Activation of Proestrogens from Hops (Humulus lupulus L.) by Intestinal Microbiota; Conversion of Isoxanthohumol into 8-Prenylnaringenin

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Hop, an essential ingredient in most beers, contains a number of prenylflavonoids, among which 8-prenylnaringenin (8-PN) would be the most potent phytoestrogen currently known. Although a number of health effects are attributed to these compounds, only a few reports are available about the bioavailability of prenylflavonoids and the transformation potency of the intestinal microbial community. To test these transformations, four fecal samples were incubated with xanthohumol, isoxanthohumol (IX), and 8-PN. Upon incubation with IX, present in strong ales up to 4 mg/L, 36% was converted into 8-PN in one fecal sample and the estrogenic properties of the sample drastically increased. In an experiment with 12 fecal cultures, this conversion was observed in one-third of the samples, indicating the importance of interindividual variability in the intestinal microbial community. Eubacterium limosum was identified to be capable of this conversion (O-demethylation) of IX into 8-PN, and after strain selection, a conversion efficiency of 90% was achieved. Finally, strain supplementation to a nonconverting fecal sample led to rapid and high 8-PN production at only 1% (v/v) addition. Up to now, the concentration of 8-PN in beer was considered too low to affect human health. However, these results show that the activity of the intestinal microbial community could more than 10-fold increase the exposure concentration. Because prenylflavonoids are present in many beers with IX being the major constituent, the results raise the question whether moderate beer consumption might contribute to increased in vivo levels of 8-PN and even influence human health.

KEYWORDS: Hop; isoxanthohumol; 8-prenylnaringenin; prenylflavonoids; intestine; bacteria; Eubacterium limosum; beer; phytoestrogens; O-demethylation

INTRODUCTION

Hops (Humulus lupulus L.) have been used for centuries as an essential raw material in beer brewing providing bitterness and flavor to beer. In the past few years, the plant has gained increasing attention as a source of prenylflavonoids, a subclass of polyphenols. These are present in the lupulin glands, found in the female hop cones. In this group, two prenylchalcones, xanthohumol (X) and desmethylxanthohumol (DMX), and three prenylflavanones, isoxanthohumol (IX), 8-prenylnaringenin (8-PN), and 6-prenylnaringenin (6-PN) (Figure 1), now receive much attention because of their possible health-promoting properties. X has been identified as a strong cancer chemopreventive agent (1), while 8-PN has been shown to be one of the most potent phytoestrogens identified so far, with a considerably higher activity than the well-known soy phytoestrogens (2, 3). These interesting properties have led to intense research aiming at deciphering various bioactivities. 8-PN showed in vivo estrogenic activity (4, 5), prevented bone loss in rats (6), inhibited angiogenesis (7) and metastasis (8), and exhibited antiandrogenic activity (9).

There is considerable interest in whether human exposure to phytoestrogens has either health risks or benefits (10−12). In the case of hop prenylflavonoids, beer is the main dietary source, with an estimated daily intake of about 0.14 mg prenylflavonoids (13). However, the concentrations detected in beer (and, therefore, the average intake) significantly depend on the brewing process, as strong ales contain up to 4 mg/L. Although X is present as a predominant prenylchalcone [up to 1% (m/m)] (14), most of it is transformed into IX by thermal isomerization during wort boiling. As a result, IX is the major prenylflavonoid found in beer in concentrations from 500 µg/L (lager/pilsner) up to 4 mg/L (strong ale) (13, 15). DMX is
equally converted into 8-PN giving final concentrations in beer up to 100 µg 8-PN/L and IX vs 8-PN ratios between 20 and 40. However, despite the high activity of 8-PN, the total estrogenicity of beers is still 500–1000-fold lower than the concentration needed for harmful in vivo activity (~100 mg/L) in rat experiments (4). Moreover, many beers are now made using hop extracts instead of whole hops, giving lower concentrations of 8-PN or even no 8-PN. Therefore, it is generally agreed that with the current knowledge, no detrimental health effects due to estrogens in beer are to be expected through moderate beer consumption (2, 4, 16). Besides beer, a number of hop-based dietary supplements are marketed, but contents may vary (17).

