Purification of a new peroxidase catalysing the formation of lignan-type compounds

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A novel peroxidase that catalyses the dimerization of ferulic acid or caffeic acid via oxidative coupling and formation of $\beta\beta'$ -linkage to the lignan-type compounds 8,8'-bis(caffeic acid) or 8,8'-bis(ferulic acid) respectively was purified from the leaves of *Bupleurum salicifolium*. The enzyme, for which the name caffeate peroxidase is proposed, was purified 2700-fold. It is a glycoprotein and has an M_r of 38000 as determined by gel filtration and SDS/PAGE. The K_m values for ferulic acid and caffeic acid were 0.24 mM and for H_2O_2 0.04 mM with caffeic acid and 0.48 mM with ferulic acid. The purified peroxidase does not exhibit activity on other phenylpropanoids tested and has no detectable phenol oxidase or NADPH oxidase activity. The caffeate peroxidase could be involved in the biosynthesis of lignans.

INTRODUCTION

Lignans are compounds widely distributed in plants and also in fluids from mammals [1–4]. Their basic chemical structure is shown in Scheme 1. It consists of two phenylpropanoid units joined through a $\beta\beta'$ -linkage. A large number of compounds derived from the fundamental structure have been reported. Some of them possess antitumoral or cytotoxic properties as well as 'digitalis-like' activity [5–9]. Information about the physiological role of these compounds or about their biosynthesis is scarce. The final stage of lignan biosynthesis could be the dimerization of phenylpropanoid compounds such as ferulic acid or caffeic acid with the formation of a $\beta\beta'$ -linkage (Scheme 1). However, to our knowledge no enzyme catalysing such a reaction has hitherto been identified. Since peroxidases are involved in a variety of dimerization reactions [10–13], we investigated the existence of a peroxidase catalysing the proposed dimerization in a plant, *Bupleurum salicifolium* (Apiaceae), in which lignans have been described [14–16]. We report in the present paper the purification of a new peroxidase able to catalyse the dimerization of either caffeic acid or ferulic acid with the formation of a $\beta\beta'$ -linkage. This new enzyme could be involved in the biosynthesis of lignans.

MATERIALS AND METHODS

Materials

Ferulic acid and caffeic acid were from Sigma Chemical Co. (St. Louis, MO, U.S.A.) and their purity was tested by t.l.c. Mature leaves from *Bupleurum salicifolium* were collected in the garden of our Faculty, washed with distilled water and drained at room temperature. The process of purification began not later than 2 h after the collection of the leaves.

Enzyme assay

The activity of the caffeate peroxidase in the dimerization of the caffeic acid or ferulic acid was determined by measuring spectrophotometrically the disappearance of ferulic acid or caffeic acid at the maximum absorption wavelength (285 nm for caffeic acid and 310 nm for ferulic acid). Throughout the process of enzyme purification the activity was monitored by determining the appearance of 8,8'-bis(caffeic acid) at its maximum absorption wavelength 348 nm. The reaction was carried out at 30 °C in a 2 ml reaction mixture containing 50 mM-Tris/HCl buffer, pH 7.4, 5 mM-EDTA, 2 mM- H_2O_2 , 1.5 mM-caffeic acid or -ferulic acid and adequate amounts of caffeate peroxidase. The activity

Scheme 1. Reaction catalysed by the caffeate peroxidase

The structures of the reaction products were determined by n.m.r. and m.s.

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of caffeate peroxidase on 3,4-dimethoxycinnamic acid, cinnamic acid and coniferyl alcohol was determined as for caffeic acid and ferulic acid, by monitoring the disappearance of the assayed substrate at its maximum absorption wavelength. The activity on guaiacol, pyrogallol, veratryl alcohol and *o*-dianisidine was determined as described in refs. [17–19]. Proteins were determined by the Bradford method [20].

Michaelis constants

For the determination of the K_m values for caffeic acid and ferulic acid the reactions were carried out as described above, with various concentrations of caffeic acid or ferulic acid and 2 mM- H_2O_2 . In the determination of the K_m values for H_2O_2 in the dimerization of these acids the concentration of caffeic acid or ferulic acid was 1.5 mM.

SDS/PAGE of proteins

Electrophoresis was carried out in a gel with an acrylamide gradient from 7.5 to 15% with 3% acrylamide in the stacking gel in accordance with the procedure of Laemmli [21]. Gels were stained by the silver method [22].

