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Monoterpene synthase activities in leaves of *Picea abies* (L.) Karst. and *Quercus ilex* L.

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Abstract

In addition to direct ecological functions in the interaction of plants with the environment, the emission of monoterpenes, especially from the foliage of evergreen trees, is of great importance for the production of ozone and photochemical oxidants in the troposphere. In the present work, we established a reproducible non-radioactive standard enzyme assay and characterized monoterpene synthase activities in needles of Norway spruce (*Picea abies* (L.) Karst.) and in leaves of holm oak (*Quercus ilex* L.). In Norway spruce, the dominant monoterpenes formed were α-pinene, camphene, and to a lesser extent β-pinene and limonene. In holm oak, α-pinene, sabinene, and β-pinene were the main products, while limonene was a minor component. Under optimum conditions, in both Norway spruce and holm oak, monoterpene formation remained constant up to 180 min and 90 min, respectively, and varied with the buffer and Mg^{2+} and Mn^{2+} concentrations used. Optimum temperature for monoterpene synthase activity was 40°C in both species; optimal pH ranged between 6.5 and 7.5 in both species. Apparent *Michaelis*-constants for the substrate GDP were ca. 17.9 ± 5.1 μM for Norway spruce and ca. 69.4 ± 22.1 μM for holm oak. Molecular weight determination by FPLC indicated that the monoterpene synthases in Norway spruce and holm oak have native molecular weights of ca. 59 and 50 kDa, respectively.

Keywords: *Picea abies*; Pinaceae; *Quercus ilex*; Fagaceae; Evergreen leaves; Enzyme characterization; Monoterpene synthases

1. Introduction

Emissions from trees provide a major source of reactive volatile organic compounds (VOC) which are essential for the production of ozone and photochemical oxidants in the troposphere (Fehsenfeld et al., 1992). Isoprene and monoterpenes are the most abundant biogenic VOC emitted from vegetation (Guenther et al., 1995) and thus contribute significantly to ozone production in areas with high anthropogenic NO_x emissions (Carter, 1994). In addition to their contribution to atmospheric chemistry, monoterpenes play important ecological roles as defense compounds against natural enemies, as well as allelopathic agents and as attractants for pollinators (for overview see Harborne, 1991). Monoterpenes are preferentially released by species containing specialized organs such as glandular trichomes, like the leaves of Lamiaceae, or resin ducts like conifer needles (Lerdau, 1991; Tingey et al., 1991). Such large storage pools in the needles of Norway spruce (*Picea abies* (L.) Karst.) (Schönwitz et al., 1990) constitute a permanent potential emission source which complements a light-dependent monoterpene emission that is closely linked to photosynthetic activity of the needles (Schürmann et al., 1993). Recent observations indicate that leaves of some oak species, e.g. the evergreen sclerophyllous holm oak (*Quercus ilex* L.), also emit large amounts of monoterpenes despite the absence of storage pools (Loreto et al., 1996a; Steinbrecher et al., 1997; Staudt

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and Bertin, 1998). This monoterpene emission is largely associated with photosynthesis, since it is stimulated by light, declines in dark, and is inhibited in the absence of CO_2 in air (Loreto et al., 1996a, 1996b).

Despite the information available on the physiological control of monoterpene emission from evergreen leaves and needles, information on the biochemical properties of the monoterpene synthases that catalyze the final steps leading from geranyl diphosphate (GDP) to the various monoterpene products in these species is still lacking.

Several monoterpene synthases of various plant species have been isolated and characterized (Alonso and Croteau, 1993; Colby et al., 1993; Savage et al., 1995; Bohlmann et al., 1998). The monoterpene synthases (or cyclases) responsible for generation of various acyclic, bicyclic or tricyclic terpenes have quite similar properties and all operate by electrophilic reaction mechanisms (Gershenzon and Croteau, 1993) as do other enzymes using prenyl-diphosphate. Different cyclization processes catalyzed by synthases lead to various monoterpenes (Gershenzon and Croteau, 1993). With native molecular weights M_r of ca. 50–120 kDa (either monomers or homodimers), rather low pI values, and significant hydrophobicity, the properties of these soluble enzymes are quite similar. The pH optima of the enzymes range from 6 to 7, and bivalent metal ions (usually Mg^{2+} or Mn^{2+}) are required as cofactor. Previously reported *Michaelis*-constants (K_m) for GDP are in the 10–20 μM range (Alonso and Croteau, 1993).

In the present work, we describe the extraction of monoterpene synthase activities and the development of a non-radioactive enzyme assay in evergreen leaves of Norway spruce and holm oak. In addition, kinetic properties of the enzyme activities of both species are also reported.

