Journal of Chromatography, 320 (1985) 435–439
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 17,365

Note

Determination of laccase activity with various aromatic substrates by high-performance liquid chromatography

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(Received October 25th, 1984)

The main limitation of spectrophotometric methods for enzyme determinations[1–6] is the dosage required of a single compound (substrate, reaction product) whose UV absorption is affected, at the same wavelength, by that of other compounds present in the mixture. This kind of determination is inapplicable to among others enzymes such as laccases, which, being one-electron oxidases acting mainly on phenols, lead to phenoxy radicals, which undergo further non-enzymatic reactions (*e.g.*, oxidation to quinones, bond cleavage, bond formation, oligomerization)[6–10]; thus a number of organic materials are formed, which are difficult to detect.

High-performance liquid chromatography (HPLC) is being increasingly used for determinations of enzyme activities[11–13], the reaction products usually being detected. In a previous paper[14] we described a method for determining the degradation of aromatic compounds by laccase, based on isocratic reversed-phase HPLC identification of the residual substrates with spectrophotometric detection. This paper reports a study of the degradation by laccase of 52 aromatics variously related to plant metabolism.

EXPERIMENTAL

Purification of laccase

Laccase was purified from a commercial strain of *Agaricus bisporus* (Somycel strain 56) as described previously[15].

High-performance liquid chromatography

Apparatus. HPLC was performed using a Perkin-Elmer Series 3B liquid chromatograph, equipped with an LC-75 spectrophotometric detector; the data were processed with a Sigma 15 chromatography data system. A Hibar LiChrosorb RP-18 (10 μm) column (25 cm × 4 mm I.D.) (Merck) was used.

Solvent. Merck solvents were used (LiChrosolv acetonitrile, analytical-reagent grade acetic acid). Water was deionized with a Millipore Milli-Q water purification system.

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TABLE I

CHROMATOGRAPHIC DATA

Compounds tested as substrates		*Eluent (water–acetonitrile–acetic acid) proportions*	*λ (nm)*	*t'_R*	*k'*	*Fraction of non-degraded substrate after 30 min (%)*
Type	*Compound*					
Phenols	Phenol	79:20:1	270	4.71	1.51	90
	4-Chlorophenol	59:40:1	280	5.08	1.90	84
	2,4-Dichlorophenol	49:50:1	285	4.34	1.69	24
	2,4,5-Trichlorophenol	49:50:1	290	7.77	2.99	79
	2-Nitrophenol	49:50:1	275	3.97	1.55	100
	4-Nitrophenol	49:50:1	315	1.79	0.70	98
	Pyrocatechol (1,2-dihydroxybenzene)	89:10:1	275	3.78	1.14	11
	Guaiacol (2-methoxyphenol)	79:20:1	275	5.68	1.81	8
	Pyrocatechol monoethyl ether (2-ethoxyphenol)	59:40:1	240	7.61	2.94	27
	Pyrocatechol monobenzyl ether (2-benzyloxyphenol)	49:50:1	280	7.03	2.97	54
	Resorcinol (1,3-dihydroxybenzene)	99:0:1	270	8.56	2.40	79
	Resorcinol monomethyl ether (3-methoxyphenol)	79:20:1	270	5.64	1.81	80
	Hydroquinone (1,4-dihydroxybenzene)	99:0:1	290	4.98	1.40	29
	Hydroquinone monomethyl ether (4-methoxyphenol)	79:20:1	270	3.98	1.28	2
	Hydroquinone monopropyl ether (4-propoxyphenol)	59:40:1	290	6.73	2.66	56
	Hydroquinone monobutyl ether (4-butoxyphenol)	49:50:1	290	3.96	1.54	50
	Hydroquinone monohexyl ether (4-hexyloxyphenol)	49:50:1	290	11.32	4.39	63
	Hydroquinone monoheptyl ether (4-heptyloxyphenol)	39:60:1	290	8.47	3.34	45
	Hydroquinone monobenzyl ether (4-benzyloxyphenol)	49:50:1	290	5.17	1.89	50
	Hydroquinone dimethyl ether (1,4-dimethoxybenzene)	49:50:1	290	4.16	1.64	100
	Naphthohydroquinone (1,4-dihydroxynaphthalene)	79:20:1	240	10.41	3.53	16
	Naphthohydroquinone monomethyl ether (4-methoxy-1-naphthol)	69:30:1	245	10.32	3.82	2
	Pyrogallol (1,2,3-trihydroxybenzene)	99:0:1	260	2.81	0.81	53
	Phloroglucinol (1,3,5-trihydroxybenzene)	99:0:1	265	3.20	1.18	26
Benzoic acids	Benzoic acid	79:20:1	275	6.68	2.14	100
	2-Chlorobenzoic acid	79:20:1	250	7.87	2.51	94
	2,4-Dichlorobenzoic acid	59:40:1	240	7.61	2.94	100
	Salicylic acid (2-hydroxy-benzoic acid)	79:20:1	295	8.06	2.56	95
	3-Hydroxybenzoic acid	89:10:1	295	7.28	2.26	82
	4-Hydroxybenzoic acid	89:10:1	260	5.25	1.62	91
	p-Anisic acid (4-meth-oxybenzoic acid)	79:20:1	255	6.43	1.95	100
	2,3-Dihydroxybenzoic acid	89:10:1	240	6.98	2.31	31

