A biotransformation strategy to reduce allergens in propolis

Running title: reduction of allergens in propolis

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Abstract

Propolis (bee glue) is a resinous, sticky, dark-coloured material produced by honeybees. Propolis today, due to its medicinal properties, is increasingly popular and extensively used in food, beverages and cosmetic products. Besides the numerous positive properties, propolis may also have adverse effects such as, principally, allergic eczematous contact dermatitis in apiarists and in consumers with an allergic predisposition. In this study, we found appropriate conditions for removing from raw propolis the caffeate esters, which are the main allergenic components. The proposed method consists of the resuspension of propolis in a food-grade solvent followed by a biotransformation based on the cinnamoyl esterase activity of *Lactobacillus helveticus*. We showed that the reduction of caffeate esters operated by *L. helveticus* did not affect the content of flavonoids, which are the main bioactive molecules of propolis. Furthermore, we verified that the biotransformation of propolis did not cause a loss of antimicrobial activity. We finally demonstrated that the ability of *L. helveticus* to hydrolyze caffeate esters in propolis is strain specific. In conclusion, the proposed strategy is simple, food-grade and effective to selectively remove allergenic molecules without affecting the bioactive fraction of propolis. This is the first study demonstrating that the allergenic caffeate esters of propolis can be eliminated by means of a bacterial biotransformation procedure.

Key words: *Lactobacillus helveticus*, propolis, cinnamoyl esterase, caffeic acid esters.
Propolis (bee glue) is a resinous, sticky, dark-coloured material produced by honeybees (*Apis mellifera*), which collect exudates from tree, mix them with wax and use the resulting material to seal and protect honeycombs. The chemical composition of propolis varies and depends mainly on the plants accessible to bees and on the season. Propolis originated from plants of the genus *Populus* (for instance, poplar), which are typical of the temperate zones of Europe, China and North America, comprises resins (20-25 %), waxes (30-40 %), volatile oils (5-10 %) and many phenolic compounds (10-30 %), which include, in particular, flavonoids. In addition, the phenolic fraction of propolis contains aromatic acids, such as cinnamic, caffeic, ferulic, *p*-coumaric acid, and their esters.

The individuation of a potential medicinal value in propolis dates back to ancient Egyptians, Greeks and Romans, who used propolis to heal wounds, sores and ulcers. Registered as an official drug in the London pharmacopoeias of the seventeenth century, propolis is today, due to its medicinal properties, increasingly popular and extensively used in food, beverages and cosmetic products. Indeed, the ethanolic extract of propolis has reportedly a wide variety of biological actions, including antimicrobial, anti-herpes and antifungal, anti-inflammatory, antioxidant, immune-stimulating, cariostatic, anticancer and anti-*Helicobacter pylori* activities. The pharmacological properties of propolis have been correlated to the presence of specific molecules. For instance, its anti-bacterial and anti-viral activities have been linked with diterpenic acids and dihydrobenzofuranic lignans, its anti-inflammatory and antioxidant activities seem to be due to flavonoids, whereas its hepatoprotective and anti-cancer activities have been proposed for several propolis constituents, including caffeoylquinic acid derivatives and phenylethyl caffeate.
Besides the above numerous positive effects, propolis, though seemingly relatively safe (calculated safe dose of about 1.4 mg per kg of body weight per day in humans; 5), may have adverse effects such as xerostomia, gastric pain and allergic eczematous contact dermatitis in apiarists (9) and in consumers with an allergic predisposition (25). Less than 10% of population is sensitive to propolis (sensitization rate between 1.2 and 6%); nevertheless, a significant increasing trend in sensitization has been observed in adolescents in recent decades (from 2 to 13.7% in the period 1995-2002; 15), possibly as a consequence of a considerably augmented use of propolis in numerous commercial products. For the same reason, a strategy to eliminate allergens from propolis would be of wide interest. Nonetheless, to the best of our knowledge, propolis with a high content in flavonoids and a reduced content of allergens is not industrially available at the moment.

