Production and Characterization of Polyclonal Antibodies against the Bitter Sesquiterpene Lactones of Chicory (*Cichorium intybus* L.)

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Polyclonal antisera against lactucin and lactucopicrin, two bitter-tasting principles of chicory (*Cichorium intybus* L.), were obtained after immunization of rabbits with KLH−lactucin and KLH−lactucopicrin conjugates, respectively. In competitive ELISA, the sera from KLH−lactucopicrin-immunized rabbits showed a very low cross-reactivity with other sesquiterpene lactones containing a guaiane skeleton, 0.5% with lactucin, 1% with a mixture of lactucin and 11β,13-dihydrolactucin, and 5% with 8-deoxylactucin and 11β,13-dihydro-8-deoxylactucin. The sera from rabbits immunized with KLH−lactucin showed 50% cross-reaction with 8-deoxylactucin and its 11β,13-dihydro derivative and only 0.4% cross-reaction with lactucopicrin. In competitive ELISAs, chicory extracts spiked with free sesquiterpene lactones showed an expected increase in inhibition level. These ELISAs seem useful in the assessment of chicory bitterness.

Keywords: *Sesquiterpene lactones; chicory (Cichorium intybus); lactucin; lactucopicrin; bitter taste; polyclonal antibodies; ELISA*

INTRODUCTION

Chicory (*Cichorium intybus* L.) is an important agricultural crop in Northern Europe. One of the quality criteria is the bitter taste which is caused by sesquiterpene lactones. Several sesquiterpene lactones have been isolated from chicory roots and leaves. The most abundant ones, lactucin, 8-deoxylactucin, and lactucopicrin and their 11β,13-dihydro derivatives, are based on a guaiane skeleton, Figure 1 (Schenck and Graf, 1939; Pyrek, 1985; Rees and Harborne, 1985; Seto *et al*., 1988; Van Beek *et al*., 1990). The bitter thresholds of these sesquiterpene lactones have been determined by Van Beek *et al*. (1990). Lactucopicrin and its 11β,13-dihydro derivative are the most bitter-tasting compounds, their threshold concentrations in water being 0.5 and 0.2 ppm, respectively. The threshold concentration of both 8-deoxylactucin and its 11β,13-dihydro derivative is 1.1 ppm, and the concentrations are 1.7 and 1.4 ppm for lactucin and 11β,13-dihydrolactucin, respectively.

Some of the sesquiterpene lactones were found both in the free form and as their glucosides. Seto *et al*. (1988) identified cichorioside B (the glucoside of 11β,-13-dihydrolactucin) and crepidiaside B (the glucoside of 11β,13-dihydro-8-deoxylactucin) in chicory. The glucoside of 8-deoxylactucin, crepidiaside A, has not yet been identified in chicory but was found in endive (*Cichorium endivia* L.) (Seto *et al*., 1988). The glucoside of lactucin, picriside A, has been identified in *Picris hieracioides* L., another member of the Compositae family (Nishimura *et al*., 1986). No threshold concentrations with respect to bitterness have been published for these glucosides.

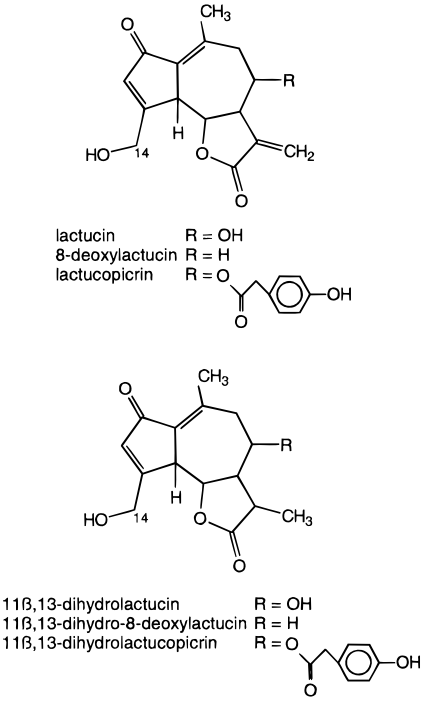

Figure 1. Structures of several guaiane sesquiterpene lactones present in chicory (*C. intybus* L.).