Analysis of the reaction products

To analyse the reaction products of the purified enzyme, caffeate peroxidase was incubated as described above but in a volume of 125 ml for 30 min. After this time the reaction mixture was acidified to pH 4.5 when the substrate was ferulic acid or pH 3.5 in the case of caffeic acid, and extracted three times with 25 ml of ethyl acetate. The samples were dried, dissolved in methanol and subjected to preparative t.l.c. on silica gel with methanol/ethyl acetate (9:1, v/v). The products were analysed by 1H -n.m.r. and ^{13}C -n.m.r. spectroscopy with [2H_6]acetone, [2H]chloroform or [2H_4]methanol as solvent and tetramethylsilane as internal reference on a Bruker WP 200 SY instrument (200 MHz), by high-resolution and low-resolution m.s. performed on a VG-Micromass ZAB-2F spectrometer and a Hewlett-Packard 5995 spectrometer respectively and by i.r. on a Perkin-Elmer G8 instrument.

Hexamethyl derivatives were obtained by treating each product from the enzymic reaction with methyl iodide in anhydrous acetone and K_2CO_3 on reflux for 9 h. The same hexamethyl derivative was also produced by treating 8,8'-bis(dimethoxycinnamic acid) obtained by the Schrecker method [23] with methyl iodide as above.

RESULTS

Isolation and purification of the caffeate peroxidase

The purification procedure is summarized in Table 1.

Homogenization and filtration through Sephadex G-25. Leaves (60 g) were mixed with 200 ml of 50 mM-potassium phosphate

buffer, pH 7.4, containing 2 mM-EDTA, 1 mM-EDTA, 1 mM-2-mercaptoethanol and 6 g of polyvinylpyrrolidone and homogenized in a Sorvall Omnimixer at 10000 rev./min in six periods of 30 s, with a 2 min interval on ice between each period.

The homogenate was filtered through cheesecloth and then centrifuged at 12000 *g* at 4 °C for 30 min. The supernatant was filtered through a Sephadex G-25 column (2.5 cm × 60 cm) equilibrated with 50 mM-sodium phosphate buffer, pH 7.4, containing 1 mM-EDTA, and the exclusion fraction was collected.

$(NH_4)_2SO_4$ precipitation. The exclusion fraction obtained from the Sephadex G-25 column was treated with solid $(NH_4)_2SO_4$. The caffeate peroxidase was precipitated between 45 and 75% of saturation at pH 7.4. The pellet containing the enzyme was resuspended in 10 ml of 50 mM-Tris/HCl buffer, pH 7.4, and $(NH_4)_2SO_4$ was removed by dialysis against the same buffer.

Ion-exchange chromatography. The dialysed sample was loaded on to a DEAE-Sepharose ion-exchange column (5 cm × 30 cm) equilibrated with 50 mM-Tris/HCl buffer, pH 8.5, and eluted with the same buffer. Under these conditions, unlike most of the protein, the peroxidase activity was not retained. The caffeate peroxidase activity fraction from the ion-exchange column was precipitated with 75%-saturated $(NH_4)_2SO_4$, the pellet resuspended and the suspension dialysed for 12 h against 50 mM-sodium phosphate buffer, pH 7.4.

Gel filtration. The dialysed sample was loaded on to a Ultrogel AcA 44 column (110 cm × 2 cm) equilibrated with 50 mM-sodium phosphate buffer, pH 7.4. The column was eluted with the same buffer. The caffeate peroxidase appeared in the exclusion volume.

Affinity chromatography. The active samples obtained from the previous step were loaded on to a concanavalin A-Sepharose column (10 cm × 0.9 cm) and eluted with a linear gradient (0–500 mM) of methyl α -D-mannoside in 200 mM-sodium phosphate buffer, pH 7.4, containing 2 mM- $MgCl_2$ and 2 mM- $NaCl$. The caffeate peroxidase was eluted at 150 mM-methyl α -D-mannoside.

By using the procedure described above the caffeate peroxidase was purified about 2700-fold, and only one band was observed on SDS/PAGE (Fig. 1).

Characterization of the purified caffeate peroxidase

Molecular size. An M_r value of about 38000 was determined by SDS/PAGE (Fig. 1) and also by gel filtration on Sephadex G-100 (results not shown).

Carbohydrate content. The caffeate peroxidase was retained by concanavalin A-Sepharose but not by wheat-germ lectin-Sepharose 6B. The total carbohydrate content of the caffeate

Table 1. Purification of caffeate peroxidase from *Bupleurum salicifolium*

For experimental details see the Results section. Enzyme activity (with caffeic acid as substrate) and protein were determined as described in the Materials and methods section. Units of activity are expressed as $\Delta A_{348}/\text{min}$.

