Electron Spin Resonance Characterization of the Radicals Produced by Enzymatic or Chemical Cleavage of Vicine†

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ABSTRACT: Vicine is a glucoside from broad beans (*Vicia faba*) that is hydrolyzed upon ingestion to the unstable aglycon divicine, the autoxidation of which has been implicated in the onset of hemolysis in favism, possibly via production of superoxide and hydrogen peroxide. The autoxidation of divicine proceeds through a series of reactions involving the formation of a radical species. In this study divicine radicals were produced either by incubation of vicine with β-glucosidase or by boiling vicine in hydrochloric acid. On the basis of electron spin resonance spectra, it was shown that the two treatments produce different radicals. By spectral simulation the acid-produced radical was demonstrated to be a deaminodivicine. The autoxidation rates of the two radicals were determined from the disappearence of their electron spin resonance signals in the presence of air: at physiological pH the enzymatically produced divicine radical was much more stable to oxygen than the chemically produced radical. The two radicals may thus be expected to behave differently in a biological system. The repercussions of these findings could be considerable, given that most of the pharmacological and biochemical studies on vicine action have been done with the chemically produced compound, which is shown here to be an unphysiological intermediate.

Vicine is a pyrimidine glucoside found in high amounts in broad beans (*Vicia faba*) together with the related compound convicine. Upon ingestion vicine and convicine are hydrolyzed by a β-glucosidase, forming the unstable aglycons divicine and isouramil, respectively (Mager et al., 1965, 1980). Both compounds undergo rapid autoxidation with formation of O_2^- as an intermediate and of H_2O_2 as one of the products (Chevion et al., 1982; Winterbourn et al., 1986; Musci et al., 1987). In individuals affected by glucose-6-phosphate dehydrogenase deficiency this may lead to the acute hemolytic crisis known as favism, involving depletion of the glutathione pool and impairment of the functioning of several key enzymes in the erythrocytes (De Flora et al., 1984; Benatti et al., 1985a; Mavelli et al., 1984, 1985). So far most attention has been given to the interaction of vicine with red blood cells, but effects on liver mitochondria have recently been reported (Graf et al., 1985), indicating a more widespread action of the drug.

The details of the metabolism of vicine and convicine, as well as the biochemical reactions causing favism, are not very well understood. As convicine is not commercially available, almost all studies in this field have been done with vicine. Two different methods have been used to produce divicine from vicine in vitro, either enzymatic cleavage by β-glucosidase or chemical hydrolysis in HCl at 100 °C. When chemically made divicine is used, the autoxidation by oxygen can be followed with a spectrophotometer; in several recent studies the kinetics of the process under various conditions have been reported. However, it was not possible to confirm the presence of a divicine radical with ESR.[1] In contrast, Albano et al. (1984) reported the ESR spectrum of a radical formed during the enzymatic breakdown of vicine, but this method is not well suited for optical studies due to the high concentration of enzyme necessary. Because of the poor resolution of the ESR spectrum, it was not possible to identify the radical; furthermore, the disappearence of the ESR signal, presumably caused by reaction with oxygen, showed a time dependence that seemed inconsistent with the oxidation rates otherwise found (Chevion et al., 1982; Benatti et al., 1984; Graf et al., 1985; Winterbourn et al., 1986; Musci et al., 1987).

To resolve these ambiguities, we have used ESR spectroscopy to study and identify the radicals prepared by chemical as well as by enzymatic hydrolysis of vicine; we have also developed a technique to measure the oxidation kinetics under identical conditions for both preparation methods.

Materials and Methods

Vicine (2,4-diamino-6-hydroxy-5-pyrimidinyl β-D-glucopyranoside) was purchased from Serva (Heidelberg, FRG) and β-glucosidase (Cat. No. G8625, Type II) from Sigma (Deisenhofen, FRG). For the chemical preparation of divicine typically 0.1 mmol of vicine in 1 mL 1 M HCl was sealed under vacuum in a Thunberg tube and heated at 100 °C for 10 min. After cooling to room temperature, the tube was opened under a stream of N_2, and a small volume of 5 M NaOH was added to bring the pH to a value between 6 and 12, whereafter the sample was transferred rapidly to a standard quartz flat cell for ESR measurements. Oxygen was allowed to enter the flat section by diffusion only. Following the measurements, the samples were retrieved for pH determination.

