Characterization of Different Commercial Soybean Peroxidase Preparations and Use of the Enzyme for N-Demethylation of Methyl *N*-Methylanthranilate To Produce the Food Flavor Methylanthranilate

Marjon J. H. Van Haandel,[†] Florence C. E. Sarabèr,[†] Marelle G. Boersma,[†] Colja Laane,[†] Yvette Fleming,[†] Hugo Weenen,[‡] and Ivonne M. C. M. Rietjens*[,†]

Department of Biomolecular Sciences, Laboratory of Biochemistry, Wageningen University, Dreijenlaan 3, 6703 HA Wageningen, The Netherlands, and Quest International, Huizerstraatweg 28, 1411 GP Naarden, The Netherlands

The potential of different peroxidase preparations for the N-demethylation of methyl *N*-methylanthranilate to produce the food flavor methylanthranilate (MA) was investigated. All tested peroxidase preparations were able to catalyze the N-dealkylation. The tested soybean preparations vary widely with respect to their heme content. Furthermore, the operational stability of purified soybean peroxidase (SP) is at least 25-fold lower than that of horseradish peroxidase and only 5-fold higher than that of microperoxidase 8. Thus, the presence of a large protein chain around a porphyrin cofactor in a peroxidase is, by itself, insufficient to explain the observed differences in operational stability. Despite its relatively low operational stability, SP proved to be the most efficient biocatalyst for the production of MA with high yield and purity, especially observed at the high temperature and low pH values at which SP appeared to be optimally active.

Keywords: *Soybean peroxidase; methyl N-methylanthranilate; food flavor; N-demethylation*

INTRODUCTION

Heme-based enzymes such as the peroxidases and the mammalian cytochromes P450 are among the most versatile enzymes in biochemistry, able to catalyze the conversion of a wide range of substrates in many different types of reactions. Among these are aromatic and aliphatic hydroxylations, heteroatom dealkylations, epoxidations, (de)halogenations, and heteroatom oxygenations (Dawson, 1988; Griffin, 1991; Ortiz de Montellano, 1992). From a biotechnological point of view the use of peroxidases is favored over that of cytochromes P450 because peroxidases can be more easily obtained and use H_2O_2 as a cheap and clean oxidant to create the so-called high-valent-iron-oxo-porphyrin intermediates required for catalysis (Chance, 1952; Dolphin et al., 1971; Groves et al., 1981; Hoffman et al., 1979; Low et al., 1996; Palcic et al., 1980; Thomas et al., 1970). Many different peroxidases are known (Diehn, 1993; Dunford and Stillman, 1976; Patterson and Poulos, 1995; Schuller et al., 1996; Sessa and Anderson, 1981). Of all these peroxidases horseradish peroxidase (HRP) is the one best studied from mechanistic and catalytic points of view (Dunford, 1991; Jones and Suggett, 1968a,b; Rodriguez-Lopez et al., 1996a,b; Van Haandel et al., 1998). However, biotechnological application of HRP is seriously hampered by the fact that the enzyme is relatively expensive and has a limited thermostability (McEldoon and Dordick, 1996). In contrast to HRP, soybean peroxidase (SP) has been reported to show better temperature behavior (McEldoon et al., 1995; McEldoon and Dordick, 1996; Toiguchi et al., 1989). Furthermore, SP is more economical for use in biotechnological applications because it can be obtained relatively easily from soybean hulls, a major byproduct of the food industry. Finally, microperoxidase 8 (MP8), being a heme-based minienzyme with a potentially wide substrate specificity and able to function at relatively high temperatures as well (Aron et al., 1986; Cunningham et al., 1991; Osman et al., 1996), may be another peroxidase of industrial interest.

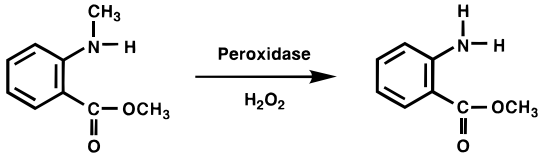

Figure 1. N-Dealkylation of MNMA to MA, the model reaction of the present study.

