Enzymatic Degradation of Cichoric Acid in *Echinacea purpurea* Preparations[⊥]

Birgit Nüsslein,[†] Michael Kurzmann,[‡] Rudolf Bauer,[§] and Wolfgang Kreis*[,†]

Lehrstuhl für Pharmazeutische Biologie der Friedrich-Alexander-Universität Erlangen-Nürnberg, Staudtstrasse 5, D-91058 Erlangen, Germany, Institut für Lebensmitteltechnologie, Lebensmittel pflanzlicher Herkunft, Universität Hohenheim, Garbenstrasse 25, D-70599 Stuttgart, Germany, and Institut für Pharmazeutische Biologie der Heinrich-Heine-Universität Düsseldorf, Universitätsstrasse 1, D-40225 Düsseldorf, Germany

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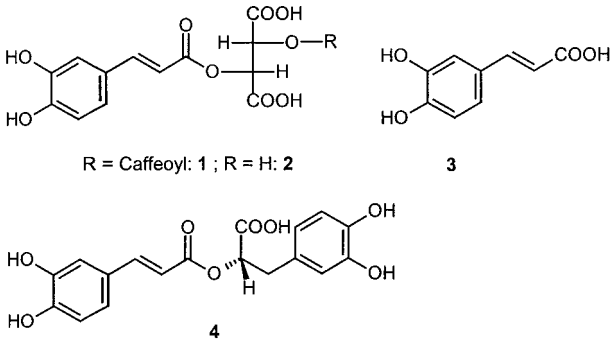
Cichoric acid (2*R*,3*R*-*O*-dicaffeoyltartaric acid) (**1**) is highly susceptible to enzymatic degradation during the preparation of *Echinacea purpurea* products. Degradation of **1** and other caffeic acid derivatives can be inhibited by antioxidants added to the extraction solvent or in buffered protein extracts saturated with nitrogen. Inhibitor studies conducted with protein extracts prepared from dried overground parts of *E. purpurea* revealed that polyphenol oxidases (PPO) but not peroxidases are responsible for the oxidative degradation of exogenous and endogenous caffeic acid derivatives. With a view to stabilizing aqueous extracts with respect to their content of **1**, the effects of ascorbic acid and ethanol were tested. Compound **1** was not stable under conditions where oxidative processes could almost be excluded. It was found that an esterase hydrolyzing the ester bonds between tartaric acid and caffeic acid is still active under PPO inhibitory conditions. Finally, addition of 40% ethanol and 50 mM ascorbic acid to aqueous extracts of "Echinaceae purpureae herba" resulted in a constant amount of cichoric acid over four weeks.

Preparations from *Echinacea purpurea* (L.) Moench (Asteraceae) represent the most frequently used herbal immunostimulants in Germany and in the United States.[1,2] Many of these are based on the pressed juice of the fresh aerial parts, harvested in full blossom. Their therapeutic effects on the common cold have been demonstrated recently in a randomized double blind study.[3] The monograph "Echinaceae purpureae herba" of the German Kommission E[4] lists the following indications for the fresh plant juice and its galenical preparations: internal use as adjuvant therapy for relapsing infections of the respiratory tract and derivative urinary tract and external use for poorly healing superficial wounds. So far, the therapeutic effects cannot be attributed to any particular constituents. However, pharmacological effects related to immune functions have been demonstrated for both high- and low-molecular-weight constituents of *E. purpurea*. Cichoric acid, alkamides, polysaccharides, and glycoproteins are regarded as the most relevant constituents.[5,6] Cichoric acid (2*R*,3*R*-*O*-dicaffeoyltartaric acid) (**1**) has been found to stimulate phagocytosis of human polymorphonuclear granulocytes in vitro[7,8] and possesses a weak antiviral effect (against vesicular stomatitis virus in L-929 cells of mice).[9] It has also been ascribed as having an inhibitory effect on hyaluronidase as well as radical-scavenging properties.[10,11] Recently it was found that **1** selectively inhibits human immunodeficiency virus type 1 integrase.[12−15] However, its bioavailability after oral application still needs to be studied.