Enzymatically prepared divicine was made by adding 5 mg of β-glucosidase to 1 mL of an N_2-flushed solution of 10 mM vicine in 50 mM NaH_2PO_4 buffer, pH 5.0, in a small test tube. After flushing with N_2 for 10 min, the tube was sealed and incubated for 30 min at room temperature; upon opening, the

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[1] Abbreviations: ESR, electron spin resonance.

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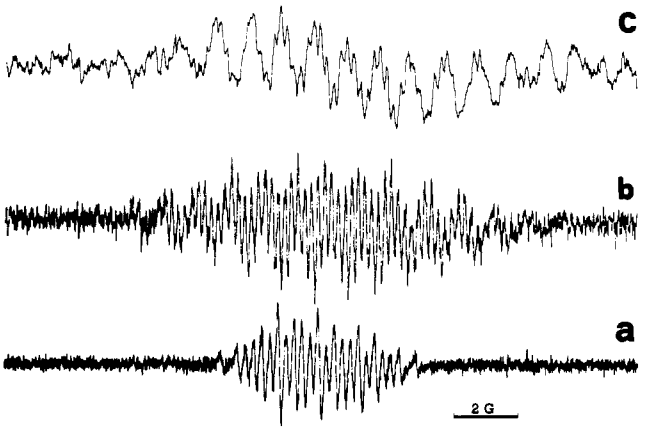

FIGURE 1: (a) High-resolution ESR spectrum of the radical produced by acid hydrolysis of vicine. (b,c) Experimental ESR spectra of the enzymatically produced divicine radical. The spectrometer settings were as follows: modulation amplitude 0.25 G, microwave power 2 mW, 16 scans accumulated in (b); modulation amplitude 0.1 G, microwave power 20 mW, 102 scans accumulated in (c). All other conditions as described under Materials and Methods.

sample was transferred immediately to a quartz flat ESR cell.

ESR measurements were made with a Bruker ESP300 spectrometer operating at 9.79 GHz, using a TM_{110}-mode cavity. High-resolution spectra were recorded with 0.1-G modulation amplitude at 100 kHz, 20-mW incident power, a time constant of 10–40 ms, and a scan time of 10–42 s for each 20 or 40 G scan (4096 data points). Normally 10–100 scans were accumulated until no further improvement of the signal to noise ratio was registered. High-frequency noise was removed by Fourier transform data analysis. Spectra for kinetics measurements were recorded by using a 320-ms time constant, 10-G modulation amplitude, and 50-mW microwave power. Under these conditions the hyperfine splittings were no longer seen, the spectrum consisting of a single broad line from which the time course of oxidation could be observed continuously or by repeated scanning.

Results and Discussion

The ESR spectra at pH 7.0 of the radicals produced from vicine by the two different preparation methods are shown in Figure 1; it is immediately obvious that the two radicals are not identical.

The chemically produced radical gives a well-resolved spectrum with relatively few lines (Figure 1a). On the other hand, the spectrum of the enzymatically produced radical displays a plethora of low-intensity lines, with the wings of the spectrum disappearing below the instrumental noise (Figure 1b). When the enzymatically produced radical is observed under low-resolution conditions, we obtain a spectrum identical with that seen by Albano et al. (1984) (Figure 1c).

The pH dependence of the spectra of the two radicals is shown in Figures 2 and 3. The ESR spectra of the radical generated by chemical breakdown of vicine are essentially unchanged up to pH 9.5 (Figure 2b,c). Above this pH, the spectrum becomes more complex, and new lines show up (Figure 2d,e), possibly from the autoxidation of the enediols produced when glucose (which is always present in the mixture) undergoes Lobry de Bruyn transformations. The spectrum at neutral pH was computer fit to a radical possessing three nitrogens, two of which are magnetically equivalent, and two equivalent protons (Figure 2a). Figure 3 shows the ESR spectra of the enzymatically produced radical at pH between 7 and 11.5. It is clear that the spectrum displays significant changes above pH 9. Such modifications of the line pattern

