Mol. Nutr. Food Res. 2005, *49*, 857 – 860 DOI 10.1002/mnfr.200500040

Biosynthetic ^{14}C-labelling of xanthohumol in hop cones

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Xanthohumol (Xn) has well-established chemopreventive potential *in vitro*. In order to carry out *in vivo* bioavailability and tissue distribution studies, ^{14}C-labelled Xn was produced by biolabelling. Supplying hop sprouts with 5 mCi [U-^{14}C]glucose led to incorporation of ^{14}C into Xn. Delivering the radioactive precursor at once resulted in Xn with a specific activity of 318 μCi · mmol^{-1}; if however the amount was supplied in aliquots over 4 days a specific activity of only 53.1 μCi · mmol^{-1} Xn was obtained.

Keywords: Hops / ^{14}C-radiolabelling / Xanthohumol

Received: March 26, 2005; revised: June 29, 2005; accepted: June 30, 2005

1 Introduction

Xanthohumol (Xn), a prenylated chalcone from hops (*Humulus lupulus*, L., Cannabaceae, see Fig. 1) showed in recent studies a broad range of interesting biological activities such as strong chemopreventive potential [1–3] and antiangiogenic activity [4]. However, despite these important *in vitro* and *in vivo* activities, little information is available regarding its bioavailability and tissue distribution in mammals *in vivo* [5].

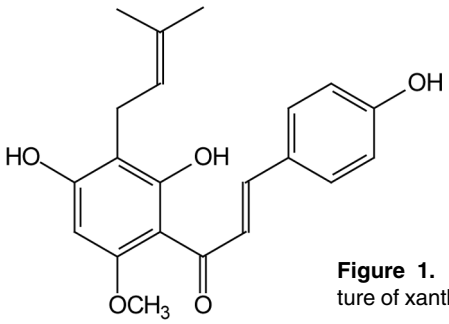

Figure 1. Chemical structure of xanthohumol.

For bioavailability studies, radiolabelled compounds have been widely used [6], because these compounds and their metabolites can be easily traced *in vivo*.

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Abbreviation: Xn, Xanthohumol

The aim of the present study was to synthesize ^{14}C-labelled Xn for *in vivo* studies in rodents. Since no chemical synthesis of Xn has been published yet, biolabelling seemed to be an appropriate method for obtaining ^{14}C-labelled Xn. This method uses the plant metabolism for the synthesis of complex natural products from radioactively labelled precursors. In previous studies we optimized the biolabelling conditions for Xn using various ^{13}C-labelled precursors [7] such as [2-^{13}C]acetate, [2-^{13}C]malonate, [ring-^{13}C]phenylalanine, [1-^{13}C]glucose and [U-^{13}C]glucose in different concentrations. The studies resulted in Xn with distinct labelling patterns and incorporation rates. The highest rate was obtained with [U-^{13}C]glucose provided at a concentration of 2.5% w/v. In accordance with the results of these studies, hop cones were fed with the general metabolite [U-^{14}C]glucose.

2 Materials and methods

2.1 Plant material and incubation experiments

Hop rhizomes (*Hallertauer Taurus*) were obtained from the Gesellschaft für Hopfenforschung, Wolnzach, Germany. The plants were grown in the garden of our institute. Feeding experiments were conducted following the experimental setting of Goese *et al.* [8]. In mid August, before the hop cones were fully grown, short hop sprouts (3–4 cm), each with two to three young hop cones, were cut from the hop plants. The sprout ends were trimmed with a razor blade before immersing them in 4 mL glass vials containing the incubation solution.

Two different incorporation experiments were performed: For both experiments 15 sprouts were treated with a total amount of 5 mCi [U-^{14}C]glucose each (245 mCi/mmol; 0.1 mCi/ml H_2O; Hartmann Analytic, Braunschweig, Germany).

Each sprout was kept in a glass tube containing 4 mL feeding solution. In experiment 1, the whole amount of 5 mCi [U-^{14}C]glucose was administered on the first day of incubation. In each tube, 3.3 mL of the radiolabelled glucose solution (333.3 μCi) was mixed with 0.7 mL modified Gamborg B5 [9] medium containing unlabelled glucose in order to reach a final concentration of 2.5% w/v glucose (labelled and unlabelled).

In experiment 2, 5 mCi [U-^{14}C]glucose were administered successively over the first 4 days of incubation in 1.25 mCi portions. The solution was also mixed with modified Gamborg B5 medium containing unlabelled glucose to a final concentration of 2.5% w/v glucose (labelled and unlabelled).

