A SPECTROPHOTOMETRIC ASSAY OF INDULIN C—A POTENTIAL NEW SUBSTRATE FOR DETERMINATION OF LIGNIN-DEGRADING ABILITIES OF MICROORGANISMS

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Received June 30, 1989
Accepted December 26, 1989

A basic spectrophotometric study of a commercially produced sodium salt of Kraft lignin (Indulin C) was performed. The method of quantitative determination of Indulin C based on its absorbance at 276 nm has been found to be applicable. The spectra were also studied by means of derivative UV-spectrophotometry, which is recommended for theoretical studies of electronic spectral behaviour of lignins and lignin model compounds. Linear dependences were found between Indulin C concentration and the second derivative of spectrum at 250 nm and 276 nm. Both methods were used for determining the lignin degrading ability of the basidiomycete *Phanerochaete chrysosporium* and their suitability is discussed.

At present practically only two methods are used for quantitative determination of microbial degradation of lignin: either the amount of $^{14}CO_2$ evolved from a radioactively labelled compound is measured[1–3], or the absorbance of the solution under defined conditions is determined using the absorption maximum corresponding to aromatic ring in UV region[4]. The radiospirometric assay is highly specific but very elaborate. The spectrophotometric method is more suitable for the purpose of routine large-scale screening of lignin degrading abilities of various organisms. This method was used by several authors[5,6] in the study of lignin biodegradation by the basidiomycete *Phanerochaete chrysosporium*, with Indulin AT and alkali isolated straw lignin as substrates. All commonly used substrates are insoluble at low and neutral pH, which causes some difficulties in handling. To simplify the assay, we tested Indulin C – a sodium salt of Kraft lignin, commercially available from Westvaco, U.S.A. As this compound is easily water-soluble, preparation of solutions required for experiments and manipulation with them is easier than in the case of substrates used as yet; but no method for determination of Indulin C is available. Therefore we studied UV spectral behaviour of Indulin C as well as the shape of the second derivative curves.

Natural lignin and lignin model compounds exhibit a characteristic maximum in the UV region at 280 nm. With changing pH a moderate bathochromic shift

occurs, which has been explained by the ionisation of phenolic HO-groups[7]. A differential curve obtained by subtracting the spectral values measured at neutral pH from those measured in alkaline region exhibits its maxima at approximately 250 nm and 300 nm (see Goldschmid[8]). The latter maximum is characteristic for non--conjugated phenolic HO-groups and was used for quantitative considerations[9]. The studies of the electronic spectra of lignins have been reported[10,11] in which computer procedure is used for resolving them into Gaussian light absorption bands. According to this method, spectra of both natural lignins and lignin model compounds (stilbenes) can be described by the model consisting of thirteen overlapping bands with constant widths and positions but with varying maximum values[12]. In our work the second derivative UV spectrophotometry has been used for determination of a new lignin model compound but it seems to be hopeful method for theoretical studies of electronic spectra of lignins, too.

In practice the absorbance at 281 nm is used in lignin determination[4], as this maximum is negligibly influenced by the change of external conditions and obeys the Lambert–Beer's law in the lignin concentration range $0-10\ g\ l^{-1}$. The changes in UV spectra, viz. the decrease in absorbance at 281 nm in the spectrum of lignin as a result of microbial degradation, can only be attributed to cleavage of the aromatic ring structures. Those lignin reactions such as demethoxylation or side-chain degradation cannot be detected by this method. The theory of UV spectra of lignin was studied in more detail by Norrström and Teder[12,13]. The use of derivative techniques in UV spectrophotometry of lignin model compounds has not yet been discussed.

EXPERIMENTAL

Indulin C. The commercial preparation Indulin C (Westvaco, U.S.A.) was purified before use by dissolving it in distilled water and extracting with chloroform. After chloroform removal the aqueous solution was lyophilized and the substance was stored in a dessiccator in the dark. The stability of Indulin C in solution was tested by measuring the viscosity and UV spectra of a 2% solution in distilled water stored at room temperature. The spectra remained unchanged and also the value of relative dynamic viscosity remained constant (1·048 ± 0·002 cP; Ostwald viscometer, 20°C, ref. H_2O) for 20 days.

Spectrophotometric measurement. Measurements in the UV region and scanning of derivative spectra were performed on a PU 8800 spectrophotometer. The pH of the solution was adjusted to 7·0–7·5 before measurement.

Organism. Phanerochaete chrysosporium strain P1, a basidiospore isolate from strain 284B (obtained from Dr Setliff, Forintek Corp., Vancouver, Canada) was maintained at 4°C on slant agars with a complete medium according to Snider and Raper[14]. For estimation of Indulin C biodegradation, a medium according to Kirk et al.[15] was used. The medium was inoculated with a suspension of conidiospores prepared by washing 9-day-old cultures of *P. chrysosporium* grown on Petri dishes with the complete medium (2% agar) at 28°C. The final spore concentration in the suspension was about 10^6 spores per ml.