TABLE I (*continued*)

Compounds tested as substrates		*Eluent (water–acetonitrile–acetic acid) proportions*	*λ (nm)*	t'_R	*k'*	*Fraction of non-degraded substrate after 30 min (%)*
Type	*Compound*					
	Gentisic acid (2,5-dihydroxybenzoic acid)	89:10:1	320	3.73	1.16	77
	Protocatechuic acid (3,4-dihydroxybenzoic acid)	99:0:1	260	13.15	3.81	37
	Vanillic acid (4-hydroxy-3-methoxybenzoic acid)	89:10:1	260	7.06	2.14	49
	Methyl vanillate	69:30:1	290	4.27	1.36	14
	Gallic acid (3,4,5-trihydroxybenzoic acid)	99:0:1	270	6.86	1.90	21
	Syringic acid (4-hydroxy-3,5-dimethoxybenzoic acid)	89:10:1	275	8.75	2.70	30
Cinnamic acids	Cinnamic acid (3-phenylpropenoic acid)	69:30:1	255	6.07	2.05	100
	p-Coumaric acid (4-hydroxycinnamic acid)	79:20:1	250	3.63	1.14	9
	Caffeic acid (3,4-dihydroxycinnamic acid)	89:10:1	325	8.63	2.65	6
	Ferulic acid (4-hydroxy-3-methoxycinnamic acid)	79:20:1	260	4.27	1.36	6
	Sinapic acid (4-hydroxy-3,5-dimethoxycinnamic acid)	79:20:1	250	4.05	1.29	0
	Chlorogenic acid (3-O-caffeylquinic acid)	79:20:1	325	5.52	1.67	1
Others	2-Hydroxyphenylacetic acid	79:20:1	275	3.30	1.04	34
	3-Hydroxyphenylacetic acid	89:10:1	275	8.80	2.54	64
	4-Hydroxyphenylacetic acid	89:10:1	275	6.58	1.93	61
	3,4-Dihydroxyphenylacetic acid	89:10:1	280	2.71	0.78	48
	3,4-Dihydroxyphenylpropionic acid	89:10:1	280	5.72	1.74	26
	2,4-D (2,4-dichlorophenoxyacetic acid)	49:50:1	290	2.79	1.10	100
	2,4,5-T (2,4,5-trichlorophenoxyacetic acid)	49:50:1	290	6.05	2.35	100
	Esculetin (6,7-dihydroxycoumarin)	89:10:1	340	7.37	2.27	1