2. Results and discussion

2.1. Determination of monoterpene synthase activity by head space analysis

This is the first report on monoterpene synthase activities in Norway spruce needles and holm oak leaves. Monoterpene synthase activities in other species have been previously tested with ^{3}H-labeled GDP, by extracting the in vitro formed monoterpenes with organic solvents and measuring the monoterpenes directly by scintillation counting (Lewinsohn et al., 1992a, 1992b) or after separation of the products on TLC or GC (for review see Alonso and Croteau, 1993). In the present work, we adapted a non-radioactive assay from the measurement of isoprene synthase activity (Lehning et al., 1999) which is based on the gas chromatographic analysis of head space containing the synthesized monoterpenes released during the assay. Using this method, monoterpene synthase activities (Fig. 1) were detected in cell-free extracts of Norway spruce and holm oak leaves. In Norway spruce (Fig. 1a), the dominant monoterpenes produced were α-pinene, camphene, and to a lesser extent β-pinene and limonene. In extracts of holm oak (Fig. 1b), α-pinene, sabinene, β-pinene, and limonene were formed. GC-MS analysis additionally revealed the formation of traces of α-thujene (9.8 min) and myrcene (11.6 min) as well as E-ocimene (13.1 min), β-phellandrene (13.2 min), and Z-ocimene (13.4 min) in holm oak. Boiling protein extracts resulted in complete loss of enzyme activity (see Fig. 1). Enzyme assays with native protein extracts without the substrate GDP showed low monoterpene background levels of 0.1–4% compared to the enzymatically formed monoter-

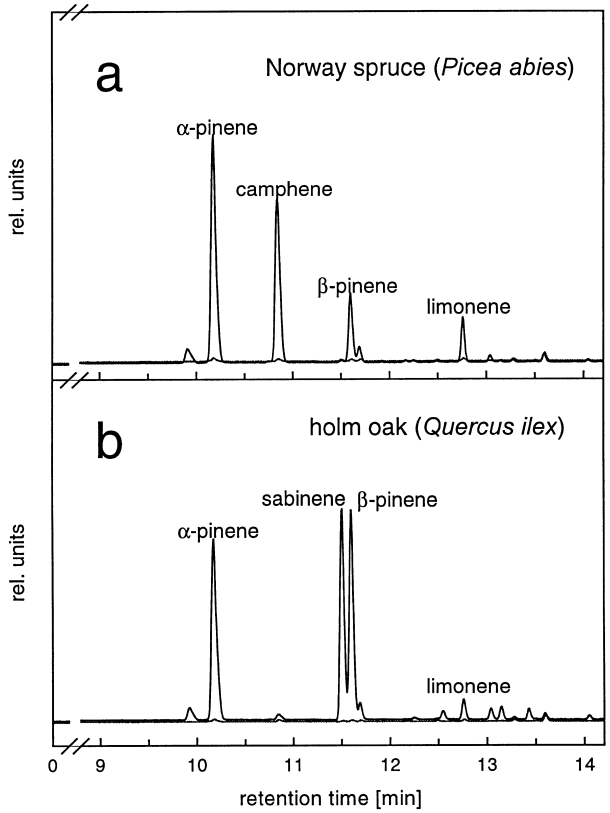

Fig. 1. Gaschromatographic analysis of monoterpenes formed in leaf extracts of (a) needles of Norway spruce and (b) holm oak leaves. For comparison, chromatograms of enzyme assays with active and heat-denaturated protein are shown. One ml samples of head space were focussed on a precolumn at room temperature. By heating the precolumn to 240°C the compounds were injected onto a capillary column and separated with a temperature program starting at 35°C and ending at 160°C. Monoterpenes were detected by flame ionization (FID) and identified by co-injection with authentic standards as well as by GC-MS analysis. Traces of α-thujene (9.8 min), myrcene (11.6 min), E-ocimene (13.1 min), β-phellandrene (13.2 min), and *Z*-ocimene (13.4 min) were detected.

penes in the presence of GDP, indicating that small amounts of monoterpenes or GDP had passed the size exclusion chromatography on the PD-10 columns.