On the following days the amount of the feeding solution taken up by the plants was replaced by Gamborg B5 medium with 2.5% unlabelled glucose. All experiments were performed at pH 7; pH was adjusted with 0.1 M NaOH. Small sections of the ends of the stems were cut with a razor blade every day in order to facilitate liquid uptake. The incubations were performed in a hood under 24 h light conditions at 22°C until complete wilting (8 days). Petri dishes containing 1 M NaOH were placed in the hood to trap respired $^{14}CO_2$ [10].

After incubation, the hop cones were cut from the stems and frozen in a glass bottle until further use.

2.2 Isolation of ^{14}C-Xn

Extraction of the hop cones and isolation of Xn were carried out as described previously [7]. Briefly, hop cones were minced with scissors and extracted with MeOH under reflux for 1 h in the two experiments. After cooling, the extracts were filtered and evaporated almost to dryness, yielding 2.1 and 2.6 g crude extract, respectively. Xn was isolated using polyvinyl-pyrrolidone/silicagur column chromatography by gradient elution from 100% MeOH to MeOH : EtOAc/0.1% formic acid 60 : 40. The yellow-coloured fractions were combined in each case, evaporated to dryness and subjected to HPLC analysis.

The radioactivity of the hop extracts and of the isolated Xn was determined with a liquid scintillation Counter (Packard Tricarb, 2200CA) by adding scintillation solution ('Ultima Gold' Perkin Elmer).

2.3 Identity, chemical and radiochemical purity of ^{14}C-Xn

The identity and purity of the isolated Xn were checked by analytical HPLC with a photodiode array detector (Agilent 1100 Series) on an RP18 (Lichrospher 100, 5 μm; 4 mm × 125 mm; Merck, Darmstadt, Germany) column using the following conditions: flow: 0.9 ml/min, solvent A: CH_3CN/0.1% TFA, solvent B: H_2O/0.1% TFA; 0–2 min 40% A, 40–60% A in 18 min, 60–95% A in 10 min, 95% A over 5 min, 95–40% A in 5 min. Ten microlitres each of a 1.1 and 2.3 mM methanolic solutions were injected and the chromatograms recorded at λ = 234, 290 and 371 nm. The identity of the isolated Xn was proven by comparing the retention time and UV spectrum (17.3 min; λ_{max} = 371.5 nm) with an authentic sample of Xn as reference. The eluent was collected directly into scintillation vials in 1 min fractions (LKB 2211 Superrac fraction collector) and subjected to scintillation counting. The chemical purity of Xn was calculated after integrating all peak areas. The radiochemical purity of Xn was determined by measuring the radioactivity of the single fractions.

3 Results and discussion

Biolabelling is a well-known method for obtaining isotopically labelled natural molecules. Especially complex compounds such as proanthocyanidins, catechins and epichatechins have already been successfully radiolabelled [10].

In previous studies, we tested different ^{13}C-labelled biosynthetic precursors in various concentrations in view of possible ^{13}C incorporation in Xn. Since the unspecific precursor [U-^{13}C]glucose led to the highest incorporation rate, we chose [U-^{14}C]glucose as precursor compound for the biolabelling experiments.

In order to reach a high yield of labelled Xn, we used hop cones of *H. Taurus* variety. This variety produces the highest amount of Xn of all commercial hop plants, delivering about 2% Xn extractable by MeOH from the cones [11]. Young cones were used, because the biosynthesis of Xn is still in progress in such cones [12].

We conducted two different feeding experiments: in experiment 1, the total amount of 5 mCi [U-^{14}C]glucose was fed on the first day of incubation while in experiment 2, the same amount of the labelled precursor was fed over 4 days. All hop cones were incubated for 8 days until complete wilting. In both experiments, 40 mg radiolabelled Xn of high chemical purity was isolated by column chromatography.