Procedure. Substrates were dissolved in 50 m*M* sodium phosphate buffer (pH 6.0) containing 10% of ethanol and their concentrations were those corresponding to substrate saturation conditions. All solutions were prepared in the absence of oxygen to avoid substrate non-enzymatic oxidations, using the buffer under vacuum, and subsequently were deaerated and flushed with nitrogen in the dark. Each dilution was reoxygenated by stirring for 20 sec immediately before each assay. A 5-μl volume of enzyme extract was mixed with 195 μl of each dilution of substrates and incubated for 1.5, 3, 6, 9, 15, 30 and 60 min at 30°C. The enzyme reaction was stopped by adding 100 μl of 30% trichloroacetic acid and other non-enzymatic reactions reduced by dipping the samples in a cryostatic bath at −40°C in the dark. Before each HPLC

assay the resulting 300-μl samples were purified using a Sep-Pak C_{18} cartridge (Waters Assoc.), washing the cartridge with acetonitrile to a final volume of 10 ml. Samples of 10 μl were then chromatographed. Elution was carried out isocratically using acetonitrile–water–acetic acid mixtures as shown in Table I at a flow-rate of 1 ml/min at room temperature. Detection was carried out at wavelengths very close to the maximum UV absorption ($|\lambda - \lambda_{max}| \leqslant 2.5$ nm). The values are given in Table I.

RESULTS AND DISCUSSION

Table I reports the chromatographic data for the compounds tested. All the determinations were performed at λ values as close as possible to the respective λ_{max} values of the substrates, in order to obtain high sensitivity during the detection.

As shown by Table I, most of the λ values are very close to each other, which confirms that a spectrophotometric assay of the laccase activities of mixtures of phenols is impracticable; moreover, the possibility of interconversions of phenolic molecules could make even UV assays run on simple substrates misleading.

The method is proposed as a general one, mainly applicable to hardly specific enzymes. This confirms the choice of the substrate as the molecular species to be tested[9,10,14] in cases when more products (and/or non-predictable compounds) arise from enzymatic reactions. The feature of this method is to make possible the sensitive assay of laccase activities on phenolic molecules bearing widely different functional groups and aromatic skeletons. It should be noted, however, that the chromatographic conditions were chosen in order to define the best HPLC conditions for the assay of laccase activities and not to develop a method for their separation.

Fig. 1 shows three typical curves of the degradation of phenolic substrates *versus* time of incubation, whereas the last column in Table I reports the amounts of non-degraded compounds after incubation for 30 min. From the data it is clear that

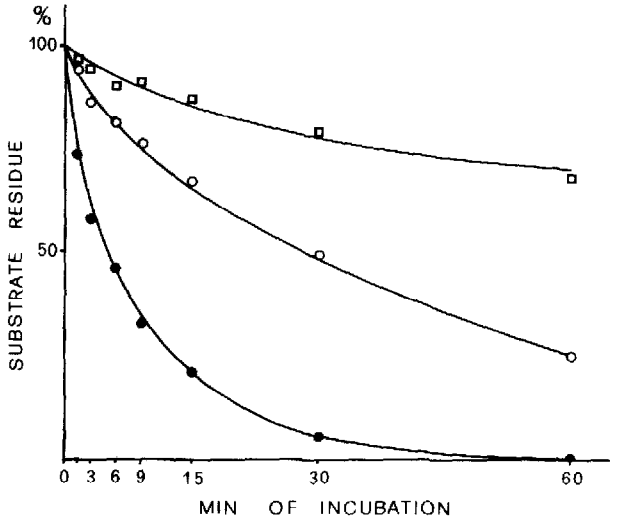

Fig. 1. Degradation curves for (●) ferulic acid, (○) vanillic acid and (□) 2,4,5-trichlorophenol.

there are differences in the biochemical behaviour owing to the chemical characteristics of the substrates and the general usefulness of the method is demonstrated. The relationship between the degradation and the structures of the substrates tested will be the subject of further study.

ACKNOWLEDGEMENTS

The authors express their gratitude to Professor G. Giovannozzi Sermanni for valuable discussions, suggestions and helpful criticism.

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