Cichoric acid (**1**) is one of the major compounds in the aerial parts of *E. purpurea*. The regular content in the aerial parts is ca. 1% (calculated from the dry matter). Besides **1**, several other caffeic acid derivatives such as caftaric acid (2-*O*-caffeoyltartaric acid; monocaffeoyltartaric acid) (**2**), 2-*O*-feruloyltartaric acid, and 2-*O*-caffeoyl-3-*O*-coumaroyltartaric acid have been found in *E. purpurea*.[7,16,17]



Various commercial preparations of *E. purpurea* pressed juice differ dramatically in their content[18,19] of **1**, which has been ascribed as the result of enzymatic degradation during processing of the fresh plant material.[7] The relevance of plant-specific enzymes in the quality control and efficacy of herbal drugs and preparations has not yet been well studied. However, it is known that plant enzymes can be active even after harvesting and after drying of the plant material. Some of these activities have been used in technical processes such as the fermentation of black tea or black pepper, the formation of secondary cardiac glycosides from *Digitalis* leaves, meat tenderizing by *Papaya* enzymes, and the liberation of aromatic compounds from precursors. As for herbal medicinal products, plant enzymes can alter the quality of the raw materials, e.g., by influencing the content of the active principles. Although such processes have often been observed, the related biochemical reactions have not been well characterized.

We here have investigated the causality of the degradation of **1** in *Echinacea* products and herein provide recommendations for the processing of stable *E. purpurea* preparations with a high content of **1**.

Results and Discussion

Endogenous cichoric acid (2*R*,3*R*-*O*-dicaffeoyltartaric acid) (**1**) was rapidly degraded when pulverized "Echinace-

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* To whom correspondence should be addressed. Tel.: +4991318528241. Fax: +4991318528243. E-mail: wkreis@biologie.uni-erlangen.de.
[†] Universität Erlangen-Nürnberg.
[‡] Universität Hohenheim.
[§] Universität Düsseldorf.

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ae purpureae herba" was soaked and stirred with sodium phosphate buffer. The time course could be followed easily by TLC analysis. Considerable amounts of **1** were still present after 30 min, whereas it was no longer detectable after 2 h of incubation. Another major compound, caftaric acid (2-*O*-caffeoyltartaric acid; monocaffeoyltartaric acid) (**2**), was degraded as well but more slowly than **1**. The content of caffeic acid (**3**), one possible degradation product, did not increase during incubation. Reducing agents, such as ascorbic acid or thiol reagents, added to the incubation buffer inhibited the degradation of both **1** and **2**. Similar effects were seen when N_2-saturated buffers without reducing agents were used and the soaked plant material was incubated in an N_2 atmosphere. As soon as O_2 was allowed to come in contact with the incubation mixture, degradation started and the supernatant turned brown (data not shown). Thus it seems as if **1** is highly susceptible to oxidation and undergoes more or less complete degradation during the preparation of fresh plant pressed juices. The degradation of **1** has also been observed during the preparation of hydroalcoholic tinctures from fresh *E. purpurea* plants.[7] Therefore, the content of **1** varies dramatically in different commerical *E. purpurea* products and even in different batches of a particular product.[18,19] Hydraulic pack presses and spindle presses are usually used for the preparation of pressed juices. Classically (cold) prepared pressed juices are devoid of **1**.

Protein extracts obtained from the herbal drug were capable of degrading **1**. For example, exogenous **1** was degraded by 82% after 10 min when protein extracts were incubated at 40 °C. It was destroyed at a much slower rate at low temperatures and was found to be stable in heat-inactivated protein extracts. It was thus concluded that enzymes are responsible for the obvious instability of **1** in aqueous extracts prepared from the drug. With a view to improving protein extraction, 0.1% Triton X-100 was added to the extraction buffer. However, the enzymatic degradation of **1** was no greater than in the controls without Triton X-100, although the amount of protein solubilized from the drug increased considerably (data not shown). Pure **1** was not available to us in large quantities. Therefore, we decided to use rosmarinic acid (**4**) as a substitute, because this compound shares some structural similarities with **1** and was available in high purity from commercial sources. As a matter of fact, **4** was degraded equally well by the protein extracts prepared from the herbal drug, and specific enzyme activities were calculated to be 0.61 and 0.55 nkat mg^{-1} protein for **4** and **1**, respectively.

