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OXIDATION OF *P*-COUMAROYLAGMATINE IN BARLEY SEEDLING EXTRACTS IN THE PRESENCE OF HYDROGEN PEROXIDE OR THIOLS

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Abstract—When *p*-coumaroylagmatine is oxidized in the presence of hydrogen peroxide in a crude extract of barley seedlings, among several products, hordatine A is formed. However, unlike the natural isomer, this is optically inactive. In the absence of hydrogen peroxide and when a thiol (glutathione, cysteine, mercaptoethanol or dithiothreitol) is added to the incubation medium, *p*-coumaroylagmatine is rapidly transformed to a thiol adduct, probably through a peroxidase-dependent co-oxidation reaction. The reactions with hydrogen peroxide or with a thiol are completely inhibited by 1 mM ascorbate.

INTRODUCTION

Hordatine A, which is a dimer of *p*-coumaroylagmatine, occurs in barley seedlings together with hordatine B, an analogous conjugate of *p*-coumaroylagmatine and feruloylagmatine; and hordatine M, a mixture of the glucosides of hordatine A and B [1]. The hordatines are optically active and the configuration of the phenylcoumaran ring has been recently determined by comparison with the similar lignan amide, grossamide [2].

It is now known that *p*-coumaroylagmatine, which is present in small amounts in barley seedlings [3], is synthesized by a *p*-coumaroyl-CoA agmatine *N*-*p*-coumaroyl transferase (EC 2.3.2.-) [4]. Racemic hordatine A has been synthesized *in vitro* by oxidative coupling of *p*-coumaroylagmatine in the presence of horseradish peroxidase, and hydrogen peroxide and a similar mechanism could be involved in the formation of hordatine A *in vivo* [1].

During a study of the *in vitro* formation of *p*-coumaroylagmatine in the presence of barley extract from *p*-coumaroyl-CoA and [U-^{14}C]-agmatine, a radioactive product, which co-chromatographed in several TLC systems with hordatine A was rapidly synthesized [4]. The time course of the reaction suggested that this is derived from *p*-coumaroylagmatine, although in these experiments no hydrogen peroxide was added to the incubation medium. We eventually found that this compound is not hordatine A but is an adduct formed as an artefact in the presence of mercaptoethanol (ME), normally added to protect the transferase.

RESULTS AND DISCUSSION

Formation of thiol adduct

[G-^{14}C]-*p*-Coumaroylagmatine was synthesized chemically and incubated with the barley extract in the conditions used for the assay of the *p*-coumaroyl-CoA agmatine transferase (Tris, 0.1 M; pH 8.5; 10 mM ME) [4]. The adduct was rapidly formed, confirming that it is derived from *p*-coumaroylagmatine. However, when ME was substituted by glutathione (GSH), cysteine, or dithiothreitol (DTT), *p*-coumaroylagmatine was still rapidly transformed, but the R_f on TLC of the product changed with the thiol (see Experimental). No reaction occurred with oxidized glutathione or without thiol. The formation of the thiol adduct was confirmed using unlabelled *p*-coumaroylagmatine and cysteine as thiol in the enzymic reaction. After chromatography, the adduct reacted not only with diazotized *p*-nitroaniline and the Sakaguchi reagent, but also with ninhydrin. No reaction occurred between *p*-coumaroylagmatine and the thiol without enzyme or on using a boiled extract.

The reaction could be monitored spectrophotometrically, as the formation of the adduct leads to a decline in the UV absorbance of *p*-coumaroylagmatine (λ_{max} 310 nm at pH 8.5). The optimum pH for the reaction was 8.5 with 50% activity at pH 9 and 7.5. It was at first thought that the enzyme involved could be a glutathione transferase, catalysing a Michael addition of the thiol to the olefinic double bond of *p*-coumaroylagmatine. A similar enzyme has been characterized in pea seedlings [5]. However, using an oxygen electrode, the reaction was found to consume molecular oxygen. No oxygen uptake could be detected when *p*-coumaroylagmatine was incubated with the barley extract without thiol and only a slow consumption was detected when mixing the thiol and the enzyme without *p*-coumaroylagmatine. When both *p*-coumaroylagmatine and the thiol were present in the incubation medium, the oxygen uptake increased with the concentration of thiol, until on reaching a ratio for thiol/*p*-coumaroylagmatine of 7, it was then *ca* 2 O_2 per molecule of *p*-coumaroylagmatine. Using standardized conditions for optimal activity (*p*-coumaroylagmatine 50 μM, thiol 2 mM, pH 8.5) GSH was found to be the best substrate among the thiols tested (relative activity GSH 100,

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cysteine 92, DTT 49, Me 12). A similar reaction occurred with *p*-coumaric acid at a slower rate (32%) indicating that the thiol addition is taking place on the hydroxycinnamic part of *p*-coumaroylagmatine. Cinnamic acid, caffeic acid and sinapic acid did not act as substrates. On spectrophotometric evidence, ferulic acid reacted only slowly (2% of the activity of *p*-coumaroylagmatine).