To exert in vivo effects claimed in vitro, dietary flavonoids need to be absorbed from the gut and reach their targets unchanged. However, up to now, no peer-reviewed data with regard to the bioavailability of prenylflavonoids are available. In general, monomeric flavonoids reach the small intestine unchanged (18), where absorption from the gut in the mesenteric circulation can take place. In vitro studies indicated extensive liver biotransformation of X (19, 20), IX (21), and 8-PN (22) upon absorption. However, the extent of dietary polyphenol absorption in the small intestine is rather limited (10–20%) (23, 24), thereby implying that a large proportion reaches the colon. Naringenin, the nonprenylated analogue of 8-PN, showed intensive microbial biotransformation in the intestine, including ring cleavage and dehydroxylation (25), followed by absorption and urinary excretion (26). The extent of degradation strongly depended on compound concentration and individual composition of the gut microbiota of the different human subjects. On the other hand, when X was fed to rats, it was mainly recovered in unchanged form from the feces (89%) (27).

As a source of 8-PN, IX, and X, moderate beer consumption leads to the intake of bioactive compounds, which could either act beneficially or be harmful depending on the final concentration reaching the target organs in the human body. In many cases, the bioavailability of phytoestrogens is strongly influenced by the composition and activity of the gut microbial community (28–30). As the microbiota can activate as well as degrade these components, the focus of the present study is to investigate the role of the microbial community toward the in vivo estrogenic properties of beer or food supplements containing hops by investigating the biotransformation or the breakdown of X, IX, and 8-PN in fecal cultures and to evaluate interindividual differences. Understanding these processes is a crucial step toward the estimation of the final bioactivity of dietary flavonoids derived from hops and the evaluation of possible effects associated with such intake.

**MATERIALS AND METHODS**

**Chemicals.** X was isolated from spent hops (i.e., the residue that remains after liquid or supercritical carbon dioxide extraction of hop cones, NateCO₂, Wolnzach, Germany) via methanol/ethyl acetate (9/1, v/v) extraction, flash chromatography fractionation, and purification by semi-preparative chromatography on a Varian Omnisphere C-18 column (250 mm × 21.4 mm, 10 µm, Varian, St.-Katelijne-Waver, Belgium) using a Gilson 322 pump (Gilson, Middleton, United States) with a Gilson UV–vis 156 detector and a Gilson 206 Fraction Collector. IX was prepared from X by isomerization under reflux in a 5% ethanolic KOH solution. IX was purified from the reaction mixture by semi-preparative high-performance liquid chromatography (HPLC). 8-PN was prepared by prenylation of naringenin with 2-methylbut-3-en-2-ol in dioxane in the presence of boron trifluoride (31). All chemicals were obtained from Sigma-Aldrich (Bormen, Belgium). Using semi-preparative HPLC, 8-PN was purified from a fraction resulting from flash chromatography containing both 8-PN and 6-PN and a B ring prenylated naringenin. The identities of IX, IX, and 8-PN were confirmed by comparison of 1H NMR and 13C NMR data (Varian 300 MHz) with literature values (32), and the purities were assessed to be more than 99% by HPLC.

**Fermentation Conditions. Bacterial Cultures.** Fecal samples were obtained from 12 healthy subjects between the ages of 20 and 35 and designated A–L. None of the subjects had a history of gastrointestinal disease and had not taken antibiotics during 3 months prior to sample delivery. Fecal slurries of 20% (w/v) fresh fecal samples were prepared by homogenizing the feces with phosphate-buffered saline (0.1 M, pH 7) containing 1 g/L sodium thioglycolate as a reducing agent. The particulate material was removed by centrifugation (1 min, 500g). *Eubacterium limosum* DSM 20543 was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany).