In addition to principles with a guaiane skeleton, sesquiterpene lactones with a germacrane or eudesmane skeleton have been identified in chicory roots (Seto *et al*., 1988). Up to now, no data have been published on the bitter taste of these types of sesquiterpene lactones.

Sesquiterpene lactones in chicory have been determined using a classical fluorometric method (Schmitt, 1940) and by high-performance liquid chromatography (HPLC) with UV detection (Leclercq, 1984; Dirinck *et al*., 1985; Price *et al*., 1990). The HPLC method was used in studies in which the levels of various guaiane

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sesquiterpene lactones in chicory have been correlated with the bitterness perceived by a sensory panel (Dirinck *et al.*, 1985; Price *et al.*, 1990, Mazijk-Bokslag *et al.*, 1991). Depending on the extraction procedure used, the highest correlation coefficients were found for lactucin glucoside ($r = 0.8$) (Price *et al.*, 1990) or for the sum of lactucin and lactucin glucoside ($r = 0.83$) (Mazijk-Bokslag *et al.*, 1991). This suggests that the total amount of lactucin, i.e. lactucin, and its converted precursor(s), such as lactucin glucoside, gives the best correlation with sensory bitterness.

As the bitter taste is an important quality criterion for breeders, producers, and at auctions, a simple, rapid assay is needed for the screening of sesquiterpene lactone levels. The analysis of both the free and glucosylated sesquiterpene lactones by HPLC is laborious because of the extraction procedures used. Immunochemical methods such as enzyme-linked immunoassay (ELISA) require less sample preparation and are suitable for screening large numbers of samples. Over 100 samples can be tested in duplicate in 1 day.

In this study we describe the production of polyclonal antibodies against two sesquiterpene lactones, lactucin and lactucopicrin. These lactones were chosen because they are important contributors to the total bitterness of chicory. The polyclonal sera were used in ELISAs for the analysis of various sesquiterpene lactones in chicory extracts.

MATERIALS AND METHODS

Materials. Bovine serum albumin (BSA) fraction V was obtained from Serva (Heidelberg, Germany). Freund's complete adjuvant containing *Mycobacterium tuberculosis* H37RA, Freund's incomplete adjuvant, and keyhole limpet hemocyanin (KLH) were from Sigma Chemical Co. (St. Louis, MO). ELISA microtiter plates were obtained from Costar (high binding, Type I; Cambridge, MA). Goat anti-rabbit IgG-alkaline phosphatase conjugate (IgG-AP) was purchased from ICN Biomedicals Inc. (Costa Mesa, CA). *p*-Nitrophenyl phosphate, silica gel 60 PF_{254} with $CaSO_4$, and cellulase Onozuka (isolated from *Trichoderma viride* L.) were from E. Merck (Darmstadt, Germany). All other chemicals and organic solvents used were reagent grade or of higher quality.

Apparatus. Centrifugal thin-layer chromatography: Chromatotron, Harrison Research, Palo Alto, CA. ELISA reader: Bio-Rad, model 3550-UV, Richmond, CA.

Isolation of Sesquiterpene Lactones. Lactucin and lactucopicrin were isolated from the milky juice of *Lactuca virosa* L. crops. To remove lipophilic material, the juice was extracted five times with a 3-fold volume of petroleum ether, separating the phases by centrifugation after each extraction. The aqueous phase was evaporated *in vacuo* at room temperature, and the residue was extracted four times with methanol (10 mL/g of residue). The methanolic extracts were concentrated by evaporation *in vacuo* at room temperature. The concentrated extract was separated into fractions using centrifugal thin-layer chromatography (TLC) (Hostettmann *et al.*, 1986). As an adsorbent 4 mm layers prepared from silica gel 60 PF_{254} containing $CaSO_4$ were used. The eluent was chloroform with increasing methanol content (0.5−4%). The separated fractions were screened using UV spectroscopy for the presence of sesquiterpene lactones (absorption maximum at 258 nm). These fractions were further analyzed by TLC to identify the fractions containing lactucopicrin and lactucin. The lactucopicrin in the corresponding fractions was of high purity according to TLC. The fractions containing lactucin were concentrated at 30 °C and further purified by gel chromatography using a LH 20 column (150 × 1.6 cm; Pharmacia, Uppsala, Sweden) and acetone as an eluent. The separated fractions were screened using UV spectroscopy (absorption maximum at 258 nm) and TLC to identify the fractions containing lactucin. Physicochemical data of the isolated compounds (UV, MS, NMR, TLC, HPLC) were in accordance with published data (data not shown) (Barton *et al.*, 1958; Pyrek, 1977).

