 μl 0.2 mM), isovaleryl-CoA (5 μl 0.2 mM) and isobutyryl-CoA (5 μl 0.2 mM) were also used.

HPLC photodiode array detection. The method used was described earlier [9].

Chalcone synthase radioassay. The method used was described earlier [9].

SDS–PAGE and immunoblotting. SDS–PAGE was performed with a 4% stacking and 12% running gel according to ref. [18]. The crude protein extracts were desalted using a membrane filter for dialysis of small-vol. samples (type VS 0.025μm from Millipore) according to the manufacturer's directions for use. The buffer used for desalting was 25 mM Tris–HCL pH 6.8. After the desalting process the vols of the protein extracts were doubled. The desalted protein extracts were then diluted by a factor of 2 with sample buffer (125 mM Tris–HCl pH 6.8, 17.4% glycerol, 4% (w/v) SDS, 1.4 M 2-mercaptoethanol and 0.0025% (w/v) bromophenol blue). After boiling for 5 min, 35-μl samples (for amounts of protein see legend for Fig. 6) and 10 μl M_r protein markers (Rainbow markers from Amersham) were loaded on to the gel. After running, the gel was stored overnight at 4°; the next day part of the gel was used for Coomassie Blue staining (G250), and the rest for blotting of the proteins on nitrocellulose. The schedule used for the immunostaining was as follows: blocking for 1 hr [TBS (50 mM Tris–HCl pH 7, 150 mM NaCl), 0.05% (w/v) Tween 80, 2% (w/v) bovine serum albumin], washing 3 × 15 min [TBS, 0.05% (w/v) Tween 80], overnight incubation with antibodies raised against parsley chalcone synthase [2 μl in 10 ml TBS, 0.05% (w/v) Tween 80, 0.2% (w/v) bovine serum albumin], washing 4 × 15 min, incubation for 4 hr with anti-rabbit IgG alkaline phosphatase conjugate (1 μl in 10 ml 'incubation buffer'), washing 4 × 15 min and staining for alkaline phosphatase.

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