In this context, here we describe a food-grade strategy to selectively and effectively reduce the allergenic molecules in propolis. Allergens in propolis are mainly caffeic acid derivatives (14) and in this study we show how to treat propolis in order to eliminate these molecules by the cinnamoyl esterase activity of a dairy bacterium, namely *Lactobacillus helveticus*. 

Materials and methods

Bacterial strains and culture conditions

Lactobacillus helveticus strains were grown overnight anaerobically at 42 °C in MRS broth (Difco, Detroit, MI, USA).

Propolis biotransformation

Raw propolis samples (collected from the Puglia region, Italy) were chilled at -20°C (since it becomes hard and very brittle at lower temperatures) finely ground in a mill and passed through a 500-μm (35 mesh) sieve. At the same time, the bacterial cell concentration of an overnight culture of L. helveticus was determined microscopically with a Neubauer-improved counting chamber (Marienfeld GmbH, Lauda-Königshofen, Germany). After that, 1 g of propolis was placed in 50 ml tubes, in which we had previously pelleted different amounts of L. helveticus cells (10⁸-10¹⁴ bacterial cells). Finally, we added 2 ml of PBS (pH 7.3), eventually containing ethanol (from 5 to 70 %) or polyethylene glycol (PEG) 400 (from 1 to 80 %). After 24 hours of incubation at 37 °C under constant agitation by a magnetic stirrer, samples were collected and analyzed by liquid chromatography/mass spectrometry.

Bacterial cell concentrations were determined microscopically with the counting chamber also after the incubation.

Analytical methods for the study of the phenolic fraction of propolis

After the biotransformation, phenolic fractions were extracted from propolis samples and analyzed as described in (10). In brief, we added 70 ml of ethyl acetate to the sample (bacteria/propolis mixture) and incubated at room temperature under agitation for 10 min. The mixture was centrifuged at 1500 g for 5 min, and the supernatant transferred into a flask. The solid residue was extracted again with ethyl acetate as described above. The extracts were
dried under nitrogen and the pellets dissolved in 100 ml methanol. After a final centrifugation at 4000 g for 1 min, supernatants were diluted and analyzed by ultra-high performance liquid chromatography coupled with tandem mass spectrometry (UHPLC-MS/MS). UHPLC-MS/MS analyses were performed with an Acquity UHPLC system (Waters, Milford, MA, US) coupled to a Quattromicro triple quadrupole mass spectrometer (Waters). A 1.7 μm C18 BEH Shield column (150 × 2.1 mm, Waters) was used for the separation at a flow rate of 0.45 ml min⁻¹. The separation was carried out at 40°C with a linear gradient elution (eluent A, 0.05% HCOOH; eluent B, 0.05% HCOOH in acetonitrile). The gradient was as follows: 20 to 30% B in 4 min, 30 to 40% B in 5 min, 40 to 60% B in 3 min, 60 to 90% B in 1 min and then 90% B for 2 min. The capillary voltage was set to 2.7 kV, the cone voltage and the energy applied during fragmentation (Elab) was specific for each compound as previously reported (10). All mass data were obtained in the negative ion mode. The source temperature was 130°C, the desolvation temperature was 380°C and argon was used at 2.1 × 10⁻³ mbar to improve fragmentation in the collision cell. Masslinx 4.0 acquired data with the Quan-Optimize option for the fragmentation study. The fragmentation transitions for the multiple reaction monitoring (MRM) were as previously reported (10). Caffeic acid (CA), 3-methyl-2-buteryl-CA (3M2B), internal standard (dihydrocaffeic acid, DHCA) and caffeic acid phenyl ethyl ester (CAPE) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Methyl caffeate was from Chromadex (Milan, Italy), ethyl caffeate from Waterstone Tech. (Carmel, IN, USA) and ethyl ferulate from Santa Cruz Tech. (Santa Cruz, CA, USA). Methanol, acetonitrile, ethyl acetate, and formic acid were supplied by Merck (Darmstadt, Germany). 2-Methyl-3-butenyl- (2M3B), benzyl- (CABE) and 3-methyl-3-butenyl- (3M3B) caffeates were synthesized as described in (10). An unpaired Student’s t test was run for statistically significant differences.
Antimicrobial assay