To ensure the reproducibility of the head space analysis, mixing experiments with monoterpene standards, protein extracts, various buffers under assay conditions at different incubation temperatures were performed (Fig. 2). Addition of 1 μl monoterpene standard mixture (0.1 mM) to Norway spruce (Fig. 2a) or holm oak leaf extracts (Fig. 2b) under standard assay conditions at 10, 40, and 60°C gave additive amounts of monoterpenes when the enzyme substrate GDP was added compared to assays without substrate. Variation of the incubation temperature from 10 to 60°C had no significant influence ($p \geq 0.05$) on the recovery of the monoterpene standards from the head space. Comparison of enzyme assays co-incubated with monoterpene standard to normal assays showed that in both cases the enzyme activities were statistically similar. In addition, replacing the protein extracts by buffer also had no influence ($p \geq 0.05$) on the recovery of monoterpene standards (data not shown), indicating that under the test conditions evaporation of monoterpenes from the assay mixture is not hampered by protein extract.

Nevertheless, for calibration of the assay (Fig. 3) 1 μl of monoterpene standard mixtures (10 μM to 1 mM in pentane) was incubated in leaf extracts at 40°C without the enzyme substrate GDP under standard assay conditions (see Section 2.4 and Table 1) for 60 min prior to the analysis of the head space. Under these conditions, the detection of monoterpenes in the head space was highly linear ($r^2 > 0.99$) for about 2 orders of magnitude. Compared to the initially mentioned procedures (Lewinsohn et al., 1992a, 1992b; Alonso and Croteau, 1993), which include several extraction and purification steps, the capillary GC analysis of the head space containing the synthesized monoterpenes is more convenient, and facilitates processing a large number of samples with a detection limit of ca. 1.5 ppmv and an accuracy of 5.2%.

2.2. Selection of optimal extraction and assay buffers

The presence of phenolic compounds, oil and resins which inactivate monoterpene synthases and other plant enzymes is a particulary severe problem in evergreen leaves of tannin-rich and essential oil bearing plants, like holm oak and Norway spruce, and has been discussed in detail by Gegenheimer (1990). Therefore, an initial objective of the present work was to maximize the level of extractable activity in order to adequately define reaction parameters and permit subsequent protein purification. Monoterpene synthase ac-

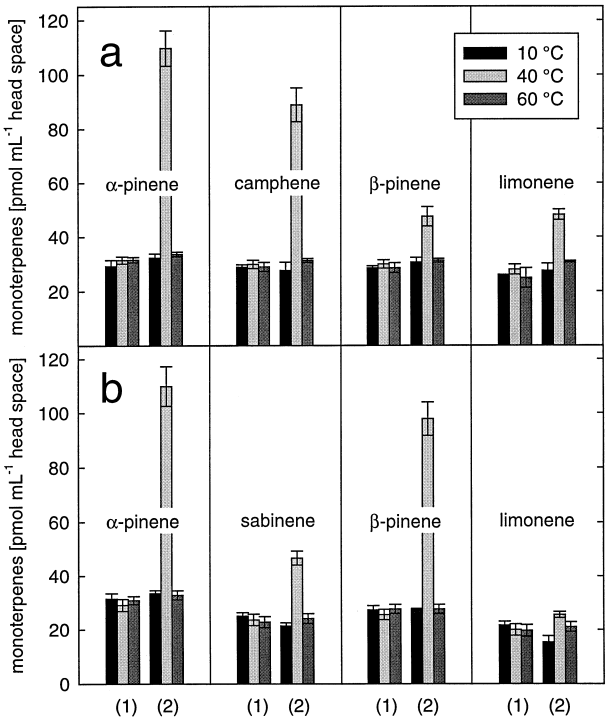

Fig. 2. Co-incubation of Norway spruce (a) and holm oak (b) leaf extracts and monoterpene standards. Leaf extracts with (**1**) monoterpene standards (1 μl of 0.1 mM solution in pentane) and (**2**) standard mixture plus enzyme substrate GDP were incubated under standard assay conditions at 10, 40, and 60°C. After 60 min, the liquid phase was removed with a gas-tight syringe and 1.0 ml samples of the head space were analyzed as described in Section 3. ($n = 5$; ±sd).

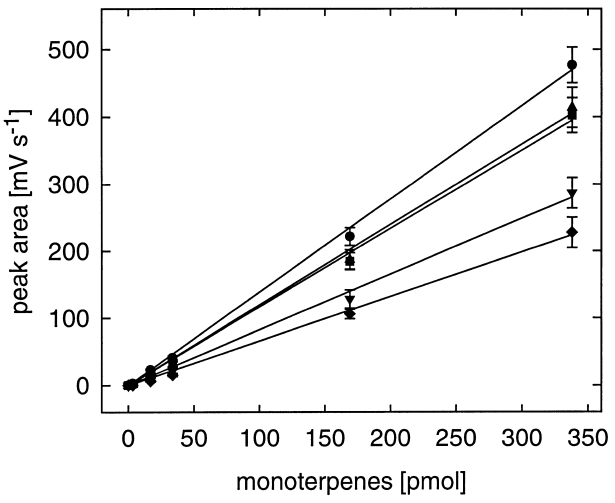

Fig. 3. Calibration curve of monoterpenes by the head space analysis. Increasing amounts of monoterpene standard mixture (ranging from 10 μM to 1 mM) were added in 1 μl pentane to 100 μl Norway spruce leaf extracts and incubated at 40°C in gas-tight vials (2 ml). After 60 min, the liquid phase was removed with a gas-tight syringe and 1.0 ml samples of the head space were analyzed as described in Section 3. ($n = 5$; ±sd; r^2-values of the linear regressions were ca. 0.99).