The results presented so far show that the degradation of **1** during the preparation of extracts from "Echinaceae purpureae herba" is definitely an enzymatic process. Initially the major involvement of an esterase was postulated, which catalyzes the hydrolysis of **1** into **2** and **3**. This was assumed because of the enhanced degradation of **1** in the presence of plant matrix, since an esterase bound tightly to the plant cell wall has recently[20] been found. However, an increased amount of **3** has never been observed in preliminary investigations. Since degradation of **1** could be inhibited by the addition of antioxidants to the extraction solvent, or in buffers saturated with nitrogen, it has been concluded that it is primarily an oxidative process. Generally, peroxidases (PO) or polyphenol oxidases (PPO) are involved in oxidative processes seen after plant harvest. To distinguish between PO- and PPO-catalyzed reactions, catalase can be used. This particular enzyme normally destroys hydrogen peroxide necessary for oxidation by a PO. However, it was found that the oxidative

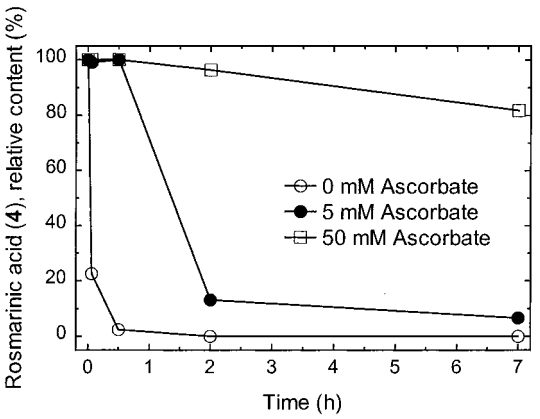

Figure 1. Influence of ascorbic acid on the degradation of **4** in protein extracts prepared from "Echinaceae purpureae herba".

degradation of the model compound rosmarinic acid (**4**) was not influenced by catalase. On the other hand, **4** was not oxidized when 0.1 mM tropolone, a potent PPO inhibitor,[25,26] was present in the incubation mixture. Therefore, it was concluded that a PPO is responsible for the oxidative degradation of caffeic acid derivatives in *E. purpurea*. PPOs are copper-containing enzymes, which are very abundant in the plant kingdom.[21] They oxidize polyphenols by consumption of molecular oxygen. The products can react with other polyphenols, with amino acids in proteins, or with other nucleophilic compounds to complex polymeric structures.[22] Therefore, the products of this reaction are difficult to capture. PPOs are usually membrane-bound enzymes, which, however, can alter their solubility during senescence. Drying the plant material can be interpreted as an aging process in this context. Indeed, we were able to extract an enzyme capable of degrading **1** and **4** from dried *E. purpurea* aerial parts. Using a spectrophotometric test[23,24] we could easily determine the PPO activity of particular extracts, and we found that it correlated very well with the degradation of **1**. Actually we found that PPO activity was very high in protein extracts obtained from "Echinaceae purpureae herba" when compared to protein extracts prepared from other herbal drugs or even potato tubers, which are known to be rich in PPO. The PPO from "Echinaceae purpureae herba" has been purified and further characterized recently.[27]

To inhibit degradation of **1** during the preparation and storage of *E. purpurea* preparations, we tested different possibilities for inhibition of the plant-derived PPO. First, the effect of ascorbic acid on the degradation of caffeic acid derivatives in protein extracts prepared from "Echinaceae purpureae herba" was investigated. Ascorbic acid possesses good antioxidant properties[28] and is physiologically safe. Ascorbic acid was added in two different concentrations, 5 and 50 mM, and the time course of the degradation of **4** was followed. Compound **4** was degraded by 77% after 5 min in controls without ascorbic acid, whereas the addition of 5 mM ascorbic acid resulted in a limited stability over time, which means the content was stable for about 30 min, after which it dropped rapidly. On the other hand, **4** was quite stable over longer periods of time when 50 mM ascorbic acid was added to the protein extract. In this case, 82% of the **4** added was still present after 7 h (Figure 1). Because of its low redox potential, ascorbic acid is more readily oxidized than **4** and **1**, and previously formed quinones are reduced again by ascorbic acid. Once ascorbic acid is completely oxidized, caffeic acid derivatives, such as **1** and **4**, are also attacked again in case PPOs are still present.

