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Ellagitannin biosynthesis: laccase-catalyzed dimerization of tellimagrandin II to cornusiin E in *Tellima grandiflora*

Ruth Niemetz, Georg G. Gross*

Molekulare Botanik, Universität Ulm, D-89069 Ulm, Germany

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Abstract

An enzyme has been purified from leaves of the weed *Tellima grandiflora* (fringe cups, Saxifragaceae) that catalyzed the O_2-dependent oxidation of the monomeric ellagitannin, tellimagrandin II, to a dimeric derivative, cornusiin E. The apparently homogeneous enzyme preparation had a M_r of ca. 160,000 (with four subunits of M_r 40,000), a pH-optimum and an isoelectric point at pH 5.2, and was most stable at pH 4.3. Inhibition studies revealed that this new enzyme, for which the systematic name 'tellimagrandin II: O_2 oxidoreductase' is proposed, is a member of the laccase (EC 1.10.3.2) family of phenol oxidases. The properties of this enzyme differed from that of a related laccase that catalyzed the transition of 1,2,3,4,6-pentagalloylglucopyranose to tellimagrandin II, the preceding step in the biosynthetic route to cornusin E.

Keywords: *Tellima grandiflora*; Saxifragaceae; Fringe cups; Cornusiin E; Ellagitannin biosynthesis; Laccase; Phenol oxidase; Tellimagrandin II

1. Introduction

Hydrolyzable plant tannins are traditionally divided into gallotannins and ellagitannins. It is now generally accepted that both groups are derivatives of a common precursor, 1,2,3,4,6-penta-*O*-galloyl-β-D-glucopyranose. For the biosynthesis of gallotannins, this ester is substituted with additional galloyl moieties to form complex metabolites that can contain up to 10 and more galloyl groups. Several acyltransferases with limited specificity have been detected in sumac (*Rhus typhina*) leaves in the past few years which catalyzed the transitions of pentagalloylglucose to various hexa- and heptagalloylglucoses (recently summarized by Fröhlich et al., 2002). Ellagitannins, in contrast, have been postulated already decades ago to be the result of intramolecular oxidation reactions between vicinal galloyl residues of the pentagalloylglucose core that lead to the 3,4,5,3′,4′,5′-hexahydroxydiphenoyl (HHDP) moieties characteristic of this class of phenolic compounds (Schmidt and Mayer, 1956). Subsequent formation of similar intermolecular linkages should provide the host of dimeric and oligomeric ellagitannins known from nature (for comprehensive reviews, see Haslam 1989, 1998).

First experimental proof of these theoretical considerations was presented by Niemetz et al. (2001) which reported that cell-free extracts from leaves of *Tellima grandiflora* (fringe cups, Saxifragaceae) oxidized pentagalloylglucose to monomeric ellagitannins. Closer investigations led to the detection of a laccase that specifically transformed this ester to tellimagrandin II (for chemical structures, see Fig. 2; Niemetz and Gross, 2003). Subsequent studies revealed the existence of an enzyme activity that further oxidized this compound to a dimeric derivative that was identified as cornusiin E (Niemetz et al., 2003). Here, we report the purification and characterization of this new enzyme that specifically catalyzes the oxidative coupling of two molecules of tellimagrandin II.

2. Results and discussion

2.1. Enzyme purification

Cell-free extracts from *T. grandiflora* were obtained by grinding frozen leaves in liquid N_2 and extraction of

* Corresponding author. Tel.: +49-731-50-22624; fax +49-731-50-22809.
E-mail address: georg.gross@biologie.uni-ulm.de (G.G. Gross).