Antimicrobial activity was determined by the agar dilution method on two different samples:

1. conventional propolis extract: 60% ethanol, 18.1 ± 1.0 mg ml\(^{-1}\) flavonoids, 7.1 ± 0.4 mg ml\(^{-1}\) phenolic acids (including 4.3 ± 0.2 mg ml\(^{-1}\) allergenic caffeate esters);

2. MIMLh5-treated propolis extract: 60% ethanol, 18.1 ± 1.0 mg ml\(^{-1}\) flavonoids, 5.2 ± 0.3 mg ml\(^{-1}\) phenolic acids (including 1.5 ± 0.2 mg ml\(^{-1}\) allergenic caffeate esters).

Furthermore, 60% ethanol solution in water was used as control. The assay was performed against five indicator microorganisms: *Staphylococcus aureus* (three strains), *Staphylococcus haemolyticus* (four), *Streptococcus pneumoniae* (six), *Candida albicans* (eight) and *Candida tropicalis* (six). Bacteria were cultivated in Mueller-Hinton medium (Difco), whereas fungi were grown on Sabouraud’s dextrose medium (Difco). In brief, the samples were serially twofold diluted in the range 0.07-9.0 mg ml\(^{-1}\) flavonoids (and equally for the ethanol control) and spiked on agar plates. Agar plates were previously inoculated with approximately 10\(^4\) CFU of the indicator strain, whose cells were collected at the mid-log phase of growth. The plates were read after 24 h of incubation at 37°C for bacteria and after 4 days at 35°C for fungi. The minimal inhibitory concentration of samples promoting growth inhibition of 50% of the strains under study were denoted MIC\(_{50}\).

Results and discussion

Few methods have been proposed to obtain propolis with a reduced proportion of allergenic substances. For instance, a recent patent reported a protocol consisting of chemical-physical purification steps to produce hypo-allergenic propolis extracts (20). However, these methods come with several drawbacks such as altered taste, reduced polyphenol content or high energy consumption during production steps. The methods also lack selectivity and they cannot
preserve the biological/pharmacological efficacy of the obtained extract. In this study, we aimed to identify a food-grade strategy to selectively and effectively decrease the allergenic molecules in propolis, preserving its bio-active components (mainly flavonoids).

Reduction of allergenic compounds in propolis

Allergenic components identified in propolis are mainly caffeic acid derivatives (14) (Fig. 1), among which 3-methyl-2-butenyl caffeate (3M2B) seems the most active (2). In specific, since the allergenic molecules of propolis contain characteristically a cinnamoyl ester bond (Fig. 1), we studied the possibility to employ a bacterial strain, namely *L. helveticus* MIMLh5, which we had recently selected over 100 lactic acid bacteria for its high cinnamoyl esterase activity (12).

To remove allergenic molecules and to make them accessible to *L. helveticus*, we first looked for a way to properly mix propolis with bacterial cells. Propolis is a very dense resinous material, sticky above room temperature and insoluble in water. Propolis is partially soluble in ethanol, the solvent commonly used in industry to prepare extracts. For this reason, we tested the stability of the cinnamoyl esterase activity of MIMLh5 at different ethanol percentages, by using 0.5 mg ml⁻¹ of chlorogenic acid. Chlorogenic acid was chosen as reference molecule in these preliminary experiments since it is a stable molecule, easy to detect chromatographically and already successfully employed to quantitatively study the cinnamoyl esterase activity of lactic acid bacteria (12). We found that above the concentration of 10 % (v/v) ethanol, the enzymatic ability of the strain MIMLh5 to hydrolyze chlorogenic acid was significantly reduced (data not shown 1, only for referees). We then dispelled raw propolis sample in a solution of PBS and 10 % (v/v) ethanol (e10PBS) in the presence of different amounts of MIMLh5 cells (10⁸-10¹⁴ bacterial cells per g of propolis). After 24 hours of incubation at 37 °C under constant agitation, we found that 10¹¹ bacterial cells reduced...
allergenic caffeic acid esters in one gram of raw propolis sample by 43%. With more bacterial cells, the reduction of allergenic caffeic acid esters increased proportionally, from 57% with $10^{12}$ cells, to 67 and 81% reduction when $10^{13}$ and $10^{14}$ cells respectively were employed (Fig. 2). Quantitative analysis revealed that all the main caffeate esters in propolis decreased during the biotransformation (Table 1), while the caffeic acid concentration increased, confirming that the reduction of caffeates resulted from the hydrolysis of the cinnamoyl ester bond (Table 1). By means of an HPLC-DAD method, we also observed that flavonoid fraction, considered the main bio-active components of propolis, was not affected (Table 1).