The rate of ^{14}C incorporation differed according to the feeding conditions. The radioactivity of the methanolic hop

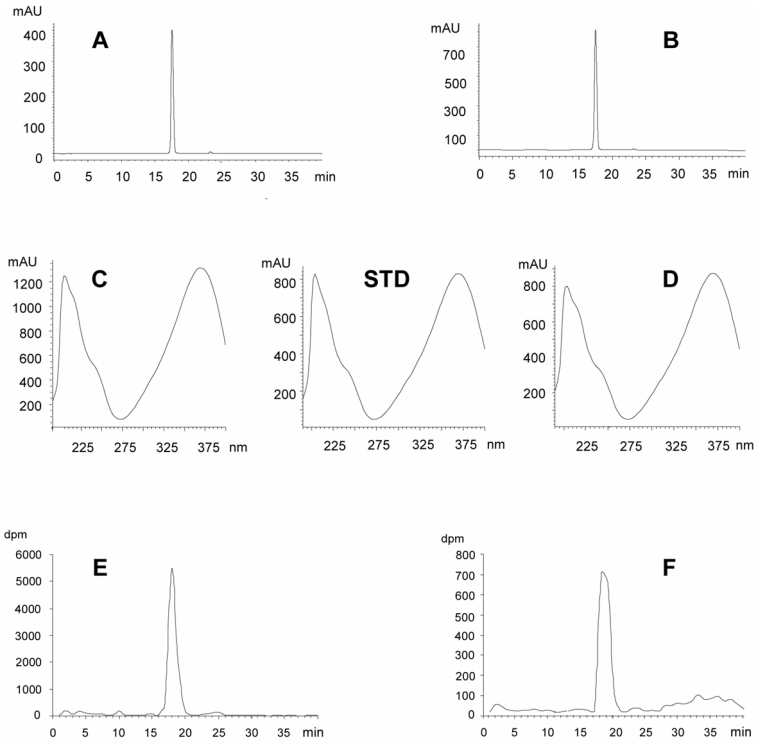

Figure 2. HPLC-UV$_{371\ nm}$ chromatograms (A and B), UV spectra (C and D) and the corresponding radiochromatograms (E and F) of Xn of experiments 1 (A, C and E) and 2 (B, D and F); for comparison the UV spectrum of an unlabelled authentic Xn sample is shown (STD).

extracts was determined as 1.85 mCi in the single application experiment 1, and as 200 μCi in experiment 2 with splitted dosage of the [U-^{14}C]glucose. This corresponds to 36.6 and 4.0% incorporation, respectively, of the applied radioactivity into methanol extractable hop compounds. From these results it is evident that the incorporation of ^{14}C was much higher in experiment 1 when the whole amount of the precursor was given on the first day of incubation. Due to the different incorporation rates, also the specific activity of Xn differed in the two experiments and was found to be 318 μCi/mmol in experiment 1 and 53.1 μCi/mmol in experiment 2.

The big variation in ^{14}C incorporation between experiments 1 and 2 might be due to strongly decreasing activities of the enzymes involved in the biosynthesis of secondary metabolites with time after the cones are separated from the plant. Alternatively, the transport of the precursor through the stems of the cuttings could be diminished.

Figure 2 shows the HPLC-UV$_{371\ nm}$ chromatograms (A and B), the UV spectra (C and D) and the corresponding plotted radiochromatograms (E and F) for the isolated Xn of experiments 1 and 2. The spectra of an authentic unlabelled sample (STD in Fig. 2), of the ^{14}C-labelled compounds (C and D) and of a mixture of both (not shown) are identical. The integration of the HPLC chromatograms allowed to calculate the chemical purity which was found to be >98% in both analyses. The radiochemical purity was determined by the relation of the sum of radioactivity in the Xn containing fractions 17–20 (see Fig. 2E and F) *versus* the sum of radioactivity in the remaining fractions. It was found to be 97.4% in experiment 1 and 80.5% in experiment 2.

In previous studies we examined the incorporation pattern of [U-^{13}C]glucose in Xn by quantitative ^{13}C NMR spectroscopy [7] and found a ^{13}C-incorporation for each carbon of Xn. Because of this observation, we assume that feeding of [U-^{14}C]glucose also leads to ^{14}C-incorporations at all carbon positions of Xn.

In this study we demonstrated a suitable method for ^{14}C-labelling of Xn. The amount of Xn (40 mg) as well as its specific activity (318 μCi/mmol in experiment 1) are sufficient for *in vivo* bioavailability and tissue distribution studies that are urgently needed to determine the target organs for its chemopreventive effects.

After Xn isolation, the remaining MeOH extract is still highly radioactive and can be used to isolate additional ^{14}C-labelled hop constituents of high biological activity like hop bitter acids [13] or prenylated flavonoids [14].

This work was supported by Deutsche Forschungsgemeinschaft (DFG). We also thank Klaus Gladel for taking care of the hop plants.

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