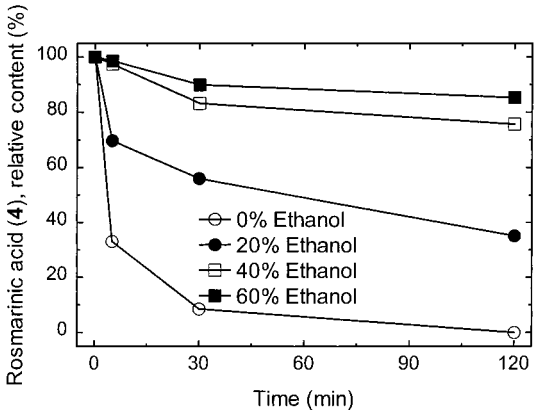

Figure 2. Influence of ethanol on the degradation of **4** in protein extracts prepared from "Echinaceae purpureae herba".

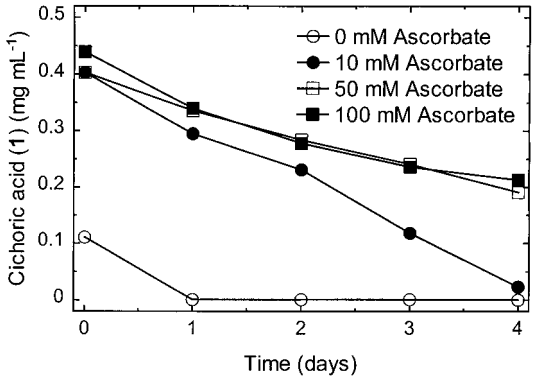

Figure 3. Time course of the degradation of endogenous **1** in various model pressed juices prepared at 4 °C with or without the addition of ascorbic acid. Ethanol was added up to a final concentration of 22%.

Since *E. purpurea* pressed juices are routinely stabilized with ethanol, we decided to test the influence of this additive on the enzymatic degradation of caffeic acid derivatives. The initial dramatic degradation of caffeic acid derivatives was lessened, in particular, when aqueous extracts were preincubated for 30 min with ethanol before adding **4**. Adding ethanol to a final concentration of 20% resulted in a model juice containing 50% more **4** than the respective control without ethanol. Still higher concentrations of ethanol stabilized the extracts even better (Figure 2).

The experiments described so far demonstrated that caffeic acid derivatives, including **1**, are degraded by PPO and that this oxidative process can be partially retarded by the addition of ascorbic acid and ethanol. This knowledge was utilized in an effort to stabilize the endogenous **1** of *E. purpurea* aqueous extracts over a long period of time. Most of the commercially available pressed juices are stabilized with 22% ethanol. Therefore, this concentration was used as a starting point when the extra beneficial effect of ascorbic acid was examined (Figure 3). Without ascorbic acid, initial concentrations of **1** ranged from 100 to 150 μg mL^{-1} and rapidly decreased with time. When buffer containing ascorbic acid was used for extraction, the initial concentration of **1** was much higher (400−500 μg mL^{-1}). However, even when 100 mM ascorbic acid was added, the concentration dropped to about 50% of the initial value after 4 weeks. More detailed analysis revealed that the overall content of caffeic acid derivatives remained quite constant, but that **2** was formed at the expense of **1**. In addition, the amount of free **3** increased over time, which has never been observed in nonstabilized preparations. This can best be explained if we assume that an esterase

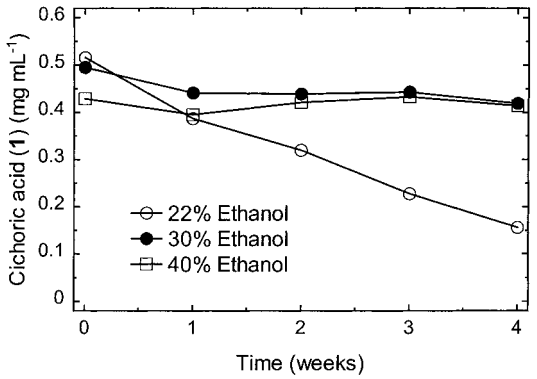

Figure 4. Time course of the degradation of endogenous **1** over a period of 4 weeks in the presence of different concentrations of ethanol. The extract was prepared at room temperature using the standard extraction buffer containing 100 mM ascorbic acid.

hydrolyzing the ester bonds between tartaric acid and **3** is still active under PPO inhibitory conditions. Its estimated activity is in the pkat range per gram drug and therefore 100 times lower than PPO activity. For this reason, it was overlooked at the beginning.