An improved propolis solubilization allowed a bigger reduction of caffeate esters

In the following part of our study, we assessed the possibility to employ a different solvent to disperse raw propolis sample, in order to improve biotransformation rate. To this aim, we considered polyethylene glycol (PEG) 400, for two main reasons: 1) PEG 400 is a food-grade additive, recently approved by the European Food Safety Authority (EFSA; 7); 2) PEG 400 reportedly solubilizes hydrophobic molecules preserving the stability of enzymes (23). In accordance to the latter statement, the stability of cinnamoyl esterase activity of *L. helveticus* MIMLh5 was unaffected up to 80% (w/v) PEG 400 in PBS (data not shown 2, only for referees). Next, we performed biotransformation experiments with 50% PEG 400 instead of ethanol and we observed a significant improvement of the reduction of caffeic acid esters. Precisely, in our experimental conditions (*i.e.* one g of propolis in 2 ml of 50% PEG 400), $10^{11}$ bacterial cells reduced the allergenic molecules by 61% (Fig. 2, Table 1). This result corresponds to a 42% increase of caffeate esters reduction as compared to the same experiment carried out in 10% ethanol. This amelioration of the biotransformation rate was also maintained at higher amounts of cells; in fact, when $10^{14}$ bacterial cells were used, about
98 % of caffeic acid esters were removed in one gram of propolis (Fig. 2). We also tested a propolis sample of different origin, specifically from China. The reduction of allergenic caffeates in Chinese propolis occurred only when a greater concentration of PEG 400 was used (80 %). Plausibly, this depends on wax concentration, which was 60 % higher in Chinese than in Italian propolis (about 40 and 25 g of wax respectively, per 100 g of raw propolis; data not shown). Therefore, optimal experimental conditions should be set for any specific propolis sample, since its composition can affect dispersion efficacy.

Different L. helveticus strains display dissimilar ability to reduce allergens in propolis

We also tested if diverse strains of L. helveticus can display a different performance during propolis biotransformation. We dispelled 1 g of propolis in 2 ml of e10PBS in the presence of four different L. helveticus strains, namely MIMLh5, SLh02, SLh13 and SLh37. L. helveticus SLh02, SLh13 and SLh37 have been included in the study because they are commercial strains with known growth performances at industrial level. Particularly, strains SLh13 and SLh37 are commercialized as dairy starters whereas SLh02 as probiotic. We used 2 × 10¹⁰ bacterial cells because this amount approximately corresponded to a 50 % activity and, therefore, it was suitable to detect possible differences in the biotransformation rate as a function of the bacterium employed. The results showed a marked difference among strains. Particularly, two of them, SLh13 and SLh37, displayed a faint activity on propolis, whereas SLh02 and MIMLh5 reduced caffeate esters markedly (Fig. 3). In a previous report, the cinnamoyl esterase activity varied markedly among L. helveticus strains and MIMLh5 displayed the highest activity (12). Furthermore, we observed that the microscopically-determined bacterial cell concentrations were the same before and after the incubation with propolis. Differences in the biotransformation rate among L. helveticus strains, therefore,
The antimicrobial activity of MIMLh5-treated (low-allergen) propolis was not significantly dissimilar from that of conventional propolis.