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the resulting powder with strong tris-borate buffer (1 M, pH 8.5) to neutralize abundant endogenous acids and to bind inhibitory phenolics. Residual contaminants were removed by subsequent stirring with Amberlite XAD ion-exchange resin, followed by ammonium sulfate fractionation. The resulting concentrated enzyme solution was desalted and further purified by gel-filtration on Sephacryl S-100 which also effectively eliminated a related tellimagrandin II-forming laccase activity. The combined active fractions obtained after rechromatography under identical conditions were subjected to hydrophobic-interaction chromatography (HIC) on butyl-sepharose and column chromatography on hydroxyapatite to yield a 344-fold purified preparation with a recovery of 23%. Native PAGE showed that the enzyme had thus been purified to apparent homogeneity (Fig. 1, lane 1). The results of a representative purification protocol are summarized in Table 1. The pure enzyme could be stored at 0–4 °C for ca. three weeks without significant loss of activity.

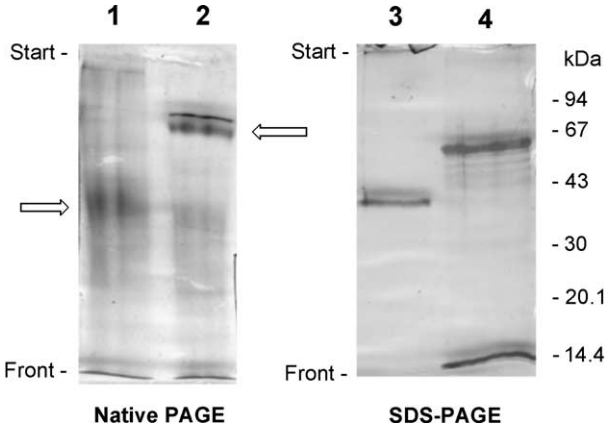

Fig. 1. Gel-electrophoretic comparison of tellimagrandin II: O_2 oxidoreductase (purified to apparent homogeneity; lanes 1, 3) and pentagalloylglucose: O_2 oxidoreductase (Niemetz and Gross, 2003; lanes 2, 4) from *Tellima grandiflora* leaves. In the native PAGE experiment (lanes 1 and 2), arrows indicate the positions of enzyme activities as determined by cutting parallel gel lanes into 1 mm segments, extraction with chilled buffer, and analysis of enzyme activities in the extracts.

Recent efforts are devoted to determine the amino acid sequence of this protein.

2.2. General properties of the enzyme

Enzyme reactions were proportional to protein concentration between 15 and 120 μg per assay, with a lag phase at 0–15 μg. No reaction occurred with enzyme that had been denatured by heat (100 °C) or by treatment with acid (1 N HCl final concentration). The enzyme was inactive below pH 2.2 and above pH 7.0, with an optimum at pH 5.2; half-maximal activities were at pH 2.8 and 5.9. After preincubation at 45 °C for 3 h, the oxidase was found most stable at pH 4.3 (half-maxima at pH 3.0 and 5.4); rapid inactivation occurred below pH 2.5 and above pH 6.5. An isoelectric point of pH 5.2 was determined for the protein by chromatofocussing on a Pharmacia Mono P column. The enzyme reaction proceeded optimally at 22 °C, with half-maximal activities at 14 and 47 °C. No reaction occurred above 70 °C and below 10 °C. After preincubation for 3 h at different temperatures, the phenol oxidase was found stable between 0 and 20 °C; it displayed only moderately decreasing stability up to 50 °C while rapid inactivation occurred at higher temperatures. An average activation energy of 116 kJ mol^{-1}, corresponding to a Q_{10} value of 5.5, was calculated for temperatures between 10 and 20 °C.

The substrate saturation curve for tellimagrandin II displayed a lag phase from 0 to ca. 5 μM tellimagrandin II, followed by normal Michaelis–Menten kinetics up to saturation at ca. 15 μM; pronounced substrate inhibition was observed above 20 μM concentration. Replots according to Lineweaver and Burk (1934) revealed a K_m value of 8.5 μM and a v_{max} of 44 pkat mg^{-1} protein. Unfortunately, determination of the substrate specificity of the enzyme was prevented by the unavailability of sufficient amounts of structurally related (labelled or unlabelled) monomeric ellagitannins and of potential dimeric reaction products required as references. Oxygen was found to serve as electron acceptor; observed enzyme activities clearly paralleled variation of pO_2