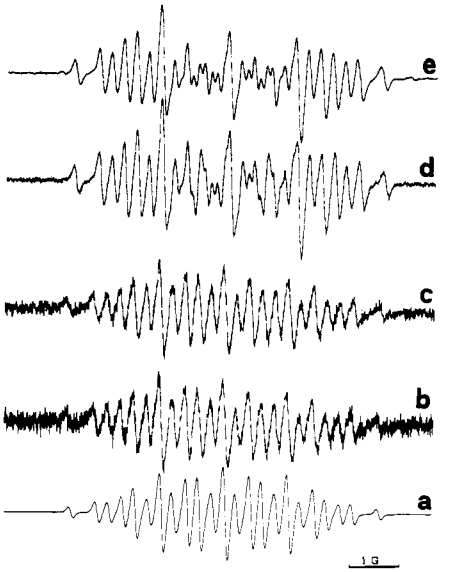

FIGURE 2: ESR spectra of the chemically prepared divicine radical at pH 7.0 (b), 9.5 (c), 10.0 (d), and 11.5 (e). (a) Corresponding computer simulation: $N_1 = 1.25$ G, $N_2 = N_3 = 0.52$ G, $H_1 = H_2 = 0.76$ G. Experimental conditions as in Figure 1a.

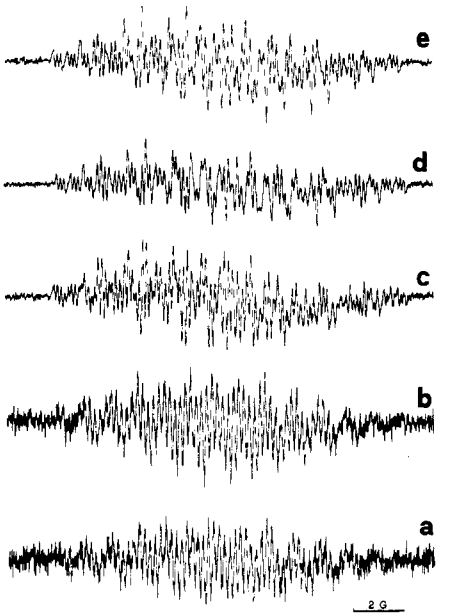

FIGURE 3: ESR spectra of the enzymatically generated divicine radical at pH 7.0 (a), 8.5 (b), 9.5 (c), 10.5 (d), and 11.5 (e). Experimental conditions as in Figure 1b.

are already evident at pH 9.5 (Figure 3c), where contributions from the enediol radicals are negligible (compare with Figure 2c). They thus reflect subtle changes in spin distribution, which are likely to depend on amino group deprotonation. From the spectrum of the enzymatically produced radical at neutral pH, it was not possible to make an unambiguous determination of the coupling constants to be used in computer simulations. However, the higher number of lines as well as the larger spectral width in the spectrum of the enzymatically versus the chemically generated radical clearly indicates that extra nuclei contribute to the observed splittings in the former case. The computer fit of the chemically made radical unambiguously shows that only three of the original four nitrogens are retained in the radical after hydrolysis in hydrochloric acid. It can therefore be suggested that this treatment not only releases the glucose moiety but also removes the magnetic coupling of an amino group to the unpaired electron. From

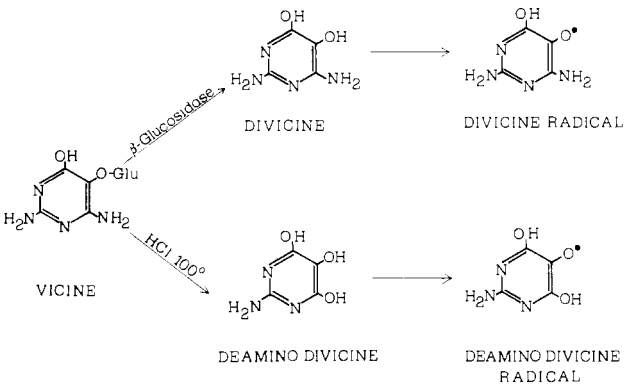

FIGURE 4: Scheme with the structure of the compounds involved in the autoxidation of the enzymatic or chemical cleavage of vicine.