In order to confirm that propolis conserved its beneficial features after treatment with *L. helveticus* MIMLh5, we assessed its best known and most widely researched property, i.e. the ability to inhibit pathogenic microorganisms. In this experiment, we compared a MIMLh5-treated (low-allergen) propolis alcoholic extract with an identical preparation, which was obtained from non-treated (conventional) propolis. The antimicrobial activity was tested against three bacterial (13 strains) and two fungal (14 strains) species, which were selected because they are ubiquitous microorganisms, often involved in opportunistic infections. The minimal inhibitory concentration (MIC)\(_{50}\) of the low-allergen propolis did not significantly differed from that of conventional propolis for any of the indicator microorganisms (Table 2). We only observed a reduced MIC\(_{50}\) (i.e. a stronger antimicrobial ability) for low-allergen propolis against *Staphylococcus aureus*. Nonetheless, this difference was not statistically significant (\(p = 0.08\); Table 2). The reduction of caffeate esters operated by the activity of strain *L. helveticus* MIMLh5, therefore, did not result in a loss of antimicrobial activity of propolis. This result is in agreement with the flavonoids content, which did not significantly change during the biotransformation.

**Conclusions**

We proposed a novel biotransformation strategy to significantly reduce allergenic molecules in propolis without affecting its bioactive fraction. This method is based on a food-grade solvent (ethanol or PEG 400) and the cinnamoyl esterase activity of *L. helveticus*, a...
dairy bacterium generally recognized as safe, included in the EFSA QPS list of microorganisms (6) and demonstrated to have probiotic properties (13).

A clinical study will be carried out in the near future, in order to confirm that the reduction of caffeate esters by means of the proposed biotransformation can actually result in attenuated allergic symptoms in propolis-sensitive people. At the moment, this strategy (recently patented; 18) is under industrial development. First promising scale-up experiments suggest that hypoallergenic high quality propolis extracts, prepared according to this strategy, will be soon industrially available.

Acknowledgments

The authors are grateful to Specchiasol S.r.l. (Bussolengo, Italy) for providing the raw propolis samples and to Sacco S.r. L. (Cadorgo, Italy) for providing strains SLh02, SLh13 and SLh37.

References


Legends

Fig. 1. Structural formula of the molecules constituting the allergenic fraction of propolis. Arrow indicates the chemical bond hydrolyzed by cinnamoyl esterase activity.

Fig. 2. Degradation of caffeic acid esters in raw propolis sample by increasing concentration of *Lactobacillus helveticus* MIMLh5 cells. Propolis has been dispersed in PBS buffer + 10 % ethanol or 50 % PEG 400. Data are reported as mean (three experiments) of the relative amount of all caffeates shown in Fig. 1 ± standard deviation (vertical bars).

Fig. 3. Relative amounts of allergenic esters of caffeic acid in propolis treated with *Lactobacillus helveticus* strains. Names of the molecules are in accordance with Fig. 1. The amount of each molecule in control sample (propolis incubated without bacteria) has been set to 100.
Table 1. Concentration of allergenic caffeic acid esters and flavonoids in the Italian propolis employed in our study. Data are shown as mg of molecule per g of propolis and concern experiments carried out by using $2 \times 10^9$ cells of MIMLh5 to treat one g of propolis dispersed in 10% ethanol or 50% PEG400. The data represent the mean of at least three independent experiments ± standard deviation. With the exception of total flavonoids and flavonoid esters, all molecules’ concentration was significantly modified by the biotransformation ($P < 0.001$, according to unpaired Student’s $t$ test). *, statistically significant differences between PEG- and ethanol-treated propolis ($P < 0.05$).