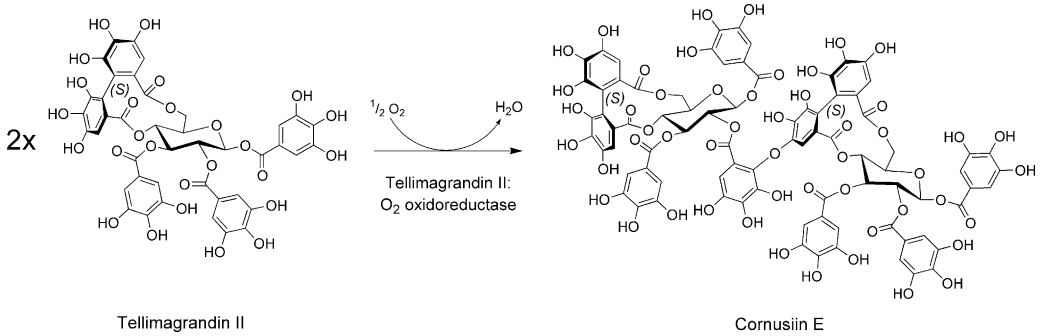

Fig. 2. Reaction scheme of the oxidative coupling of the monomeric ellagitannin, tellimagrandin II, to the dimeric derivative, cornusiin E, by the laccase (EC 1.10.3.2)-type phenol oxidase, tellimagrandin II: O_2 oxidoreductase, from leaves of *Tellima grandiflora* (fringe cups, Saxifragaceae).

Table 1
Purification of tellimagrandin II: O_2 oxidoreductase from *Tellima grandiflora* leaves[a]

Step	Total protein (mg)	Total activity (pkat)	Specific activity (pkat mg^{-1})	Purification (-fold)	Recovery (%)
$(NH_4)_2SO_4$, 30–80% ppt.	150	4.1	0.027	–	100
Desalted $(NH_4)_2SO_4$ pellet	107	5.8	0.054	2	141
1st Sephacryl S-100	13.2	13	0.98	36	317
2nd Sephacryl S-100	1.8	3.2	1.8	67	78
Butyl-Sepharose	0.36	1.3	3.6	133	32
Hydroxyapatite	0.10	0.93	9.3	344	23

[a] Crude extracts were not assayed because of excess contaminants preventing HPLC analysis of reaction products.

values in the assays, as determined for different N_2/O_2 ratios between 8:2 (normal atmosphere, 100% activity) and 100% N_2 (0% activity). No differences between light and dark assay conditions were observed. Common redox cofactors (NAD(P)H, NAD(P), FMN, FAD) had no effect on the reaction rates.

Gel-filtration experiments with a calibrated Sephacryl S-100 column revealed an apparent M_r value of ca. 160,000 for the native enzyme. After denaturing SDS-PAGE, only one protein band of M_r 40,000 was detected (Fig. 1, lane 3) which suggests that the native enzyme existed as a homotetramer. Comparison of the electrophoretic mobilities of the enzyme, after PAGE under denaturing and non-denaturing conditions, with those of a related laccase, pentagalloylglucose: O_2 oxido–reductase (Niemetz and Gross, 2003), revealed clear evidence for the non-identity of these two proteins (Fig. 1).

2.3. Inhibitor studies

As reported above, the coupling of tellimagrandin II to cornusiin E displayed an absolute requirement for O_2 as electron acceptor, suggesting that this reaction was probably catalyzed by some sort of phenol oxidase. However, considering that media containing polyphenolics, in particular galloylated compounds, have been found to artificially generate hydrogen peroxide (Long et al., 2000), the participation of a peroxidase could not be excluded a priori. To gain more information on the nature of the enzyme, a series of inhibitor experiments was conducted whose results are summarized in Table 2. Hydrogen peroxide caused a very strong inhibition, indicating that the enzyme was not a peroxidase. Carbon monoxide, in contrast, had virtually no effect; together with the absence of any requirement for NADPH and the lacking association with the microsome fraction it was concluded that the enzyme did not belong to the cytochrome P450-depending monooxygenases but was a member of the vast class of oxygen dependent (poly)phenol oxidases. This class is commonly divided into (1) *o*-diphenol:O_2 oxidoreductases (EC 1.10.3.1) which are known under several trivial names, like *o*-diphenol oxidase, catecholase, etc., and that also comprise the 'tyrosinases' (EC 1.14.18.1), and (2) the related *p*-diphenol:O_2 oxidoreductases (EC 1.10.3.2). The latter are known as 'laccases' that are widely occurring in fungi, while they have been characterized less frequently in higher plants (Mayer and Staples, 2002).