**Culture Medium.** All fermentation experiments were performed in brain heart infusion broth (37 g/L, Oxoid, Hampshire, United Kingdom) with 0.5 g/L L-cystein HCl. Fecal bacteria as well as *E. limosum* require

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**Figure 1.** Structures of hop prenylflavonoids. By thermal isomerization, the chalcones X and DMX are converted into the respective flavanones IX (from X) and 8-PN and 6-PN (from DMX).
Conversion of Isoxanthohumol into 8-Prenylarangerin


Table 1. Microbial Transformation of X, IX, and 8-PN after Incubation with Fecal Samples A–D

<table>
<thead>
<tr>
<th></th>
<th>X (% recovery)</th>
<th>IX (% recovery)</th>
<th>8-PN (% recovery)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>start</td>
<td>Start</td>
<td>Start</td>
</tr>
<tr>
<td>A</td>
<td>74.9 (10.7)</td>
<td>5.9 (0.8)</td>
<td>ND§</td>
</tr>
<tr>
<td>B</td>
<td>80.5 (2.5)</td>
<td>9.1 (2.4)</td>
<td>ND§</td>
</tr>
<tr>
<td>C</td>
<td>73.6 (3.1)</td>
<td>2.2 (0.1)</td>
<td>5.3 (0.2)</td>
</tr>
<tr>
<td>D</td>
<td>65.4 (4.2)</td>
<td>11.7 (1.6)</td>
<td>ND§</td>
</tr>
<tr>
<td></td>
<td>X</td>
<td>IX</td>
<td>8-PN</td>
</tr>
<tr>
<td>A</td>
<td>90.1 (6.3)</td>
<td>ND§</td>
<td>ND§</td>
</tr>
<tr>
<td>B</td>
<td>83.0 (5.1)</td>
<td>ND§</td>
<td>ND§</td>
</tr>
<tr>
<td>C</td>
<td>19.0 (2.9)</td>
<td>36.4 (7.4)</td>
<td>ND§</td>
</tr>
<tr>
<td>D</td>
<td>86.0 (4.5)</td>
<td>ND§</td>
<td>ND§</td>
</tr>
<tr>
<td></td>
<td>X</td>
<td>IX</td>
<td>8-PN</td>
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<tr>
<td>D</td>
<td>ND§</td>
<td>ND§</td>
<td>7.9 (1.3)</td>
</tr>
</tbody>
</table>

§ The compounds were incubated for 8 days at a concentration of 25 mg/L, and results are presented as average (± SD) molar percentage recovery of X, IX, or 8-PN relative to the dosed amount of flavonoid. ND, not detected.

RESULTS

Biotransformation of Hop Prenylflavonoids. Incubation of X, IX, and 8-PN with Human Fecal Samples. The capacity of the cultures obtained from fecal samples A, B, C, and D (further referred to as cultures A–D) to degrade or transform the hop prenylflavonoids X, IX, and 8-PN was tested by incubating the cultures with 25 mg/L of each individual compound for a period of 8 days. Results are presented as molar % recovery of X, IX, or 8-PN, relative to the dosed amount (Table 1). X proved to be relatively stable with a recovery of above 65%. In all cultures incubated with X, IX was detected (few %). However, as this was also observed after incubation of autoclaved fecal material with X (data not shown), no bacterial mechanisms are involved in the isomerization of X into IX. In culture C, also 5% of 8-PN was recovered. In cultures A, B, and D, IX was very resistant to transformation as more than 80% was recovered. In culture C, however, the bacterial community was able to transform IX into 8-PN, as IX strongly disappeared and about 35% of 8-PN was recovered. This transformation did not occur when testing autoclaved fecal material. Remarkably, 90% of the initial amount of 8-PN was degraded in cultures A and D, while more than 50% of 8-PN was still recovered in cultures B and C. These results indicate that different fecal cultures are characterized by varying capacities to transform the hop prenylflavonoids.

Microbial Conversion of IX into 8-PN by Fecal Cultures. The most interesting data obtained from Table 1 are the results from culture C. A considerable amount of IX was transformed into 8-PN, showing the role of IX as a pro-estrogen, which can
be activated by the intestinal microbial community. To further
investigate these transformations, cultures E–L were incubated
with 25 mg/L IX and samples were taken after 72 h (Figure 2).
In cultures E, J, and K, varying amounts of 8-PN were
recovered, and especially in cultures J and K, a lower recovery
of IX was found in comparison to the non-8-PN-producing
cultures. These results stress the significance of the difference
in microbial community composition and activity, since conver-
sion of IX into 8-PN was evident in only four of 12 cultures.