Step	Activity (units)	Protein (mg)	Specific activity (units/mg)	Purification (fold)	Recovery (%)
Sephadex G-25	236	876	0.27	1	100
$(NH_4)_2SO_4$ (45–75% satn.)	176	160	1	3.7	74
DEAE Sepharose	107	1.8	59	218	45
Ultrogel AcA 44	96	0.6	160	593	40
Concanavalin A-Sepharose	51	0.07	728	2696	21

Fig. 1. SDS/PAGE of the caffeate peroxidase preparation at different steps of the purification process

SDS/PAGE of the enzyme preparation at different steps of the purification process was carried out as described in the Materials and methods section. Proteins were stained by the silver method. Lane A, DEAE-Sephacel chromatography step; lane B, Ultrogel AcA 44 exclusion-chromatography step; lane C, concanavalin A-Sepharose affinity-chromatography step, showing a band of M_r 38 000 corresponding to the caffeate peroxidase. The M_r markers (left-hand lane) used were BSA (M_r 66 000), egg albumin (M_r 45 000), glyceraldehyde-3-phosphate dehydrogenase (M_r 36 000) and carbonic anhydrase (M_r 29 000).

peroxidase was determined with the use of mannose as standard [24]. The value obtained was of 0.2 mg equivalents of mannose/mg of purified enzyme.

Effect of pH. The caffeate peroxidase has optimum activity at pH 7.4 with both caffeic acid and ferulic acid; 100 mM-sodium phosphate buffers in the range pH 5.8–7.0 and 100 mM-Tris/HCl buffers in the range pH 6.5–9.5 were used.

Characterization of the reaction products

The reaction catalysed by the caffeate peroxidase described is shown in Scheme 1. The reaction product and ferulic acid (Scheme 1, Ia and IIa), when this was the substrate, were analysed by ^1H -n.m.r. spectroscopy. The spectrum of the reaction product does not show the doublets at δ 6.5 and δ 7.1 p.p.m. attributable to protons on unsaturated carbon atoms in the α - and β -positions respectively with respect to carboxy groups, ruling out the possibility that the dimerization is carried out through a biphenyl linkage. One singlet is observed at δ 7.62 p.p.m., this being due to protons in the β -position with respect to carboxy groups, as well as two signals at δ 3.82 and δ 3.88 p.p.m., which are due to two methoxy groups. These data are in agreement with structure (IIa) (Scheme 1), corresponding to 8,8'-bis(ferulic acid). The mass spectrum agrees with this structure, since the molecular ion appears at m/z 386.1008 ($\text{C}_{20}\text{H}_{18}\text{O}_8$) and the loss of one and two molecules of CO_2 characteristic of dicarboxylic acids at m/z 342 and 298 is observed, m/z 342 being the base peak of the spectrum.

When caffeic acid (Ib, Scheme 1) was the substrate, the ^1H -n.m.r. spectrum of the product of the reaction presents a singlet at δ 8.0 p.p.m., which is due to protons attached to the double bond adjacent to the carboxy group.

The mass spectrum shows a molecular ion at m/z 358.0681

Fig. 2. (a) ^1H -n.m.r., (b) ^{13}C -n.m.r. and (c) m.s. spectra of hexamethyl derivative (III) from the 8,8'-bis(caffeic acid) obtained with caffeate peroxidase

The spectra obtained for the synthetic hexamethyl derivative of the 8,8'-bis(caffeic acid) [23] are identical with those obtained for the compound synthesized by the caffeate peroxidase with further chemical methylation.

($\text{C}_{18}\text{H}_{14}\text{O}_8$) and the loss due to two molecules of CO_2 . These data are in accordance with the structure (IIb, Scheme 1) corresponding to 8,8'-bis(caffeic acid).

The products of the reaction were methylated, and the same hexamethyl derivative was obtained. The i.r. spectrum of this compound shows a strong absorption at 1700 cm^{-1} , which is due to an $\alpha\beta$ -unsaturated ester group. The absorption of aromatic rings is also observed at 1592 cm^{-1} . The m.s. spectrum (Fig. 2c) shows the molecular ion at m/z 442.1623 ($\text{C}_{24}\text{H}_{26}\text{O}_8$).