From the experiments shown so far it may be concluded that ascorbic acid is beneficial for the stability of **1** in *E. purpurea* model juices, but that other treatments or additives are recommended for stabilizing the extracts. Therefore, we modified the ethanol content of the model juices. Compound **1** was not stable in preparations containing 22%, 30%, or 40% ethanol without ascorbic acid and decreased to about 10% of the initial concentration after 1 week. On the other hand, **1** was stable in heat-treated samples, with the initial content being lower than in the ethanol-treated samples, which may best be explained by a short period between extraction and boiling, when caffeic acid derivatives could be degraded.

Extracting the drug in the presence of 50−100 mM ascorbic acid and stabilizing the aqueous extract with more than 30% ethanol yielded model juices with a high initial content of **1** and good long-term stability. The addition of 50 mM ascorbic acid to the extraction medium could not prevent degradation when only 22% ethanol was used for stabilization (Figure 4). In this particular case **1** decreased to about 30% of the initial concentration after 4 weeks. Compound **1** was not even stable in the presence of 30% ethanol and eventually decomposed to **3** and **2**. On the other hand, model juices containing a minimum of 50 mM ascorbic acid and 40% ethanol were as stable as the heat-treated controls.

To summarize, the addition of ascorbic acid and an increase in the ethanol content can inhibit enzymatic degradation of **1** during the preparation of *E. purpurea* products and therefore can lead to products containing high concentrations of **1**. Excessive access to oxygen should be avoided during all the steps of the manufacturing process. Besides the options described here for preservation of **1**, blanching may also be adequate to inhibit the enzymes degrading **1** during processing of fresh or dried crushed plant material.[30] The effects of formulation and temperature on the stability of **1** during the storage of *Echinacea* products have been studied recently.[30,31] The results of these studies are well in accordance with our own findings and can all be explained by the presence of **1**-degrading *Echinacea* enzymes and their activation in a humid atmosphere, in aqueous solution, or under inappropriate temperature conditions.

Experimental Section

General Experimental Procedures. TLC was performed on Silica 60 F_{254} plates (Merck, Darmstadt) using a Camag Linumat IV apparatus (Desaga, Heidelberg). A mixture of ethyl acetate−formic acid−acetic acid−water (100:11:11:27) served as the developing solvent. Caffeic acid derivatives were detected under $UV_{365\ nm}$ after spraying the plates with 1% 2-aminoethyl diphenylborinate (natural product reagent A) in ethyl acetate (w/v) and 5% PEG 4000 in dichloromethane (w/v). HPLC was performed on a Waters system (Waters, Eschborn) equipped with an RP-18 column (Nucleosil C_{18}, 5 μm, 250 × 4 mm; Macherey-Nagel, Düren). Compounds **1** and **2** were isolated from the roots of Echinacea purpurea,[7] and **4** was obtained from Extrasynthese (Genay, France). Polyvinylpolypyrrolidone was obtained from ISP (Hedinger, Esslingen), and 10DG gel columns from Bio-Rad (München). All chemicals used for HPLC were of gradient grade.

Plant Material. "Echinaceae purpureae herba" was obtained from Sandfort (harvest 1996 ES-104) and from Martin Bauer, Vestenbergsgreuth (harvest 1997).

Fermentation of the Herbal Drug. One part of pulverized "Echinaceae purpureae herba" was stirred with 9 parts of 50 mM phosphate buffer (pH 7.0) at room temperature. An autoclaved drug (121 °C, 1 atm) which served as the control was treated in the same way. In several experiments 50 mM ascorbic acid, dithiothreitol, or β-mercaptoethanol was added as reducing agent. When necessary, oxygen-free conditions were provided by incubating samples, previously soaked in nitrogen-saturated buffer in an exsiccator flushed with a stream of N_2. One part of a sample (soaked drug) was mixed with 9 parts of methanol and thoroughly mixed, and the resulting suspension then centrifuged for 10 min at 4 °C (13 000 rpm). The supernatant was subjected directly to TLC analysis.