In Vitro Estrogenic Response of the Incubations. As 8-PN is
known to have strong estrogenic activity, the conversion of IX
into 8-PN and the degradation of 8-PN were monitored by
measuring the estrogenic response of the incubations of cultures
A–D with IX and 8-PN at time zero and after 8 days (Figure
3). As no increase of the estrogenic response was detected for
the cultures A, B, and D upon incubation with IX, data are not
shown. In contrast, the high estrogenic activity was determined
culture C, strongly indicating transformation of IX into an
estrogenically active reaction product. The estrogenic response
of all cultures incubated with 8-PN decreased after the incuba-
tion because the dose–response curve shifted to higher con-
centrations. This suggests the degradation of 8-PN into non-
estrogenic derivatives. The strongest decrease was seen in
culture A as the EC50 value rose from 120 to 800 nM. The
lowest decrease was seen for culture B, while for cultures C
d and D a shift of 0.5 log units of the EC50 values was detected.
As HPLC analysis showed that more than 90% of the initially
dosed 8-PN disappeared in culture D, a more pronounced shift
in estrogenic activity was expected for this sample. Therefore,
the higher remaining activity could be due to other degradation
products with estrogenic activity. This will be investigated in
further research. In this experiment, 8-PN was 25-fold less active
than E2 based on their EC50 values.

Incubation of X, IX, and 8-PN with E. limosum. As E.
limosum is an intestinal bacterium capable of O-demethylation
and degradation of flavonoids, this species was tested for its
capacity to transform or degrade X, IX, or 8-PN. To investigate
long-term stability, the bacterium was incubated with 25 mg/L
of each compound for a period of 13 days. Results are presented as molar % recovery of X, IX, or 8-PN, relative to the dosed amount of individual compound (Table 2). Again, conversion of X into IX was a spontaneous process, as similar results were obtained with autoclaved bacterial cultures. E. limosum was able to transform IX into 8-PN, but because incubated 8-PN could almost fully be recovered after 13 days, further degradation did not occur. The small proportion of 6-PN recovered is probably due to isomerization of 8-PN via DMX as an intermediate.

Production of 8-PN by E. limosum. Transformation Kinetics. To investigate the transformation kinetics of the conversion of IX into 8-PN, the same E. limosum was incubated with IX in three parallel experiments and the concentrations of IX and 8-PN were monitored for a period of 13 days (Figure 4). Although incubation conditions were nearly identical, the 8-PN production already started after 3 days in incubation 3, while a very long lag phase (10 days) was noted in incubations 1 and 2. Moreover, the transformation rate was higher in incubation 1 than in incubation 2. This shows that transformation kinetics differed depending on the grown E. limosum culture.

Strain Selection. Because of the differences in transformation rate, it was attempted to select the most efficient IX-converting strain performing three successive selection rounds. This consisted of six parallel incubations of E. limosum with 25 mg/L IX and incubation for 8 days. Next, the culture that produced the highest amount of 8-PN was selected and used as inoculum for the next round of six parallel incubations (Table 3). While in the first selection round, the lowest production was only 2%, an increase of up to 82% was apparent after three selection steps, and the most efficient culture transformed all of the dosed IX into 8-PN. The mean production of all six incubations in each round increased from 22.5 up to 90.5%, and the standard deviation (SD) decreased from 20 to 7% after the selection procedure. This means that, after only three rounds, a strain was selected, which converted almost all IX (high mean) and was also stable (low SD).

E. limosum Supplementation to Fecal Cultures. From Table 1 and Figure 2, it was concluded that interindividual differences ultimately determined the capacity to transform IX into 8-PN. Therefore, we supplemented the most efficient E. limosum strain from the selection experiments to the nonproducing culture B to examine the capacity of this strain to start up production of 8-PN in the complex environment of an intestinal suspension. The strain was added to the culture in proportions ranging from 0% (solely fecal sample) up to 100% (axenic E. limosum culture) and incubation with 90 μM IX (n = 3).