Mixtures of lactucin and 11β,13-dihydrolactucin (1:2) and of 8-deoxylactucin and 11β,13-dihydro-8-deoxylactucin (1:1) were a gift from Dr. J. M. Hay of AgResearch (Palmerston North, New Zealand). Both mixtures were isolated from chicory roots.

Preparation of Conjugates. Both lactucin and lactucopicrin were conjugated to BSA and KLH. Lactucin (4 mg) or lactucopicrin (5 mg) in 200 μL of pyridine:acetonitrile (1:1, v/v) was activated by coupling of 50 μL of *p*-nitrophenyl chloroformate in acetonitrile (43 mg/mL) to the hydroxyl group on C_{14}. For the preparation of KLH conjugates, 50 μL of the activated hapten solution was allowed to react with 4.3 mg of KLH in 3 mL of distilled water at room temperature for 1 h. The reaction mixture was neutralized with 1.5 M HCl. For the preparation of BSA conjugates, 200 μL of the activated hapten solution was allowed to react with 40 mg of BSA in 3 mL of distilled water and neutralized with 1.5 M HCl after incubation at room temperature for 1 h. All conjugates were dialyzed against PBS (0.01 M phosphate buffer, pH 7.5, containing 0.85% NaCl).

Production of Polyclonal Antibodies in Rabbits. Two rabbits (no. 453 and 455) were immunized subcutaneously (sc) with *ca.* 500 μg of KLH−lactucin in 1.0 mL of PBS emulsified with 1.0 mL of Freund's complete adjuvant. To obtain lactucopicrin specific sera, two other rabbits (no. 456 and 457) were injected with KLH−lactucopicrin in the same concentrations as for KLH−lactucin. Booster injections (sc) with *ca.* 250 μg of conjugate in 2.0 mL of PBS/Freund's incomplete adjuvant (1:1, v/v) were given at 4 week intervals. Blood samples were taken 1 week after each booster injection. Serum was obtained by centrifugation at 2000*g* for 10 min. Antibody titers were determined by ELISA as described below.

ELISA for the Determination of Antibody Titers. BSA−lactucin or BSA−lactucopicrin conjugate was used as the solid-phase immobilized antigen for sera from the rabbits immunized with KLH−lactucin or KLH−lactucopicrin, respectively. Each well of a 96-well ELISA microtiter plate was coated with 1 μg of BSA conjugate in 100 μL of PBS. The plate was incubated at 4 °C overnight. The wells were washed three times (350 μL/well) with PBS-Tween (0.01 M PBS, pH 7.5, containing 0.1% Tween-20), blocked with 200 μL of PBS-BSA (0.01 M PBS, pH 7.5, 1% BSA), and incubated at 4 °C overnight. The plates were stored at −20 °C. Prior to use, the plate was washed three times (350 μL/well) with PBS-Tween to remove excess of blocking agent. To each well was added 100 μL of a serial dilution of anti-lactucin or anti-lactucopicrin serum (in PBS). The plate was incubated at 37 °C for 1 h and washed three times (350 μL/well) with PBS-Tween. Subsequently, goat anti-rabbit IgG-AP in a 1:1000 dilution in PBS (100 μL/well) was added. After incubation at 37 °C for 1 h, the plate was washed as described above, and freshly prepared substrate buffer (10 mg of *p*-nitrophenyl phosphate in 10 mL of a 50 mM carbonate buffer, pH 9.6, with 0.5 mM $MgCl_2$) (100 μL/well) was added. After incubation at 37 °C for 45 min, the absorption at 405 nm was determined.