The structure of the thiol adduct was not determined. It was found, however, that catalase (5 units/ml) inhibits the reaction by 70% (measured by the loss of absorbance at 310 nm), indicating that peroxides and more probably hydrogen peroxide could be produced in the process. Furthermore, ascorbic acid, which is a known substrate of peroxidases [6, 7] was found to inhibit the reaction (measured by the loss of absorbance at 310 nm), causing 50% inhibition at 20 μM; 100% at 1 mM. On the basis of these results, it is thought that peroxidases present in the barley extract can generate reactive species which may be quinone methides. These have been postulated as intermediate products of peroxidase activity [8], and are known to be very reactive [9]. These intermediates could be trapped by the thiol in a chemical reaction similar to the co-oxidation of thiols with olefins [10]. Peroxides could then be produced which would allow the reaction to proceed.

In view of the stoichiometry of the reaction, it is possible that more than one molecule of thiol could be added on to the *p*-coumaroyl moiety. This explanation, however, remains speculative as the product of the reaction has not been identified. Enzyme-catalysed formation of thiol adducts with phenolics have already been reported. The aerobic oxidation of *p*-hydroquinone by horseradish peroxidase in the presence of a thiol has been described [11]. In a similar reaction, tyrosinase was also found to catalyse the conjugation of cysteine with pyrocatechol. Tyrosinase oxidizes pyrocatechol to *o*-quinone which then combines with cysteine non-enzymatically [12]. Hydrogen peroxide, however, was not involved in either of these reactions.

In vitro *synthesis of hordatine A*

The *p*-coumaroyl-CoA agmatine transferase exhibits a peak of activity 3 days after the beginning of germination [13]. Furthermore [U-^{14}C]-agmatine was found to be incorporated in the hordatines *in vivo* in 3-day-old seedlings [4], indicating that the enzyme catalysing the dimerization should be present in this material, assuming that hordatine A synthesis proceeds through direct dimerisation of *p*-coumaroylagmatine. [G-^{14}C]-*p*-Coumaroylagmatine was not metabolized at all when supplied *in vivo* for 2 hr to the seedlings under the conditions used for feeding [^{14}C]-agmatine [4], probably because it did not penetrate into the tissue. When *p*-coumaroylagmatine was incubated with a dialysed extract of 3-day-old barley seedlings in the presence of hydrogen peroxide, hordatine A was formed, together with other products which appeared as two yellow bands of low mobility on paper chromatography. Hordatine A was identified with the natural product isolated from the plant by co-chromatography in solvents 1 and 2, ion-exchange chromatography, UV spectrum, PMR and FAB mass spectrometry (see Experimental), but was optically inactive. Slow addition of hydrogen peroxide was necessary to obtain a high yield. The reaction could be monitored spectrophotometrically (292 nm). The complete transformation of *p*-coumaroylagmatine was achieved after a decline in absorbance of *ca* 55%. Ascorbic acid (1 mM) completely inhibited the reaction, but only for a limited period of time when hydrogen peroxide was added in excess.

Many isoperoxidases can be separated from extracts of barley seedlings [14, 15]. Synthesis of optically active hordatine A *in vitro* may therefore require the separation and use of one specific isoperoxidase present in the extract. The direct dimerization of *p*-coumaroylagmatine is not of course the only possible mechanism of formation of hordatine A, but remains the more likely in view of the existence of an active agmatine *p*-coumaroyl-CoA transferase requiring an activated cinnamic acid as substrate. *p*-Hydroxyphenylpropionyl-CoA is inactive as a substrate for this enzyme [Negrel, J. and Smith, T. A., unpublished] suggesting that the CoA derivatives of the phenylcoumaran ring would also be inactive. In addition, the ease of obtaining racemic hordatine A in the presence of hydrogen peroxide and peroxidase suggests that this is the mechanism for hordatine synthesis *in vivo*.

EXPERIMENTAL

Barley (*Hordeum vulgare* L., cv Proctor) was grown as previously described [4].