**DISCUSSION**

In this study, the conversion of hop prenylflavonoids by human intestinal microbiota was investigated. These plant-derived compounds are considered as one of the four principal groups of phytoestrogens in food and are mainly ingested through beer consumption. Because 8-PN concentrations in beer are generally very low, possible health effects due to exposure
to these substances are usually considered negligible. However, this view could change when considering that IX, which is quantitatively the most important prenyllflavonoid in beer, may act as a pro-estrogen in food due to microbial O-demethylation. This could lead to enhanced concentrations of 8-PN and to a possibly important exposure after beer consumption. However, there seem to be important interindividual differences, as only one-third of the tested human fecal samples showed IX activation. The intestinal bacterium *E. limosum* was able to perform this conversion, and after strain selection, the bacterium rapidly converted all IX into 8-PN. Strain supplementation to nonconverting fecal samples led to rapid and efficient 8-PN production, even at low ratios.

The possibility that IX would act as a pro-estrogen was recently detected in vivo by Schaefer et al. (35). After dosing 10 mg of IX to two men, increased 8-PN concentrations, corresponding to up to 5% conversion, were detected in urinary samples. According to the authors, this in vivo activation would be an oxidative demethylation process in the liver, based on the findings of Nikolic et al. (21). Although the prenyl chain of IX was the primary target of liver modifications, the latter researchers also recovered small amounts of 8-PN after the incubation of IX with liver microsomes. This contrasts with the findings of Coldham et al. (36) who could not find IX activation by the liver. However, the liver is not the only transformation site inside the human body. The human colon contains ~10^{12} microorganisms/cm^3, with an enormous metabolic potential. Bacterial enzymes may catalyze many reactions including hydrolysis, dehydroxylation, demethylation, ring cleavage, and decarboxylation. The importance of this microbial community in the metabolism of phytoestrogens has been clearly established. Decroos et al. (37) recently isolated a microbial consortium capable of transforming the soy phytoestrogen daidzein into equol, and Wang et al. (38) found two bacteria responsible for the conversion of lignans. Moreover, several intestinal bacteria possess β-glucosidases, which are necessary for the hydrolysis of phytoestrogen glucosides (30). Therefore, the gut microbiota are now considered as an important factor influencing phytoestrogen bioavailability (29).

Our results confirm a similar activity related to the group of prenyllflavonoids, since IX can be converted into the potent phytoestrogen 8-PN. These transformation products can subsequently be absorbed from the colon, as it is established for soy isoflavones (39), to exert biological activity inside the body. While 8-PN was only one out of a large number of liver transformation products of IX (21), the human intestinal microbiota selectively converted IX into 8-PN with high conversion efficiencies (>35%). These results show that the impact of microbial activities on in vivo conversion of IX will most likely be more pronounced than possible liver transformations. However, as only one-third of the samples converted IX, interindividual variability in microbial community activity could strongly influence this in vivo conversion. Finally, Schaefer et al. (35) noted that urinary 8-PN excretion after beer consumption was slower than expected (up to several days), which could very well be due to delayed conversion of IX into 8-PN in the intestine, followed by absorption and renal excretion. Enter-hepatic circulation is another important factor determining the retention of IX and 8-PN.

Up to now, little is known about intestinal transformations of prenyllflavonoids. Nokandeh et al. (27) dosed 1000 mg X/kg body weight to rats and isolated 22 metabolites from the feces. The majority (89%) of the recovered flavonoids was unchanged X, which is in agreement with our data, as we detected only very minor degradation of X. The rest was composed of small amounts of different metabolites including some IX. Avula et al. (40) performed a similar experiment with rats and detected mainly unchanged X next to a number of unidentified metabolites. However, no published data were available with regard to the availability of IX and 8-PN to gut microbiota. In our experiments with fecal samples, IX was either partially transformed into 8-PN or remained very stable during the incubations. 8-PN, on the other hand, was more sensitive to degradation as, in some experiments, 90% of the dosed amount disappeared. One possible explanation for this difference in stability between IX and 8-PN is the protective effect of the methoxy group on the A ring of IX, as this is the only structural difference with 8-PN. In general, the first step in the degradation of flavonoids is the opening and scission of the heterocyclic ring (41) and the absence of a free hydroxyl in positions 5, 7, or 4' would protect the compound from this cleavage (42, 43). An important consequence regarding optimum bioavailability could be a slow release of 8-PN from IX in the colon and absorption through the gut epithelium, taking into account that 8-PN is prone to undergo decomposition.