Protein Extraction. All steps were carried out on ice or at 4 °C. One part of pulverized drug was stirred for 30 min with 1 part of polyvinylpolypyrrolidone and 18 parts of N_2-saturated 50 mM Na−P_i (pH 6.0). In several experiments 0.1% Triton X-100 was added to improve protein extraction. After centrifugation for 15 min at 20 000 rpm the supernatant was filtered through 4 layers of mull. The crude protein extract was then passed through a 10DG gel column (previously equilibrated with 50 mM Na−P_i, pH 6.0). Protein was determined according to Bradford,[29] using BSA as the protein standard.

Photometric Determination of Polyphenol Oxidase. The standard assay contained 500 μL of buffer (0.5 mM SDS in 50 mM Na−P_i, pH 6.0), 100 μL of a 0.5 M L-proline solution, 275 μL of Na−P_i pH 6.0, and 25 μL of protein crude extract. The reaction was started by addition of 100 μL of 4-methylcatechol solution (25 mM). In separate experiments 0.1 or 0.5 mM tropolone (final concentrations) or 280 U catalase was added 5 min prior to the catechol substrate. After 3 min the reaction was stopped with 1000 μL of methanol, and product formation was calculated from the absorption at 525 nm, resulting from the formation of a pink prolin-catechol adduct (ϵ_{525} = 1550 L × mol^{-1} × cm^{-1}). Heat-inactivated protein extracts served as controls.

Degradation of Caffeic Acid Derivatives in Protein Crude Extracts. Assays were carried out at 40 °C. Protein extract (230 μL) was mixed with 20 μL of the respective substrate dissolved in dimethyl sulfoxide (final concentration 100 μM). In separate experiments ascorbic acid (5 or 50 mM), tropolone (0.01, 0.1, or 1 mM), ethanol (20%, 40%, or 60%), or catalase (280 U/mL) were added to the incubation mixture 5 min prior to the addition of the respective substrate. Heat-inactivated protein extracts served as controls. The reaction was stopped by the addition of 750 μL of methanol. After centrifugation for 10 min at 13 000 rpm the supernatant was withdrawn and subjected to HPLC analysis.

Stability of 1 in Aqueous Extracts. Samples, 2.00 g each, of the herbal drug were stirred at room temperature or 4 °C for 30 min with 20 mL of Na−P_i pH 6.0. In separate experiments ascorbic acid (10, 50, or 100 mM) was added to the extraction buffer. After centrifugation for 15 min at 20 000 rpm and 4 °C, the mush was filtered through 2 layers of mull and the resulting model pressed juice stabilized with ethanol (final concentration: 22, 30, or 40%). Aliquots were diluted with methanol and precipitated, and particulate material was spun down. The supernatant was subjected to HPLC analysis.

High-Performance Liquid Chromatography. One part of a sample (soaked drug or protein extract) was mixed with 9 parts of methanol and thoroughly mixed, and the resulting suspension or solution then centrifuged for 10 min at 4 °C (13 000 rpm). The supernatant was subjected to HPLC analysis. Caffeic acid derivatives were eluted and separated from each other with 5% acetonitrile−water containing 0.1% *o*-phosphoric acid (solvent A) and 75% acetonitrile−water containing 0.1% *o*-phosphoric acid (solvent B), applying the following gradient: start (6% B), 2 min (6% B), 4 min (12% B), 19 min (12% B), 22 min (30% B), 36 min (30% B), 40 min (100% B), 45 min (100% B), 47 min (6% B), 50 min (6% B). Samples containing **4** were analyzed with a modified gradient program: start (30% B), 20 min (30% B), 22 min (100% B), 26 min (100% B), 28 min (30% B), 35 min (30% B). The flow rate was 1.0 mL min^{-1}, and caffeic acid derivatives were detected at 330 nm and quantified on the basis of external standards.

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