TLC was on cellulose CC41 using solvents: (1) *n*-BuOH–EtOH–H_2O (4:1:2) (R_f *p*-coumaroylagmatine 0.63, hordatine A 0.50, adduct of *p*-coumaroylagmatine with ME 0.5—with cysteine 0.3; with GSH 0.15; with DTT 0.5; (2) *n*-BuOH–HOAc–H_2O (4:1:5 upper) R_f *p*-coumaroylagmatine 0.77; hordatine A, 0.59).

[G-^{14}C]-*Agmatine* was prepared from [G-^{14}C]-arginine as previously described [4].

Synthesis of p-*coumaroylagmatine*. *p*-Coumaroyl-*N*-hydroxysuccinimide ester was synthesized as described in [16]. Agmatine sulphate (205 mg, 0.9 mmol) was dissolved in 20 ml H_2O and adjusted to pH 8 with $NaHCO_3$ and then *p*-coumaroyl-*N*-hydroxysuccinimide ester (261 mg, 1 mmol), dissolved in 20 ml Me_2CO, was added. The mixture was left in the dark at room temp for 24 hr. After acidification with 0.5 ml 17 M HOAc, the Me_2CO was evaporated and the mixture extracted with 1 vol. EtOAc. The aq. phase was then applied to a CG 50 column (H^+ form, 100–200 mesh, 2.5 × 5 cm) and washed with 100 ml H_2O. The *p*-coumaroylagmatine containing some unreacted agmatine was then eluted with 4 M HOAc. After evaporation, the residue was dissolved in a minimum vol. of 8 M HOAc and passed through a cellulose column in *n*-BuOH–HOAc–H_2O (5:2:3) to remove the agmatine. After evaporation, *trans*-*p*-coumaroylagmatine acetate was recrystallized from MeOH on standing at −15° (yield 65% from agmatine). ^{1}H NMR (200 MHz, CD_3OD): δ1.5–1.7 (4H, *m*, CH_2–CH_2), 1.92 (3H, *s*, OAc), 3.1–3.4 (*m*, overlapping solvent signal, CH_2–N), 6.42 (1H, *d*, J = 15.5 Hz), 6.79 (2H, *d*, J = 8.6 Hz, 7.41 (2H, *d*, J = 8.6 Hz and 7.45 (1H, *d*, J = 15.5 Hz). The 200 MHz ^{1}H NMR spectrum of *p*-coumaroylagmatine confirmed the presence of a *trans* double bond (J = 15.5 Hz, typical of a *trans* olefinic coupling). The rest of the spectrum is also in accordance with the expected structure.

[G-^{14}C]-*p*-coumaroylagmatine was synthesized from [G-^{14}C]-agmatine using the same procedure with an excess of *p*-coumaroyl-*N*-hydroxysuccinimide ester at pH 8. It was purified by TLC in solvent 2 and electrophoresis on a cellulose plate (1 hr, 400 V; pyridine–HOAc–H_2O, 1:5:94). The electrophoresis separated the *cis* and *trans* forms (mobility relative to an agmatine standard = 1:*cis* 0.5:*trans* 0.44). The *trans*-*p*-coumaroylagmatine was eluted from the cellulose using 8 M HOAc.

Enzyme preparation. The shoots of 3-day-old barley seedlings were homogenized with a pestle and mortar with sand in 4 vol. 0.1 M Tris buffer pH 8.5. The extract was centrifuged at 10000 *g* for 20 min and the supernatant dialysed against the same buffer (0.01 M).

Formation of the thiol adduct was monitored spectrophotometrically at 310 nm (0.9 ml Tris 0.1 M buffer pH 8.5, 100 μl enzyme extract, *p*-coumaroylagmatine 50 nmol, 30°). The reaction was started by addition of 2 μmol of thiol (10 μl).

Oxygen consumption was monitored using a Clark type O_2 electrode in a total vol. of 2 ml at 30°. (1.8 ml Tris 0.1 M pH 8.5 buffer, 200 μl enzyme extract, *p*-coumaroylagmatine 100 nmol, thiol 4 μmol).

Synthesis of hordatine A. The enzyme extract was prepared as described above but using 0.1 M NaPi buffer pH 6. After dialysis the extract obtained from 5 g of the shoots of barley seedlings was mixed with *trans*-*p*-coumaroylagmatine acetate (200 mg, 0.592 mmol) in a total vol. of 100 ml 0.01 M NaPi buffer pH 6 at 30°. H_2O_2 (40 ml; 6 mM) was then added dropwise (20 ml/hr) while stirring. The reaction was followed spectrophotometrically (292 nm) and stopped with HOAc. After evaporation *in vacuo* under red. pres. the residue was dissolved in 8 M HOAc and purified on descending PC in solvent 1 (yield 30% from *p*-coumaroylagmatine).