<table>
<thead>
<tr>
<th>Molecule</th>
<th>Control (mg g$^{-1}$)</th>
<th>Biotransformation with $2 \times 10^9$ cells of MIMLh5 g$^{-1}$ (mg g$^{-1}$)</th>
<th>10% ethanol</th>
<th>50% PEG400</th>
</tr>
</thead>
<tbody>
<tr>
<td>3M3</td>
<td>2.66 ± 0.11</td>
<td>1.69 ± 0.10</td>
<td>1.53 ± 0.10</td>
<td></td>
</tr>
<tr>
<td>3M2</td>
<td>4.33 ± 0.19</td>
<td>2.53 ± 0.14</td>
<td>2.41 ± 0.14</td>
<td></td>
</tr>
<tr>
<td>2M2</td>
<td>2.43 ± 0.13</td>
<td>1.52 ± 0.10</td>
<td>1.35 ± 0.09</td>
<td></td>
</tr>
<tr>
<td>CABE</td>
<td>6.76 ± 0.41</td>
<td>4.83 ± 0.30</td>
<td>4.36 ± 0.30</td>
<td></td>
</tr>
<tr>
<td>CAPE</td>
<td>34.62 ± 1.42</td>
<td>26.01 ± 1.12</td>
<td>21.84 ± 1.10*</td>
<td></td>
</tr>
<tr>
<td>Total amount</td>
<td>50.80 ± 2.59</td>
<td>36.58 ± 1.91</td>
<td>31.50 ± 1.73*</td>
<td></td>
</tr>
<tr>
<td>Total flavonoids</td>
<td>208.00 ± 11.90</td>
<td>204.00 ± 12.64</td>
<td>212.00 ± 13.81</td>
<td></td>
</tr>
<tr>
<td>Flavonoid esters</td>
<td>46.00 ± 2.44</td>
<td>45.08 ± 2.44</td>
<td>46.80 ± 2.55</td>
<td></td>
</tr>
<tr>
<td>Caffeic acid</td>
<td>3.80 ± 0.17</td>
<td>4.40 ± 0.19</td>
<td>4.51 ± 0.22</td>
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</tr>
</tbody>
</table>
Table 2. Minimal inhibitory concentration (MIC) of propolis preparations. The means (± standard deviation) of three independent experiments are shown. Ethanol solutions, tested as control, did not affect microbial growth. The p-values refer to the statistic difference between the two propolis samples according to the unpaired Student’s t test.

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>mg ml⁻¹ flavonoids</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Conventional propolis</td>
<td>MIMIL₅-treated propolis</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>1.50 ± 0.52</td>
<td>1.04 ± 0.28</td>
</tr>
<tr>
<td><em>Staphylococcus haemolyticus</em></td>
<td>1.00 ± 0.35</td>
<td>0.87 ± 0.35</td>
</tr>
<tr>
<td><em>Streptococcus pneumoniae</em></td>
<td>0.26 ± 0.09</td>
<td>0.26 ± 0.07</td>
</tr>
<tr>
<td><em>Candida albicans</em></td>
<td>3.00 ± 1.04</td>
<td>2.55 ± 0.94</td>
</tr>
<tr>
<td><em>Candida tropicalis</em></td>
<td>2.55 ± 0.94</td>
<td>2.7 ± 0.78</td>
</tr>
</tbody>
</table>
Fig. 1

<table>
<thead>
<tr>
<th>R</th>
<th>Compound</th>
</tr>
</thead>
<tbody>
<tr>
<td>CH₃</td>
<td>3-methyl-2-butenyl caffeate (3M2B)</td>
</tr>
<tr>
<td>CH₃</td>
<td>3-methyl-3-butenyl caffeate (3M3B)</td>
</tr>
<tr>
<td>CH₂</td>
<td>2-methyl-2-butenyl caffeate (2M2B)</td>
</tr>
<tr>
<td>CH₂CH₃</td>
<td>phenethyl caffeate (CAPE)</td>
</tr>
<tr>
<td>CH₂</td>
<td>benzyl caffeate (CABE)</td>
</tr>
</tbody>
</table>

 caffeic acid (CA)
Fig. 2

Residual % of caffeates

\[ \log_{10} \text{of bacterial cells per gram of propolis} \]

- △ 10 % ethanol
- • 50 % PEG 400