In the ^1H -n.m.r. spectrum (Fig. 2a) a singlet is observed at δ 7.87 p.p.m., which is due to protons in the β -position with

respect to carboxy groups, and three singlets at δ 3.84, δ 3.74 and δ 3.63 p.p.m., which correspond to six methoxy groups. The ^{13}C -n.m.r. spectrum (Fig. 2b) shows at δ 167.57 p.p.m. the signal corresponding to a carbonyl group of the ester. At δ 124.40 p.p.m. appears the signal corresponding to a tetrasubstituted α -carbon atom and at δ 142 p.p.m. that corresponding to trisubstituted β -carbon atom. All these data are in agreement with structure (III) (Fig. 2a inset). Further support to corroborate the structure proposed comes from the hexamethyl derivative of the 8,8'-bis(cafeic acid) and 8,8'-bis(ferulic acid) synthesized by Stobbe condensation according to the Schrecker method [23] and further methylation. This synthesis leads unequivocally to product (III). The ^1H -n.m.r., m.s., i.r. and ^{13}C -n.m.r. spectra are the same for the compounds obtained by both the enzymic and the chemical methods. All these results allow us to conclude that the isolated caffeate peroxidase catalyses the dimerization through the formation of a $\beta\beta'$ -linkage of caffeic acid as well as of ferulic acid.

Time course of the reaction

In the spectrum of the substrates and products in the reaction catalysed by the caffeate peroxidase monitored with respect to time, a decrease in the absorbance at 285 nm and an increase in that at 348 nm due to the disappearance of caffeic acid and the appearance of 8,8'-bis(cafeic acid) is observed. A similar spectrum was obtained when the substrate used was ferulic acid (results not shown).

Michaelis constants

The K_m values obtained were 0.24 mM for caffeic acid and ferulic acid and 0.04 mM for H_2O_2 with caffeic acid and 0.48 mM with ferulic acid. When the reactions were monitored as described in the Materials and methods section, the saturation curves were hyperbolic, and the K_m values were calculated by the Lineweaver-Burk method.

Substrate-specificity

The activity of the caffeate peroxidase was examined with several substrates, always with H_2O_2 as acceptor (Table 2). The caffeate peroxidase is active on different compounds such as guaiacol, pyrogallol, coniferyl alcohol and *o*-dianisidine as well as caffeic acid and ferulic acid, but is not active on veratryl alcohol. The caffeate peroxidase is unable to catalyse the transformation of phenylpropanoids such as 3,4-dimethoxycinnamic acid, cinnamic acid and coumaric acid. The enzyme does not

possess NAD(P)H oxidase, phenol oxidase or tyrosine hydroxylase activity (results not shown).

DISCUSSION

The structure analysis of the reaction products 8,8'-bis(cafeic acid) and 8,8'-bis(ferulic acid) shows that the purified peroxidase catalyses an oxidative coupling through a $\beta\beta'$ -linkage of two units of caffeic acid or ferulic acid. Some peroxidases are able to dimerize ferulic acid, phenol and tyrosine [10-14], but through the formation of an *o,o'*-linkage as well as other types of linkages.

The name caffeate peroxidase is proposed for the purified peroxidase because the enzyme has more activity on caffeic acid than on ferulic acid and the K_m for H_2O_2 with caffeic acid is lower (0.04 mM) than with ferulic acid (0.48 mM).

A mixture of isoenzymes of horseradish peroxidase (purchased from Sigma Chemical Co.) does not catalyse the dimerization of caffeic acid or ferulic acid through a $\beta\beta'$ -linkage (results not shown). These results indicate that the caffeate peroxidase is specific for the catalysed oxidative coupling through a $\beta\beta'$ -linkage as described in this paper.

It cannot be concluded whether or not the K_m values obtained for ferulic acid and caffeic acid are within a physiological range because the concentrations of these products in the plant studied are unknown. The K_m values for H_2O_2 are 0.04 mM with caffeic acid and 0.48 mM with ferulic acid, suggesting that caffeic acid would be the more physiological substrate.

The enzyme is a peroxidase that does not use O_2 as electron acceptor (results not shown). As shown in Table 2 the caffeate peroxidase is able to catalyse the transformation of various compounds, always with H_2O_2 as electron acceptor. No activity was detected on phenylpropanoids such as cinnamic acid, coumaric acid or 3,4-dimethoxycinnamic acid, and these results indicate that the enzyme dimerizes only those cinnamic acid derivatives that carry a hydroxy group at the 3-position and a hydroxy or methoxy group at the 4-position.