Competitive ELISA for Characterization of Serum Specificity. The optimum ratio of immobilized antigen and antibody was determined by ELISA. Wells were coated with various conjugate dilutions and titrated with serial dilutions of the antibodies (in PBS). A ratio resulting in an absorbance between 1.0 and 1.5, and with lowest conjugate and antibody concentration, was used for coating plates to perform competitive ELISAs. The plates were blocked and washed according to the protocol used for monitoring antibody titers (see above). Subsequently, 50 μL of any of the purified sesquiterpene lactones (in PBS) at different concentrations were added to the wells followed by 50 μL of antiserum at the appropriate dilution in PBS. The plates were incubated at 37 °C for 1 h and treated further as described in the ELISA protocol.

Application of ELISA to Chicory Head Extracts. Fresh chicory heads were ground in a household juice centrifuge. The collected juice was treated with cellulase Onozuka (1 mg/mL of juice) at 40 °C for 3 h (Seto *et al.*, 1988), filtered through a

Table 1. Cross-Reactivity of Polyclonal Sera Raised against KLH−Lactucin (No. 453 and 455) and KLH−Lactucopicrin (No. 456 and 457)[a]

	cross-reactivity (%)			
serum no.	lac	dhlac + lac	dh8d + 8d	lacp
453	100	100	10	<0.4
455	100	100	50	0.4
456	<0.5	1	5	100
457	ls	ls	100	100

[a] lac = lactucin; dhlac + lac, a mixture of 11β,13-dihydrolactucin and lactucin (2:1); dh8d + 8d, a mixture of 11β,13-dihydro-8-deoxylactucin and 8-deoxylactucin (1:1); lacp, lactucopicrin; ls, limited solubility of the sesquiterpene lactones, which made it impossible to calculate the cross-reactivity.

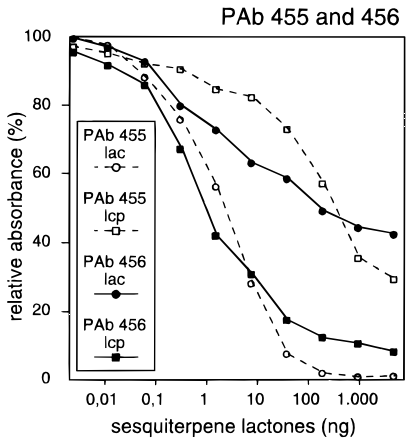

Figure 2. Inhibition curves of serum 455, raised against lactucin, and serum 456, raised against lactucopicrin, with serial dilutions of lactucin (lac) and lactucopicrin (lcp).

0.45 μm filter unit, and used in a competitive ELISA as described above. The purified sesquiterpene lactone dilutions in the first step were replaced by a serial dilution of chicory head extract.

RESULTS

Production and Characterization of Polyclonal Antibodies against Lactucin. Thirteen weeks after initial immunization, antibody titers were 2×10^6 (serum 453) and 8×10^6 (serum 455). The specificity of the polyclonal sera was determined by competitive ELISA. For serum 453, 20 ng of lactucin/well (400 ng/mL) gave a 50% inhibition of binding (IC_{50}) using plates coated with BSA−lactucin, at a concentration of 5 ng/well.

The cross-reactivities of serum 453 with a mixture of lactucin and dihydrolactucin (1:2), an equimolar mixture of 8-deoxylactucin and dihydro-8-deoxylactucin, and lactucopicrin were also determined by competitive ELISA (see Table 1). The cross-reactivity with the lactucin/dihydrolactucin mixture is about 100%. The cross-reactivity with the 8-deoxylactucin/dihydro-8-deoxylactucin mixture and with lactucopicrin was 10% and less than 0.4%, respectively.

For serum 455 the IC_{50} for lactucin is 2 ng/well (40 ng/mL). The serum showed a cross-reactivity of 100% with the mixture of lactucin and its dihydro derivative, 50% with 8-deoxylactucin and dihydro-8-deoxylactucin, and 0.4% with lactucopicrin (see Table 1). In Figure 2 the inhibition curves of serum 455 with lactucin and lactucopicrin are shown.