tivities in holm oak leaves were extracted best with a 700 mM Mops buffer (pH 7.3) containing antioxidants (200 mM ascorbic acid, 50 mM 2-mercaptoethanol) and a mixture of additives for adsorption of phenolic compounds (8.3% (w/v) Dowex 1 × 2, 1% (w/v) PVP-30, 1.5% (w/v) PEG-1500). Omission of PVP-30 or Dowex 1 × 2 during extraction, or lowering the molarity of the buffer (to 100 mM) caused nearly complete loss of enzyme activity. Omission of PEG-1500 lowered the enzyme activity by ca. 13%. PVP-30 could not be replaced by insoluble polyvinylpyrrolidone polymer PVPP (at 1% (w/v) of the buffer). Screening for the best assay buffer showed that with 50 mM KP_i as buffer reagent monoterpene synthase activity could be enhanced by 85 ± 3% compared to other buffers (Tris, Hepes, Mops, each 50 mM) tested.

Measurements of monoterpene synthase activities in Norway spruce needles were based on the extraction procedure of monoterpene synthases from wood or bark tissue of conifers (Lewinsohn et al., 1992a). With this extraction buffer (50 mM Hepes pH 7.3 containing 10% (v/v) glycerol, 2% (w/v) PVP-30), antioxidants (5 mM each of Na-ascorbate, Na-disulfite, DTT) monoterpene synthase activity was detected in Norway spruce needles. Replacement of Hepes as extraction buffer by Tris, KP_i, Mops or Bis–Tris did not significantly enhance enzyme activity. Among the assay buffers tested, highest enzyme activity was found with 50 mM Mops.

Addition of the proteinase inhibitors phenylmethylsulfonyl fluoride (PMSF) and benzamidine hydrochloride (BA) (each 1 mM) as well as the presence of molybdate (1 mM) as inhibitor of phosphatases in the extraction and assay buffer did not influence monoterpene synthase activities.

2.3. Bivalent cation requirement of monoterpene synthase activities

In general, monoterpene synthase activities are strongly dependent on bivalent metal ions (usually Mg^{2+} and/or Mn^{2+}) (Alonso and Croteau, 1993; Bohlmann et al., 1998). Presence of these cations at concentrations of 0.1–20 mM was also found to be essential for monoterpene synthase activity in Norway spruce and holm oak. From work on other terpene synthases, it is proposed that the function of bivalent cations may be to neutralize the negative charge of the allylic diphosphate moiety by complex formation (Croteau, 1988; Alonso and Croteau, 1993). In Norway spruce, enzyme activity was stimulated by Mg^{2+} and Mn^{2+} with different preferences for Mg^{2+} or Mn^{2+} as cofactor for the synthesis of α-pinene/camphene and β-pinene/limonene, respectively. Maximum monoterpene synthase activities were achieved by the following combination of Mg^{2+} and Mn^{2+} in the assays: addition of Mn^{2+} up to a concentration of 2.5 mM stimulated formation of α-pinene and camphene by 50%, while at higher Mn^{2+} concentrations α-pinene and camphene synthesis was inhibited. Formation of β-pinene and limonene was maximally stimulated at 5 mM Mn^{2+} by a factor of ca. 3, compared to tests with Mg^{2+} alone.

In holm oak, monoterpene synthase activity strongly depended on the presence of Mg^{2+} in the assay. Replacement of Mg^{2+} by Mn^{2+} reduced enzyme activities by ca. 80%. When both cations were added, increasing amounts of Mn^{2+} up to 20 mM strongly inhibited the synthesis of α-pinene, sabinene, β-pinene, and limonene. Since the bivalent cation requirement differed between Norway spruce and holm oak leaves, standard enzyme assays of Norway spruce extracts were performed with 2.5 mM Mn^{2+} and 20 mM Mg^{2+}, while monoterpene synthase activities of holm oak leaves were measured in the presence of 20 mM Mg^{2+} (see Table 1).