Since lignans have been isolated from *Bupleurum* [17-19] as well as the enzymes phenylalanine ammonia-lyase, cinnamate hydroxylase and coumarate hydroxylase (results not shown) involved in the biosynthesis of caffeic acid from phenylalanine, it could be suggested that the caffeate peroxidase here reported is involved in the biosynthesis of the lignans. The proposed pathways would be phenylalanine $\rightarrow$ cinnamic acid $\rightarrow$ coumaric acid $\rightarrow$ ferulic acid or caffeic acid $\rightarrow$ lignans.

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Table 2. Substrate-specificity of the caffeate peroxidase

Activity on caffeic acid and ferulic acid was determined as described in the Materials and methods section. Activity towards cinnamic acid, coumaric acid and coniferyl alcohol was determined similarly except that the activity was monitored at the wavelength of maximum absorption of the assayed substrate. No change of absorbance was interpreted as no activity.

Substrate	Peroxidase activity ($\Delta A/\text{min per ml}$)
Caffeic acid	0.303
Ferulic acid	0.150
3,4-Dimethoxycinnamic acid	None
Cinnamic acid	None
Guaiacol [17]	0.016
Coumaric acid	None
Pyrogallol [17]	0.034
Veratryl alcohol [18]	None
Coniferyl alcohol	0.110
<i>o</i> -Dianisidine [19]	0.09

REFERENCES

- McRae, W. D. & Towers, G. H. N. (1984) *Phytochemistry* **23**, 1207-1220.
- Stitch, S. R., Toumba, J. K., Groen, M. B., Funke, C. W., Leemhuis, J., Vink, J. & Woods, G. F. (1980) *Nature (London)* **287**, 738-740.
- Setchell, K. D. R., Lawson, A. M., Mitchell, F. L., Adlercreutz, H., Kirk, D. N. & Axelson, M. (1980) *Nature (London)* **287**, 740-742.
- Setchell, K. D. R., Lawson, A. M., Conway, E., Taylor, N. F., Kirk, D. N., Cooley, G., Farrant, R. D., Wynn, S. & Axelson, M. (1981) *Biochem. J.* **197**, 447-458.
- Loike, J. D. & Horwitz, S. B. (1976) *Biochemistry* **15**, 5443-5448.
- Markkanen, T., Mäkinen, M. L., Maunuksela, E. & Himanen, P. (1981) *Drugs Exp. Clin. Res.* **7**, 711-718.
- Wozniak, A. J. & Ross, W. E. (1983) *Cancer Res.* **43**, 120-124.
- Long, B. H., Musial, S. T. & Brattain, M. G. (1984) *Biochemistry* **23**, 1183-1188.
- Fagoo, M., Braquet, P., Robin, J. P., Esanu, A. & Godfraind, T. (1986) *Biochem. Biophys. Res. Commun.* **134**, 1064-1070.

10. Markwalder, H. U. & Neukom, H. (1976) *Phytochemistry* **15**, 836–837
11. Danner, D. J., Brignac, P. J., Jr., Arceneaux, D. & Patel, V. (1973) *Arch. Biochem. Biophys.* **156**, 759–763
12. Bayse, G. S., Michaels, A. W. & Morrison, M. (1972) *Biochim. Biophys. Acta* **284**, 34–42
13. Brizat, P., Winkler, G., Kalchhauser, H. & Breitenbach, M. (1986) *J. Biol. Chem.* **261**, 4288–4294
14. González, A. G., Trujillo, J. M., Estévez, R. & Pérez, J. A. (1975) *An. Quím.* **71**, 109–110
15. González, A. G., de la Rosa, R. & Trujillo, J. M. (1986) *Tetrahedron* **42**, 3899–3904
16. González, A. G., Pérez, J. P. & Trujillo, J. M. (1978) *Tetrahedron* **34**, 1011–1013
17. Morita, Y., Iwamoto, H., Aibara, S., Kobayashi, T. & Hasegawa, E. (1986) *J. Biochem. (Tokyo)* **99**, 761–770
18. Tien, M. & Kirk, T. K. (1984) *Proc. Natl. Acad. Sci. U.S.A.* **81**, 2280–2284
19. Saeki, K., Ishikawa, O., Fukuoka, T., Nakagawa, H., Kai, Y., Kakuno, T., Yamashita, J., Kasai, N. & Horio, T. (1986) *J. Biochem. (Tokyo)* **99**, 485–494
20. Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254
21. Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
22. Heukeshoven, J. & Dernick, R. (1985) *Electrophoresis* **6**, 103–112
23. Schrecker, W. A. (1957) *J. Am. Chem. Soc.* **79**, 3823–3827
24. Clarke, J. & Shannon, L. M. (1976) *Biochim. Biophys. Acta* **427**, 428–442

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