Production and Characterization of Polyclonal Antibodies against Lactucopicrin. Thirteen weeks after initial immunization, the antibody titers of rabbits immunized with KLH−lactucopicrin were 3×10^7 (serum 456) and 2×10^7 (serum 457). The specificity and cross-reactivity of both sera 456 and 457 were tested with competitive ELISA. The IC_{50} for lactucopicrin was 1 ng/well and 1 μg/well for sera 456 and 457, respectively. The cross-reactivity of serum 456 with lactucin is 0.5% and with the mixtures of lactucin/dihydrolactucin and 8-deoxylactucin/dihydro-8-deoxylactucin 1% and 5%, respectively (see Table 1). The inhibition curves of serum 456 with lactucin and lactucopicrin are shown in Figure 2. The cross-reactivity of serum 457 is 100% with the mixture of 8-deoxylactucin and its dihydro derivative. Since the solubility of sesquiterpene lactones is limited to about 5000 ng/well (100 μg/mL), the cross-reactivity to lactucin and the mixture of lactucin and its dihydro derivative could not be calculated.

Competitive ELISA with Chicory Extracts. Competitive ELISAs with chicory extracts and chicory extracts spiked with lactucin or lactucopicrin were performed with sera no. 455 and 456. The amounts of the various sesquiterpene lactones present in the extract were determined by HPLC (results not shown).

Serum 455 recognizes lactucin-like sesquiterpene lactones (lactucin, 8-deoxylactucin, and their dihydro derivatives, see Table 1), and, therefore, the sum of the amounts of lactucin-like sesquiterpene lactones in the extract was taken as a measure of serum 455 reactive principles and is represented at the *x* axis. A 100 ng lactucin equivalent inhibited serum 455 to the maximum inhibition level (see Figure 2). Addition of 100 ng of lactucin to every dilution of the extract resulted in an increase of the inhibition to the expected level in comparison with the extract inhibition. Addition of 100 ng of lactucopicrin showed only a slight increase of inhibition (Figure 3A). This increase is comparable with the inhibition showed by serum 455 toward 100 ng of lactucopicrin (see Figure 2).

Serum 456 is specific for lactucopicrin. The amount of lactucopicrin in the extract is taken as a measure for serum 456 reactive principles. Extract dilutions spiked with 100 ng of lactucopicrin showed an increased inhibition to the maximum inhibition level as expected from the inhibition curve of serum 456 with lactucopicrin (see Figures 3B and 2). Spiking with the same amount of lactucin showed only a minor increase of inhibition as compared to the original extract dilutions (Figure 3B).

DISCUSSION

In the present study polyclonal antibody sera with relative high specificity for lactucopicrin and for both lactucin and 8-deoxylactucin were obtained. To our knowledge it is the first time that antibodies have been developed against sesquiterpene lactones. Among sera of rabbits immunized with KLH−lactucin conjugate, polyclonal serum 455 recognizes the group of lactucin-like sesquiterpene lactones, *i.e.*, lactucin, 8-deoxylactucin, and their dihydro derivatives. Rabbit serum 456, obtained after immunization with KLH−lactucopicrin conjugate, showed a high specificity for lactucopicrin. Data obtained with competitive ELISAs showed that, in chicory extracts spiked with free sesquiterpene lactones, the expected increase in inhibition over the extract inhibition level was found (Figures 2 and 3). In addition, if we combine the various standard (Figure 2) and sample dilution (Figure 3, nonspiked extract curves) curves, it is clear that the slopes of these curves are very