Indulin C biodegradation. Sterile water solution of Indulin C was added to an inoculated medium to a final Indulin C concentration of $1\cdot0 \text{ g l}^{-1}$ in the culture, pH 4·2. After that, 15 ml aliquots of the resulting mixture were dispensed into 250 ml Erlenmayer flasks and incubated at 28°C. On the third and then every third or fourth day the cultures were flushed with pure oxygen. At the beginning of the cultivation and then on days 6, 13, 20, 27 and 34 aliquots were taken to follow the biodegradation of Indulin C. To culture samples was added 20 ml of 0·1M-NaOH solution and the cultures were extracted for 25 h on a rotary shaker at 200 r.p.m. Solid particles were separated by centrifugation and UV spectra of properly diluted supernatants were taken after pH adjustment to 7·0–7·5 (0·1M-HCl).

RESULTS AND DISCUSSION

Indulin C exhibits spectra analogous to those of Indulin AT and native lignin samples in the UV region, i.e. absorption maximum at 280 nm, which is subject to a strong bathochromic shift (Fig. 1). The influence of solution basicity on the absorption maximum shift is in Table I. When the pH drops below 5, precipitation of Indulin C occurs. In the pH region between 5·5 and 8·0 the absorption maximum is stable; in the concentration range of $0\cdot01 \text{ g l}^{-1}$ to $0\cdot1 \text{ g l}^{-1}$ it obeys the Lambert–Beer's law and is suitable for quantitative determination. A differential absorbance curve at pH 13 vs pH 7 exhibits maxima at 298 nm and 246 nm. According to Goldschmid's method[9] it is possible to attribute to Indulin C 1·97% of phenolic groups. The second derivative spectra seem to be very interesting; they show sharp maxima at 228 nm, 250 nm and 304 nm, a slight maximum at 370 nm and a sharp maximum at 276 nm corresponding to the maximum in UV spectrum (Fig. 2). In the concentration range of $10-100 \text{ mg l}^{-1}$ we found a linear correlation between the response (units) and the concentration (g l^{-1}) for the 276 nm peak:

$$D_{276} = (83\cdot2 \pm 1\cdot33)\, c$$

and for the 304 nm peak:

$$D_{304} = (58\cdot6078 \pm 0\cdot6028)\, c\,.$$

The response at 250 nm is too steep to be suitable for determining 10-fold lower concentrations, i.e. in the range of $1-10 \text{ mg l}^{-1}$. The relationship is characterized by the equation:

$$D_{250} = (202\cdot805 \pm 10\cdot52)\, c\,.$$

For the purpose of measurement of routine samples we studied the influence of external factors on the shape of UV spectra. A 30 min sterilization of a sample had no effect on the shape of the spectra. No interference appeared in the region between 230 nm and 400 nm caused by using different media. However, intracellular compounds released into the solution during homogenization influenced the shape of the

UV spectra considerably: the absorbance value at 276 nm measured against distilled water was 20% higher than that referred to the blank. In the derivative spectrum the maxima at 250 nm and 304 nm and the minimum at 276 nm remained unchanged in comparison with the blank (Fig. 3). It is well-known that a considerable adsorption of lignin model compounds to the fungal mycelium takes place in the course of a biological experiment. That is why the release of Indulin C during the extraction was followed; the maximum extraction concentration was that reached after 25 h

TABLE I
Effect of pH on Indulin C spectra

pH	λ_{max}, nm
5·5—8·0	276
9·0	277
9·8	278
11·0	280
12·0	282
13·0	284
14·0	286

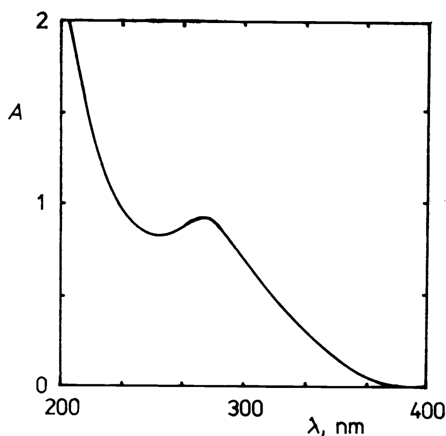

FIG. 1
UV spectrum of Indulin C. $c = 0{\cdot}06\ g\ l^{-1}$, pH 7·0, reference: H_2O

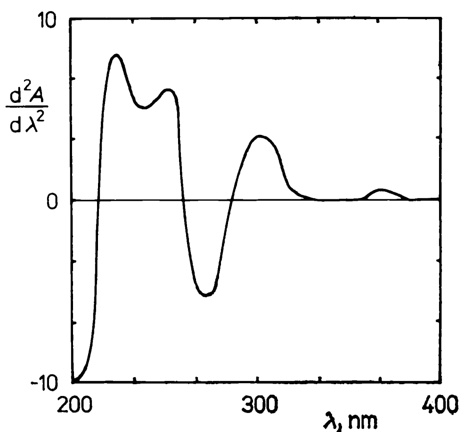

FIG. 2
The second derivative UV spectrum of Indulin C. $c = 0{\cdot}06\ g\ l^{-1}$, pH 7·0, reference: H_2O

agitation, mycelial homogenization, sonication and centrifugation. Table II shows that practically 100% of the maximum value is reached on overnight shaking.