In addition to the strict dependency on bivalent cations, Bohlmann et al. (1997) reported a further requirement of monoterpene synthases for the monovalent cation K^+ from grand fir (*Abies grandis* L.). In the present work, additional supplementation of K^+ of up to 600 mM has no effect on monoterpene synthase activities in both species. Moreover, the repla-

Table 1
Kinetic properties of monoterpene synthase activities in leaf extracts from Norway spruce needles and holm oak leaves

	Norway spruce	Holm oak
pH optimum	Broad optimum between pH 6.5 and 7.5	Sharp optimum at pH 6.7
Temperature optimum	40°C; activities measurable up to 50°C	40°C; activities measurable up to 60°C
Activation energy E_a (Arrhenius plot)	α-Pinene: 60.5 kJ mol^{-1}	α-Pinene: 62.6 kJ mol^{-1}
	Camphene: 78.2 kJ mol^{-1}	Sabinene: 78.2 kJ mol^{-1}
	β-Pinene: 39.8 kJ mol^{-1}	β-Pinene: 70.3 kJ mol^{-1}
	Limonene: 120.5 kJ mol^{-1}	Limonene: 114.2 kJ mol^{-1}
Apparent *Michaelis*-constants (K_m) according to Hanes	α-Pinene: 17.7 μM	α-Pinene: 55.0 μM
	Camphene: 13.2 μM	Sabinene: 57.3 μM
	β-Pinene: 26.2 μM	β-Pinene: 57.8 μM
	Limonene: 14.6 μM	Limonene: 107.6 μM

cement of the bivalent cations by the monovalent cation K^+ in the assay caused a complete loss of enzyme activity in both species.

2.4. Kinetic properties of monoterpene synthase activities

Monoterpene synthase activity proceeded linearly for 180 min in Norway spruce and 90 min in holm oak leaf extracts (Table 1). With increasing protein contents in the assay mixture, a linear increase in monoterpene synthase activity up to 20 and 60 μg protein per assay in Norway spruce and holm oak was observed, respectively.

The monoterpene synthase activities of Norway spruce and holm oak leaves showed properties similar to those of enzymes from other species (Croteau, 1988; Alonso and Croteau, 1993; Lewinsohn et al., 1992a, 1992b). In Norway spruce (Table 1), apparent *Michaelis*-constants (K_m), calculated from Hanes plots (Bisswanger, 1994), ranged between 13.2 μM for camphene and 26.2 μM for β-pinene, while in leaf extracts of holm oak higher K_m-values were measured ranging from 54.3 μM for sabinene to 107.6 μM for limonene. Nevertheless, the buffer system chosen influenced the apparent *Michaelis*-constants measured here. When Hepes was used as assay buffer for Norway spruce leaf extracts, slightly lower constants were found ranging from 7.1 μM for limonene to 14.6 μM for β-pinene. Especially, in holm oak leaf extracts, the use of Hepes as assay buffer considerably lowered the apparent K_m to values ranging from 5.5 μM for limonene to 8.0 μM for β-pinene, but on the other hand the maximum enzyme velocity was reduced by 50% compared to KP_i as assay buffer.

The effect of pH in the assay mixture was examined using different buffer systems (Mes/KOH pH range 5.4–6.7; Hepes/KOH pH range 6.5–7.3; Tris/HCl pH range 6.9–8.4). In Norway spruce (Table 1), a broad pH optimum between pH 6.5 and 7.5 was observed for monoterpene synthase activities. The pH optimum of monoterpene synthase activities in holm oak was sharper with an optimum value at pH 6.7. Enzyme activities decreased drastically at pH values higher or lower than 6.7 (see Table 1).

Among several environmental variables tested, temperature had a dominant effect on monoterpene emission of plants (Tingey et al., 1991). Monoterpene synthase activities of both species also responded strongly to assay temperature (Table 1). In Norway spruce, the enzyme activities responsible for α-pinene and camphene formation increased exponentially between 20 and 40°C by a factor of 5.1 and 7.5, respectively, while synthesis of β-pinene and limonene was stimulated in this temperature range by a factor of 2.1 and 2.3, respectively. At higher temperatures, monoterpene synthase activities dropped rapidly and reached zero at about 50°C. The temperature optima of ca. 40°C for monoterpene synthases from Norway spruce needles were similar to the previously described temperature optimum for the (−)-1*S*, 5*S*-pinene synthase that was isolated from the hard wood of grand fir (Lewinsohn et al., 1992b).