Identification of hordatine A. Ion exchange chromatography was performed as described in [17]. UV spectra [1, 18] were recorded in 96% EtOH, ^{1}H NMR: δ1.5–1.7 (8H, *m*, CH_2CH_2), 1.92 (6H, *s*, OAc), 3.1–3.4 (*m*, overlapping solvent signal, CH_2N), 4.21 (1H, *d*, J = 7.6 Hz), 5.92 (1H, *d*, J = 7.6 Hz, 6.51 (1H, *d*, J = 13.7 Hz), 6.81 (*d*, J = 8.3 Hz) overlapping 6.7–6.9 (*m*; together 3H), 7.17 (2H, *d*, J = 8.3 Hz) and 7.35–7.50 (3H, *m*).

The 200 MHz ^{1}H NMR spectrum of the *p*-coumaroylagmatine dimer compared well with the NMR data for natural hordatine A [1]. Slight differences in chemical shifts may be due to the change in solvents (CD_3OD in this work, D_2O in ref. [1]). The presence of the characteristic pair of doublets ascribed in ref. [1] to the two dihydrobenzofuran methine protons confirms that the dimerization has proceeded to give hordatine A. The FAB-MS of hordatine A was run in glycerol (*m/z* 573 (7) $[M+Na]^+$; 551 (27) $[MH]^+$; 395 (10) $[M-156]$; 277 (84); 191 (67); 173 (100); 157 (49); 147 (52); 116 (76)). A second but minor set of molecular ions at *m/z* 625 (11), 647 (3) due to an unidentified impurity could also be detected.

^{1}H *NMR spectra* were determined in CD_3OD using a JEOL Fx-200 spectrometer.

Optical rotation was determined with a Perkin-Elmer 241 MC polarimeter in a 1 ml cell using the D-line of the Na lamp.

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REFERENCES

1. Stoessl, A. (1967). *Can. J. Chem.* **45**, 1745.
2. Yoshihara, T., Yamaguchi, K. and Sahamura, S. (1983) *Agric. Biol. Chem.* **47**, 217.
3. Stoessl, A. (1965) *Phytochemistry* **4**, 973.
4. Bird, C. R. and Smith, T. A. (1981) *Phytochemistry* **20**, 2345.
5. Diesperger, H. and Sandermann, H. (1979) *Planta* **146**, 643.
6. Chmielincka, J., Ohlsson, P. I., Paul, K. G. and Stigbrand, T. (1971) *FEBS Letters* **17**, 181.
7. Demirevska, K. N. and Bakardjieva, N. (1976) *Can. J. Botany* **54**, 90.
8. Gaspar, T., Penel, C., Thorpe, T. and Greppin, H. (1982) *Peroxidases* 1970–1980, University of Geneva.
9. Wagner, H. U. and Gompper, R. (1974) in *The Chemistry of Quinonoid Compounds* (Patai, S., ed.) Part 2, Ch. 18. John Wiley, New York.
10. Oswald, A. A. and Wallace, T. J. (1966) in *The Chemistry of Organic Sulphur Compounds* (Kharasch, N. and Meyers, C. Y., eds) Vol. 2, Ch. 8. Pergamon Press, Oxford.
11. Esterbauer, H. and Schwarzl, E. (1977) *Z. Naturforsch.* **32c**, 650.
12. Sanada, H., Suzue, R., Nakashima, Y. and Kawada, S. (1972) *Biochim. Biophys. Acta* **261**, 258.
13. Bird, C. R. and Smith, T. A. (1983) *Phytochemistry* **22**, 2401.
14. Anstine, W., Jacobsen, J. V., Scandalios, J. G. and Varner, J. E. (1970) *Plant Physiol.* **45**, 148.
15. Mitra, R., Jagannath, D. R. and Bhatia, C. R. (1970) *Phytochemistry* **9**, 1843.
16. Negrel, J. and Smith, T. A. (1984) *Phytochemistry* **23**, 31.
17. Bird, C. R. and Smith, T. A. (1981) *J. Chromatogr.* **214**, 263.
18. Stoessl, A. and Unwin, C. H. (1970) *Can. J. Botany* **48**, 465.