Two methods were suggested for quantitative determination of Indulin C. The first is quite analogous to the currently used techniques of UV spectrophotometric determination and consists in measuring the absorbance at 276 nm in a neutral solution against a blank; the second is based on evaluating second derivative spectra or a response at 276 nm which shows a good reproducibility and a high value of the

TABLE II

Per cent of extractable Indulin C during extraction

Procedure	Sample					$\langle x \rangle$
	1	2	3	4	5	
Cultivation	65·6	62·9	67·5	65·4	65·5	65·4
90-min shaking	78·7	82·9	78·8	76·6	81·0	79·6
5-h shaking	86·9	85·7	100·0	89·6	89·7	89·9
25-h shaking	95·1	91·4	109·6	100·0	100·0	98·7
Homogenization, 10-min sonication, centrifugation	100·0	100·0	100·0	100·0	100·0	100·0

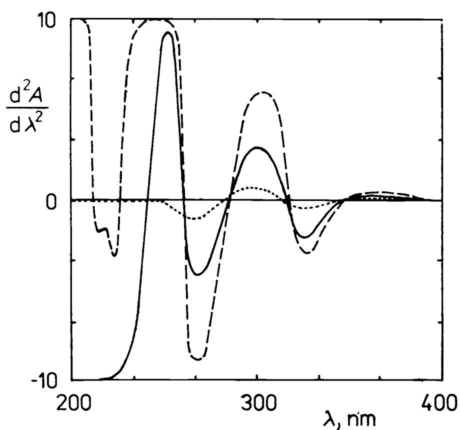

FIG. 3

The second derivative UV spectra of various Indulin C concentrations measured against a blank after homogenization of the mycelium. ········ 0·01 g l^{-1}, ——— 0·05 g l^{-1}, ----- 0·10 g l^{-1}

coefficient of determination of the response-concentration ratio. The experimental data are given in Tables III and IV.

TABLE III

Calibrations; equation for calibration curve $y = k_1 x + k_0$

Concentration of Indulin C g l^{-1}	A_{276}	Units d^2A (276 nm) $d\lambda^2$
0	0	0·24
0·01	0·144	1·60
0·02	0·272	2·40
0·03	0·432	3·12
0·04	0·584	3·84
0·05	0·736	4·56
0·06	0·880	5·28
0·07	1·008	6·08
0·08	1·184	6·80
0·09	1·312	7·60
0·10	1·472	8·48
k_1	14·6327 ± 0·1165	77·9637 ± 3·7511
k_0	0	0·6473 ± 0·2219
r^2	0·9996	0·9959

TABLE IV

Comparison of results of a classical and a derivative method. Values are averages of three cultures ±s.d.

Day of cultivation	Average contents of Indulin C in cultures			
	classical method		derivative method	
	g l^{-1}	%	g l^{-1}	%
0	0·93 ± 0·08	100	0·93 + 0·08	100
6	0·93 ± 0·15	100	0·92 ± 0·12	99
13	0·78 ± 0·08	84	0·86 ± 0·17	92
20	0·72 ± 0·08	77	0·75 ± 0·18	81
27	0·71 ± 0·06	76	0·72 ± 0·08	77
34	0·69 ± 0·10	74	0·70 ± 0·18	75

Large differences were found among values determined by derivative techniques. The considerable dispersion of values can apparently be ascribed to the high sensitivity of the method and the difference between blank composition and the background of the solution measured: the spectrum of compounds released into the solution on mycelium homogenization probably changes during the cultivation which influences the peak shift or the magnitudes of the response. This problem could be solved by construction of a series of calibration curves for each sample taken (i.e. each day of estimation), but the efficiency of this procedure is doubtful. In this respect the second derivative UV spectra of lignin and lignin model compounds can be useful because they make it possible to follow the region from 200 nm to 250 nm much more accurately than with classical UV spectrophotometry. As the second derivative spectra can be taken easily with high reproducibility, they can be recommended for theoretic speculations including structural relationships in electronic spectra. The investigation of the second derivative spectra seems to be simpler than the mathematic modelling based on the measurement of A vs λ curves. The derivative method has only a limited importance for routine Indulin C determination; in this case the measurement of A_{276} in a neutral solution against a blank is quite satisfactory because of its rapidity and simplicity.

The authors thank Dr Petr Rychlovský from the Department of Analytical Chemistry, Charles University Prague, for his assistance in the evaluation of the 2nd derivative UV *spectra.*

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Translated by K. Sigler.