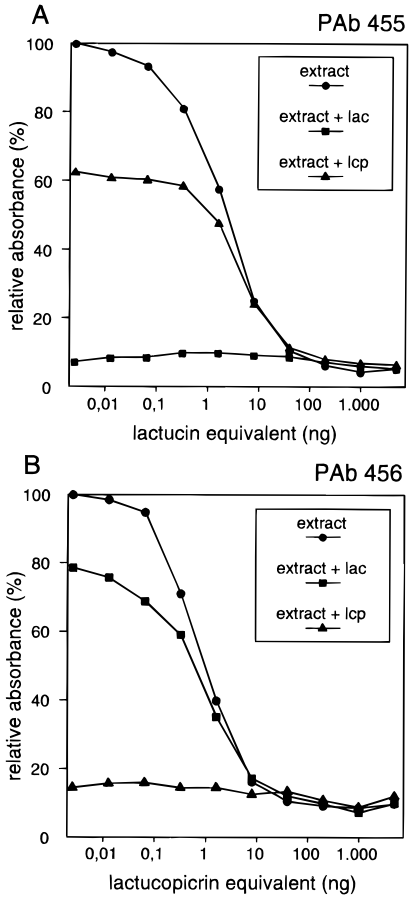

Figure 3. Competitive ELISA of chicory extract dilutions spiked with 100 ng of lactucin (lac) or 100 ng of lactucopicrin (lcp) using serum 455, raised against lactucin (A), and serum 456, raised against lactucopicrin (B).

similar for lactucin and lactucopicrin, respectively. These data suggest that other principles from the matrix of an extract do not interfere with the recognition of the sesquiterpene lactones by antibodies. In conclusion, with the screening of an extract with the various sera, an assessment of the amount of the different sesquiterpene lactones can be made.

The sensitivity of these ELISAs is comparable to the HPLC method described by Leclercq (1984). The detection limit for the HPLC method is 0.6 ng of lactucin and for the ELISAs about 0.1 ng/well (2 ng/mL).

The levels of various guaiane sesquiterpene lactones in chicory have been correlated with the bitterness perceived by a sensory panel (Dirinck *et al*., 1985; Price *et al*., 1990; Mazijk-Bokslag *et al*., 1991). After grinding chicory under liquid nitrogen, Dirinck *et al*. (1985) separated free sesquiterpene lactones from the more polar principles such as their supposed glucosides, by extraction of the ground chicory with diethyl ether preceding HPLC analysis. A correlation coefficient of 0.69 was found for lactucin concentrations and sensory bitterness scores. Price *et al*. (1990) separated free sesquiterpene lactones from the more polar principles by extraction with chloroform. After extraction the chloroform phase containing the free sesquiterpene lactones was analyzed with HPLC. The aqueous phase, containing the polar principles, presumably glucosides, was treated with cellulase preceding HPLC analysis. The highest correlation coefficient was found for lactucin present in the cellulase-treated fraction ($r = 0.8$), while a very low coefficient was found for free lactucin ($r = 0.28$). No correlation coefficient was given for the sum of lactucin and lactucin glucoside. Mazijk-Bokslag *et al*. (1991) used an extraction with acidified methanol (pH 3.5) in which glucosides are hydrolyzed. A correlation coefficient of 0.83 was found for lactucin. This indicates that the total amount of lactucin (lactucin and its converted precursor(s), such as lactucin glucoside) shows the best correlation with sensory bitterness. However, nothing is known about the bitter perception of the glucosides. Leclercq (1992) suggests that those compounds may also be bitter. It is not known whether glucosides are bitter themselves or that, during consumption, these glucosides are converted to their bitter-tasting aglucons by endogenous chicory enzymes.

The extraction procedure used is important for the assessment of the amount of sesquiterpene lactones in extracts. Based on our own HPLC data, fresh chicory juices do not contain measurable amounts of free sesquiterpene lactones (data not shown). However, a number of unidentified peaks with short retention times were observed. Incubation of chicory juices with cellulase increased the amount of free sesquiterpene lactones and decreased the amount of unidentified peaks. This suggests that these peaks correspond to sesquiterpene lactone glucosides which are converted to their free form by cellulase. In addition to this conversion of glucosides, the increase of free sesquiterpene lactones may be caused by the breakdown of cell walls upon cellulase incubation which would facilitate the extraction of the compounds (Leclercq and Netjes, 1985). Another explanation could be the release of endogenous enzymes converting glucosides and/or other sesquiterpene lactone precursors (Leclercq, 1992).

In a preliminary experiment we have shown that the inhibition values of cellulase-treated and nontreated extracts were comparable in competition ELISA (data not shown). This suggests that the specific antibodies are able to recognize both free sesquiterpene lactones and their more polar counterparts. In a following publication the use of these ELISAs to investigate the consequences of various extraction procedures on the levels of free and glucosidic sesquiterpene lactones will be described. Based on the data of sensory bitterness, these ELISAs, therefore, seem useful for the assessment of the bitterness of chicory.

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