One approach to discriminate between these two enzyme groups is the use of selective inhibitors. Among the tested inorganic compounds (cf. Table 2), hydroxylamine has been reported to inhibit laccases stronger than catecholases (Ferrar and Walker, 1996); the *T. grandiflora* enzyme displayed strong inhibition with this compound. Laccases, unless catecholases, are insensitive to inhibition by carbon monoxide (Griffith, 1994) which had no effect in our study. In contrast, laccases have a higher affinity for the respiratory poisons azide and cyanide than tyrosinases (Leech and Daigle, 1998), and these compounds exerted moderate to strong effects on the enzyme described here. Cinnamic acids, which were clearly inactive in our experiments, have been reported as good inhibitors of catecholases but not of laccases (Walker and McCallion, 1980; Ferrar and Walker, 1996; Pedreño and Ros Barceló, 1999). Metal chelators appear to have ambivalent effects: the pharmaceutical desferal (for explanation of acronyms and trivial names, see footnote to Table 2) has been reported to inhibit laccases, but not catecholases (De Pinto and Ros Barceló, 1996), while the opposite effect has been observed for the copper chelators DIECA and tropolone (Kahn and Andrawis, 1985; Ferrar and Walker, 1996; Pedreño and Ros Barceló, 1999). We found strong inhibition with desferal for the tellimagrandin II oxidizing enzyme, but DIECA and tropolone were inactive. Mimosine, a toxic non-protein amino acid that has many biologically relevant effects, including that of acting as a metal chelator, has been reported to strongly inhibit phenolases, while being inactive with laccases (Ferrar and Walker, 1996); it displayed no effect in our studies. Detergents have also been used to differentiate between the two classes of phenol oxidases. Walker and McCallion (1980) reported that the cationic detergent CTAB inhibited laccases but did not affect catecholases; we found a pronounced reduction of enzyme activities in the presence of this agent. Significant inhibition was also observed in our tests with sulfur compounds (2-mercaptoethanol, reduced glutathione, Na hydrogene sulfite) that have

Table 2
Inhibitor studies with tellimagrandin II: O_2 oxidoreductase[a]

Agent class	Compound	Concentration	Inhibition (%)
Inorganics	Nitrogen, replacing oxygen	100%	100
	Carbon monoxide	$CO:O_2 = 1:1–7:1$	0
	Hydrogen peroxide	2.5–7.5 mM	100
	Hydroxylamine	0.1–0.8 mM	0–100
	Na azide	0.1–0.8 mM	44–64
	Na cyanide	0.1–0.8 mM	0–100
Cinnamic acids	Cinnamate	0.25–10 mM	0
	p-Coumarate	0.25–10 mM	0
	Ferulate	0.25–10 mM	0
Chelators	Desferal[b]	5–55 mM	8–100
	DIECA	0.03–3.3 mM	0
	Mimosine	0.1–1 mM	0
	Tropolone	0.1–1 mM	0
Detergents	CTAB	0.25–10 mM	0–100
Sulfur compounds	2-Mercaptoethanol	0.1–0.8 mM	0–100
	Glutathione, reduced	0.1–0.8 mM	0–100
	Na hydrogene sulfite	0.1–0.8 mM	50–100

[a] Determinations were performed under standard assay conditions.
[b] Trivial names and acronyms: CTAB, cetyltrimethylammonium bromide; desferal, desferrioxamine mesylate; DIECA, Na diethyldithiocarbamate; mimosine, β-(*N*-3-hydroxypyridone-4)-α-aminopropionic acid; tropolone, 2-hydroxy-2,4,6-cycloheptatriene-1-one.

been found to inhibit both catecholases and laccases (Ferrar and Walker, 1996).