To identify possible microbial degradation pathways of prenyllflavonoids, a comparison can be made with naringenin, lacking the prenyl side chain. This flavanone has been extensively studied and is mainly present in citrus fruits in a glycosidic form (44) and in tomatoes as aglycone (45). Degradation of naringenin by intestinal bacteria starts with heterocyclic ring cleavage, followed by dehydroxylation yielding phloroglucinol and 3-phenylpropionic acid (25). The extent of this degradation strongly depended on the nature of the fecal sample, which resembles the results obtained after the incubation of the fecal samples with 8-PN. This again shows that interindividual differences in the microbial community of the intestine are decisive on the fate of 8-PN and related flavonoids in the human body.

Microbial O-demethylation (8-PN from IX) is known for a number of anaerobic bacteria that can even use methyl ethers for respiratory growth. These bacteria either use fumarate (Desulfotobacterium) as an electron acceptor (46) or are so-called homoacetogens converting CO_{2} into acetate (47). *E. limosum* is a homoacetogenic bacterium, which is frequently isolated from human feces (48). It is known to be involved in the biotransformation of specific isoflavonoids (49), and a related *Eubacterium* strain was identified as a producer of the lignan enterodiol (38). Instead of trying to isolate a bacterium with the ability to demethylate IX into 8-PN, we investigated the activity of this *E. limosum*. The strain proved to be able to produce 8-PN and did not further degrade it, as this compound could be completely recovered following the incubation of *E. limosum* with 8-PN. After strain selection, the bacterium even quantitatively converted IX into 8-PN after only 1 day of incubation. Because IX can easily be produced from X, a microbial fermentation process of IX could facilitate efficient large scale production of 8-PN. On the other hand, strain supplementation to a nonconverting fecal sample led to rapid and high IX conversion. This could imply that cosupplementation of IX and a transforming bacterium leads to a “slow release” 8-PN production in situ in the intestine, after which the produced 8-PN can be absorbed and transported to its targets.

Several reports now correlate possible health benefits with intentional phytoestrogen uptake (10). Yet, at the same time, questions rise whether environmental exposure to phytoestrogens could impose health risks such as endocrine disruption. Moreover, overall health effects may result from a combination of
phytochemicals with multiple and perhaps additive or interfering activities. Up to now, only isoflavones and lignans are considered relevant phytoestrogens in the human diet (40), especially because 8-PN concentrations in beer are considered to be too low for positive or negative health effects (4,16). However, the intestinal microbial community may have a crucial role in the in vivo conversion of IX into 8-PN. Taking into account an accumulation factor of 2 based on slow elimination of 8-PN (35), a daily consumption of 1 L of beer containing 2 mg IX/L would lead to a daily 8-PN burden of 1.4 mg. As 8-PN is only about 10 times less active as compared to E2, the daily exposure of about 0.14 mg E2 equivalents is well within the range of positive biological activity (e.g., protective effect on bone metabolism) without negative side effects (e.g., proliferation of the endometrium). Although this example is very simplified and does not account for differences in bioavailability, it clearly indicates the intriguing role of the gut microbial community in the exposure to biologically active compounds. As microbial O-demethylation of IX could readily lead to a 10-fold increase of the ingested 8-PN, supposing an IX vs 8-PN ratio in beer of 30 (13), large population studies are warranted in order to acquire insight into the in vivo bioavailability of hop prenylflavonoids throughout the intestine and the extent of microbial transformations in the colon. Such studies are essential to validate our findings on the capacity of individuals to produce 8-PN from IX, giving rise to possible health effects of IX consumption.

ABBREVIATIONS

8-PN, 8-prenylnaringenin; IX, isoxanthohumol; X, xanthohumol; DMX, desmethylxanthohumol; HPLC, high-performance liquid chromatography; E2, 17β-estradiol; LC-MS/MS, liquid chromatography—mass spectrometry/mass spectrometry.

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