In contrast to the sharp decline of enzyme activities at temperatures higher than the optimum temperature in Norway spruce, the temperature response curves of monoterpene synthases in holm oak were bell-shaped between 20 and 60°C with maximal enzyme activities at ca. 40°C. This high temperature optimum of monoterpene synthases in holm oak leaves of ca. 40°C is likely to trigger the in vivo temperature optimum for monoterpene emission between 35 and 45°C (Loreto et al., 1996a; Ciccioli et al., 1997; Staudt and Bertin, 1998) of this species.

Based on these results, standard enzyme assays were run for 60 min with substrate concentrations (corresponding to 5-times apparent *Michaelis*-constants) of 100 μM GDP for Norway spruce and 250 μM GDP for holm oak. In addition, the protein concentrations in the assay were kept to ca. 10 and 30 μg for Norway spruce and holm oak, respectively. Measurement of phosphatase activities with [1−^{3}H]-GDP performed in parallel to the non-radioactive enzyme assays showed that under standard assay conditions approx. 13% and 3% of GDP was converted to oxygenated products (i.e. geraniol), while approx. 6% and 8% of GDP was found in the volatile monoterpene fraction in Norway spruce and holm oak, respectively. These data clearly show that under standard monoterpene assay conditions no substrate limitation for the monoterpene synthases can occur by competing phosphatase activities, as indicated by the time linearity of the monoterpene formation (see Table 1), and the fact that phosphatase inhibition has no influence as mentioned above (see. Section 2.2).

2.5. Molecular weight determination

Monoterpene synthases are soluble, although most of them are associated with plastids in vivo (Colby et al., 1993; Bohlmann et al., 1997; Turner et al., 1999). To date, the vast majority of monoterpene synthases characterized, e.g. the (−)-1*S*, 5*S*-pinene synthase from grand fir (Lewinsohn et al., 1992b), the (−)-4*S*-limonene synthase from *Mentha spicata* L. (Colby et al., 1993), or the 1,8-cineol synthase from *Salvia officinalis* L. (Wise et al., 1998), are monomers with native molecular weights M_r of ca. 56–72 kDa, although dimeric forms are also found, e.g. the γ-terpinene synthase (M_r ca. 96 KDa) from *Thymus vulgaris* L. (Alonso and Croteau, 1991) or the bornyldiphosphate synthase (M_r ca. 135 KDa) from *S. officinalis* (Wise et al., 1998).

Calibrated gel permeation chromatography with leaf extracts of Norway spruce and holm oak (after a 100,000 *g* centrifugation to remove microsomal membranes) revealed single peaks of activity at elution volumes corresponding to M_r of ca. 59 and 50 kDa for Norway spruce (Fig. 4a) and holm oak (Fig. 4b), respectively, indicating that the native proteins are also functional monomers.

Since many monoterpene synthases generate multiple products from GPD (Bohlmann et al., 1997; Wise et al., 1998), as e.g. the (−)-4*S*-limonene synthase found in the wood of grand fir generates (−)-limonene and to a lesser extent (−)-α-pinene, (−)-β-pinene and (−)-β-phellandrene (Bohlmann et al., 1997), our measurements reflect the overall enzymatic synthesis of acyclic (myrcene), monocyclic (limonene) and bicyclic monoterpenes (e.g. α-pinene), and do not allow any conclusions on the distinct number of monoterpene synthases present in holm oak or in Norway spruce protein extracts. Nevertheless, depending on different temperature response curves and different preferences for Mg^{2+} ions in Norway spruce, two groups of enzymes, i.e. (a) forming α-pinene and camphene and (b) forming β-pinene and limonene, can be distinguished in this species. In holm oak, the biochemical characteristics of the limonene-forming enzyme differed from those of the α-pinene, sabinene, and ß-pinene producing activities and therefore also give indications for the presence of several monoterpene synthases.

Future work on Norway spruce and holm oak will focus on the isolation of the different monoterpene synthase proteins and genes, which are prerequisites for studying the regulation of enzyme activities and gene expression in order to understand the contribution of monoterpene synthases to the observed monoterpene emission of plants.