It is evident from these inhibitor studies that the tellimagrandin II oxidizing enzyme from *T. grandiflora* belongs to the laccase (EC 1.10.3.2) subgroup of phenol oxidases.

2.4. Conclusions

Evidence has been presented in this communication that coupling of two molecules of tellimagrandin II to the dimeric ellagitannin, cornusiin E, is catalyzed by an oxygen-dependent laccase-type phenol oxidase. The pH and temperature sensitivities of this enzyme, as well as its molecular weight characteristics, revealed clear differences to a related enzyme from *T. grandiflora* that has been recently found to catalyze the oxidation of 1,2,3,4,6-pentagalloylglucose to tellimagrandin II (Niemetz and Gross, 2003), i.e. the reaction that is providing the substrate for the subsequent dimerization step to cornusiin E. The laccase described here should thus receive the provisional systematic name 'tellimagrandin II:oxygen oxidoreductase'; the reaction catalyzed by this new enzyme is depicted in Fig. 2.

3. Experimental

3.1. Plant material

Young leaves (2–4 months old) from greenhouse grown *T. grandiflora* (Pursh) Lindley (fringe cups, Saxifragacea) plants were washed with dist. H_2O, frozen in liquid N_2 and stored at −20 °C in evacuated plastic bags where they could be kept for more than 6 months without apparent loss of enzyme activity.

3.2. Chemicals and general procedures

Tellimagrandin II was prepared enzymatically; cornusiin E was isolated from leaves of *T. grandiflora* (Niemetz and Gross, 2003). Carbon monoxide was obtained by dehydration of formic acid with concentrated H_2SO_4 (C. Paetz, Halle, unpublished). IEP determination was done by chromatofocussing on Mono P (Pharmacia; column size 200×5 mm i.d.) as described by Niemetz and Gross (2003). Native and SDS-PAGE analyses were performed on 8 and 10% separating gels, pH 8.8, respectively, as described by Niemetz and Gross (1998) but without addition of detergent. Standard enzyme assays (utilizing unlabelled tellimagrandin II as substrate) were incubated and analyzed by RP-18 HPLC as previously described (Niemetz and Gross, 2003; Niemetz et al., 2003). Protein concentrations were measured colorimetrically (Bradford, 1976) with BSA as standard; very dilute solutions were analyzed by UV photometry (Kalckar, 1947).

3.3. Enzyme purification

Enzyme from leaves of *T. grandiflora* was extracted and pre-purified as reported earlier (Niemetz and Gross, 2003; Niemetz et al., 2003). The desalted $(NH_4)_2SO_4$ pellet was subjected to gel-filtration on Sephacryl S-100 (Pharmacia; column size 40×2.4 cm

i.d.) in 50 mM acetate buffer, pH 5.0. After rechromatography under identical conditions, active frs were combined, supplemented with 1.25 M $(NH_4)_2SO_4$ and subjected to HIC on butyl-sepharose (Pharmacia 'fast flow'; column size 17×16 mm i.d.) equilibrated with 50 mM acetate buffer (pH 5.0) plus 1.25 M $(NH_4)_2SO_4$. The column was successively washed with 1.25 M $(NH_4)_2SO_4$ in acetate buffer (pH 5.0), followed by eluting the enzyme with acetate buffered 0.625 M $(NH_4)_2SO_4$. This fraction was chromatographed on hydroxyapatite (Bio-Gel HTP, Bio-Rad; column size 10×12 mm i.d.) equilibrated in 50 mM acetate buffer, pH 5.0. After washing with this buffer (which eliminated the bulk of inactive protein) and with 0.3 M Na phosphate buffer, pH 5.0, the enzyme was eluted with 0.4 M phosphate buffer.

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