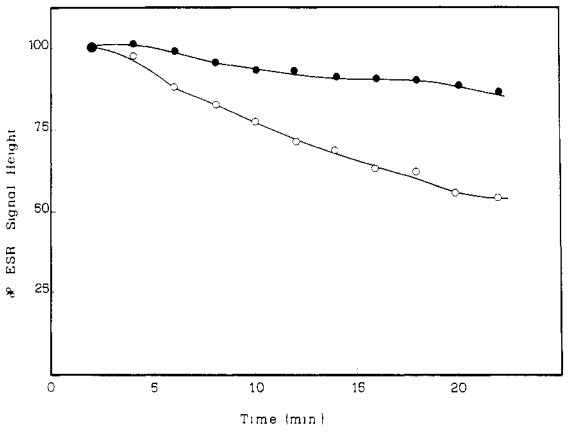

FIGURE 5: Time-dependent decay of the radicals produced by the two different preparation methods at pH 7.0. The disappearence of the ESR signal was followed as described under Materials and Methods; the flat cell was left untouched during the experiments. Typical results from one experiment are shown for the radical produced by β-glucosidase (●) or by HCl hydrolysis (○).

the magnitude and distribution of the coupling constants (see Figure 2a), one can predict that the amino group in position 4 has been lost during the acid cleavage. As a matter of fact, this is the only way to obtain a molecule with two magnetically equivalent nitrogens on the ring and an extra nitrogen with a weaker spin density, besides the two equivalent protons of the amino group. Figure 4 shows the structures of the compounds that are likely to be involved in the formation of the two radicals. The details of the reaction scheme are not well-known; unfortunately, the degradation products of the radicals are unstable themselves and have so far evaded characterization by traditional analytical techniques. It is worth noting that the chemical cleavage does not generate isouramil, the other aglycon involved with divicine in the onset of hemolysis in favism. Isouramil is still a deaminodivicine, but lacks the amino group substituent in position 2.

The question is now whether the two radicals differ in their reactivities. The time-dependent disappearance of the ESR signals of the radicals was studied by using extreme spectrometer settings, reducing the spectrum to one broad line and thereby allowing a direct comparison of the rates of decay. Although the exact time course varied somewhat from sample to sample, due to the uncontrolled oxygen diffusion in the ESR flat cell, there is a pronounced difference in the stability of the two radicals. At physiological pH, the enzymatically produced radical is much more stable than the chemically generated radical (Figure 5). At high pH, the stability of the two species tends to converge and both radicals decay rather quickly.

In previous studies preparation of divicine using β-glucosidase has been applied only in a few cases (De Flora et al., 1983; Mavelli et al., 1984, 1985). In particular, all our current knowledge of the reaction mechanism of divicine has been obtained in studies using the chemically produced compound, which should be considered an artifactual intermediate that does not occur under physiological conditions. It therefore becomes obvious that the reaction mechanism of vicine needs to be reinvestigated. The ESR technique used here will be a powerful tool for the forthcoming studies in this area. In fact, one direct observation can be made already: the isotropic ESR spectrum shows that the divicine radical is not bound to the enzyme, in spite of the high protein concentrations used. An interesting approach could be represented by isouramil; having only one amino group, this compound might be more stable toward the acid treatment. However, the only reported attempt to detect an ESR signal was unsuccessful (Chevion et al., 1982).

In conclusion, we have succeeded for the first time in getting high-resolution ESR spectra of the radicals produced by enzymatic or chemical degradation of vicine. The spectral analysis demonstrates that the widely used chemically produced divicine is an unphysiological compound, which is less stable than the naturally occurring divicine radical. It remains to be established to what extent the previous work on the vicine reaction mechanism and the actual involvement of O_2^- and H_2O_2 in its in vivo effects should be reconsidered.

Registry No. HCl, 7647-01-0; vicine, 152-93-2; divicine radical, 116265-59-9; deamino divicine radical, 116265-60-2; β-glucosidase, 9001-22-3.

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