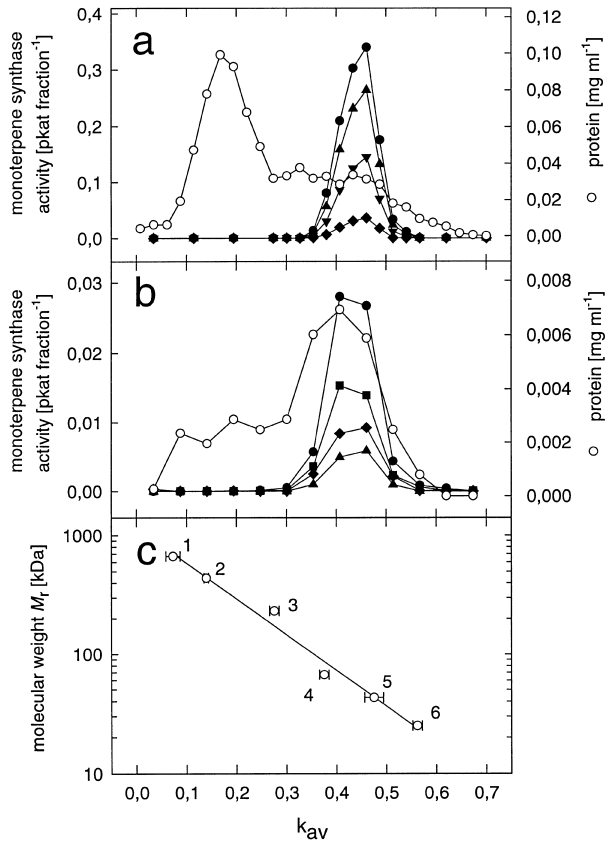

Fig. 4. Molecular weight determination of monoterpene synthase activities in Norway spruce (a) and holm oak (b) by FPLC on Superdex-200 HR. Monoterpene synthase activities were determined by incubating aliquots of 88 µl under standard conditions (●–● α-pinene, ■–■ camphene, ▲–▲ β-pinene, ◆–◆ limonene, ▼–▼ sabinene; ○–○ protein). Calibration of the column (c) was performed with the molecular weight markers (1) ferritin: 440 kDa, (2) catalase: 232 kDa, (3) aldolase: 158 kDa, (4) bovine serum albumine: 67 kDa, (5) ovalbumine: 43 kDa, (6) thyroglobulin: 25 kDa ($n = 2$; ±sd).

3. Experimental

3.1. Chemicals

Buffer reagents and other chemicals were obtained commercially and were of analytical grade. The synthesis of geranyl-diphosphate (GDP) was carried out according to Keller and Thompson (1993). The product was identified by ^{1}H- and ^{31}P-NMR spectroscopy (AC-400 FT-NMR spectrometer, Bruker, Karlsruhe, Germany) with D_2O as solvent.

3.2. Plant material, growth and irradiation conditions

Norway spruce seeds were purchased from the Staatliche Samenklenge Laufen (Bavaria, Germany). The seeds were germinated as described by Rosemann et al. (1991) for Scots pine, and were cultivated in a growth chamber at 24/15°C day/night temperature, and a 14 h light period from 6 a.m. to 8 p.m. (Sylvania VHO F195W cool white, Erlangen, Germany) at a photosynthetic photon flux density [PPFD] of 60 µmol m^{-2} s^{-1}. After thirty days of cultivation, the primary needles of the seedlings were harvested, shock frozen in liquid nitrogen and stored at −80°C until use.

Current year leaves of naturally grown holm oak trees were sampled on August 4, 1998 in the plant garden of the Centre d'Ecologie Functionelle et Evolutive du CNRS, Montpellier (France). Leaves were cut from the twigs, shock frozen in liquid nitrogen, transferred to Garmisch-Partenkirchen on dry ice, and stored at −80°C until use.

3.3. Preparation of protein extracts

Leaf material (250 mg) of holm oak or Norway spruce was homogenized under liquid nitrogen with a

mortar and pestle and suspended in 6 ml protein extraction buffer [for Norway spruce: 50 mM Hepes/KOH pH 7.3 containing 10% (v/v) glycerol, 2% (w/v) PVP-30, antioxidants (5 mM each of Na-ascorbate, Na-disulfite, DTT); for holm oak: 700 mM Mops/HCl pH 7.3 containing 1.5% (w/v) PEG-1500, 1% (w/v) PVP-30, 8.3% (w/v) Dowex 1 × 2, 20 mM $MgCl_2$, antioxidants (200 mM ascorbic acid, 50 mM 2-mercaptoethanol)] and stirred in an ice bath for 20 min. Cell debris were sedimented by centrifugation (18,000 *g* for 20 min) at 4°C. Aliquots of 2.5 ml of the supernatant were desalted on PD-10 columns [Pharmacia, Uppsala, Sweden; equilibration and elution buffer for Norway spruce 50 mM Mops/HCl pH 7.3 or for holm oak 50 mM KP_i, pH 7.3, each buffer containing 10% (v/v) glycerol and antioxidants (10 mM Na-ascorbate, Na-disulfite, DTT, each)] resulting in a final volume of 3.5 ml which was stored at −80°C. Protein was determined according to Bradford (1976) with BSA as a standard.

3.4. Monoterpene synthase standard enzyme assay

For standard enzyme assays, 91 μl of protein extracts were transferred into gas-tight 2 ml crimp seal vials (Supelco, Bellefonte, USA) after addition of 4 μl 0.5 M $MgCl_2$ (final concentration: 20 mM) for holm oak and 4 μl 0.5 M $MgCl_2$/ 62.5 mM $MnCl_2$ (final concentration: 20 mM $MgCl_2$, 2.5 mM $MnCl_2$) for Norway spruce. The enzyme reaction was started by the addition of 5 μl of a 2 mM or 5 mM geranyldiphosphate (GDP) solution (final concentration: 100 μM for Norway spruce and 250 μM for holm oak) and incubated for 60 min at 40°C. The enzyme reaction was terminated by removing the reaction mixture from the closed vial with a syringe, and washing the vial with 150 μl assay buffer. Samples of the head space (1.0 ml) were trapped at 27°C on a pre-column filled with Tenax TA 60/80 (Alltech, Unterhaching, Germany), and reaction products were separated by capillary gas chromatography (GC) after heating the precolumn to 240°C. The GC system (Chrompack CP 9000, Chrompack, Frankfurt/M., Germany) was equipped with a head space-volume-autosampler (HSS, Gerstel, Mülheim, Germany), a temperature-programmed-injection system (KAS 3, Gerstel, Mülheim, Germany) and a capillary column (DB 1701, 30m, ID 0.25 mm, film thickness 1 μm; J&W Scientific, Folsom, CA, USA). Separation was performed with a temperature program starting at 35°C, increasing after 0.5 min at a rate of 30°C min^{-1} to 78°C, remaining for 4 min at this condition, the temperature was then raised at a rate of 9°C min^{-1} to 160°C, and then at a rate of 35°C min^{-1} to the final temperature of 250°C. The products were detected with a flame ionization detector (FID; temperature: 270°C), and monoterpenes were identified by co-injection with authentic standards (Roth, Karlsruhe, Germany) and by GC-MS analysis (ATD 400, Autosystem XL GC, Turbo Mass MS spectrometer, Perkin Elmer, Beaconsfield, UK). The amounts of monoterpenes formed in vitro were calculated using the relative response factor obtained from standard mixtures of known concentrations (see Fig. 3). Protein extracts without the substrate GDP were carried out in parallel in order to correct for non-enzymatic background.

3.5. GDP phosphatase assay

Measurement of phosphatase activity was performed according to the method of Alonso et al. (1992). Tests with [1−^{3}H]-GDP (3.7 kBq $nmol^{-1}$, Biotrend, Köln, Germany) were done in Eppendorf tubes under similar conditions and compared to the non-radioactive monoterpene synthase assay (see Section 3.4). Following the extraction of monoterpenes (in 2 × 0.5 ml hexane) and of oxygenated products (i.e. geraniol liberated from the substrate by endogenous phosphatases) (in 2 × 0.5 ml diethyl ether) 250 μl aliquots of hexane and diethyl ether eludates were transferred to scintillation vials containing 15 ml Quicksafe A (Zinsser Analytik, Frankfurt, Germany) for determination of [^{3}H] content (Beckman LS 3801, München, Germany; [^{3}H] efficiency = 51.5%).

3.6. FPLC gel permeation chromatography

The native molecular weight of monoterpene synthase proteins from Norway spruce and holm oak were determined from 100,000 *g* centrifugation (1 h) supernatants of crude leaf protein extracts, concentrated by ultrafiltration (Microcon-10, Amicon, Witten, Germany) to final protein concentrations of c. 3 mg ml^{-1}. 200 μl of theses fractions were separated on a Superdex-200 HR column (Pharmacia, Uppsala, Sweden) by FPLC (controller LCC-500, pumps P-500, fraction collector Frac-100, Pharmacia, Uppsala, Sweden). The proteins were eluted in 50 mM Mops/HCl pH 7.3 (for Norway spruce) and 50 mM KP_i, pH 7.3 (for holm oak), each buffer containing 10% (v/v) glycerol and antioxidants (10 mM Na-ascorbate, Na-disulfite, DTT, each) at a flow rate of 0.4 ml buffer min^{-1}. The protein concentration in each fraction (0.25 ml) was determined according to Bradford (1976). Monoterpene synthase activities were determined as described in Section 3.4. Calibration of the Superdex-200 HR column was performed with the high and low molecular weight protein kits from Pharmacia (Uppsala, Sweden).

3.7. Statistical analysis

Statistical analysis was performed with SPSS version

8.0 for Windows. Differences between mean values of the samples were compared using a one way analysis of variance (ANOVA).

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