On the basis of these considerations, the objective of the present study was to investigate the biocatalytic potential of SP, HRP, and MP8 for the production of fine chemicals. Literature data report on a wide range of soybean preparations and isoenzymes (Diehn et al., 1993; McEldoon et al., 1995; Gijzen et al., 1993; Sessa and Anderson, 1981) and, therefore, we compared different commercially available samples with respect to their heme content and number of heme-containing proteins, to determine the best source for SP purification. As a model reaction the N-demethylation of methyl *N*-methylanthranilate (MNMA), leading to the production of methylanthranilate (MA) (Figure 1), was used. This model reaction was chosen for the following

* Author to whom correspondence should be addressed (telephone 31-317-482868; fax 31-317-484801; e-mail ivonne.rietjens@p450.bc.wau.nl).

[†] Wageningen University.

[‡] Quest International.

10.1021/jf9909656 CCC: $19.00
Published on Web 04/19/2000

reason: MNMA from citrus leaves is readily available and a relatively cheap source, whereas MA is more expensive than MNMA. Therefore, the investigated reaction provides an industrially relevant route for the natural production of an important topnote flavor in Concord grape.

MATERIALS AND METHODS

Chemicals. MNMA (ex. citrus leaves), MA (ex. grapes), *N*-methylanthranilate, and anthranilate were obtained from Quest International (Naarden, The Netherlands). H_2O_2 (30% in water) was from Merck (Darmstadt, Germany). HRP and cytochrome *c* from horse heart were obtained from Boehringer (Mannheim, Germany). MP8 was prepared by proteolytic digestion of horse heart cytochrome *c* essentially as described before (Aron et al., 1986; Kraehenbuhl et al., 1974). Dried soybeans were obtained from Mervo Products (Hengelo, The Netherlands). Soybean flakes consisting of brown flakes made of soybean hull were obtained from Quest International. Soybean pellets consisting of brown dried pellets from soybean hull and soybean extract were obtained from ADUMIN (Tel Aviv, Israel). Soybean flour was a pale yellow flour-like powder commercially obtained as provaflor soybean flour from Cargill (Amsterdam, The Netherlands). SP was either purified or obtained from Enzymol International (Columbus, OH). HPLC analysis proved both purified SP preparations to be identical.

Solubilization of the Different Soybean Samples. The different soybean samples were preincubated at *x*% (w/v) (x = 1 for extract and flour, 17 for pellets, and 10 for flakes) in 0.1 M potassium phosphate (pH 7.6) for 1 h at 0 °C and then homogenized by mixing during 2 min using a vortex. The mixture obtained was centrifuged using an Eppendorf centrifuge for 10 min at 4 °C (13000*g*). The supernatant thus obtained was used for further characterization of the heme content, measurement of peroxidase activity, and/or purification of SP. For the dry beans a different procedure was followed. This sample was preincubated at 20% (w/v) in 0.1 M potassium phosphate (pH 7.6) for 2.5 h and then homogenized during 15 min at maximum speed using a blender (Braun, Frankfurt, Germany). The mixture obtained was centrifuged for 10 min at 4 °C (16000*g*). The supernatant obtained was filtered using glasswool and then centrifuged for 20 min at 4 °C (27000*g*). The supernatant thus obtained was used for further characterization of the heme content and measurement of peroxidase activity. On the basis of the solubilization protocol used, the studied soybean fractions contain especially soluble forms of peroxidases.

Purification of SP. Soybean extract was used as the starting material and prepared as described above only using 20 mM Tris-HCl (pH 7.4). Purification was performed by a single affinity chromatography step on concavalin A−Sepharose (Pharmacia Biotech, Uppsala, Sweden), performed essentially as described previously (Gillikin and Graham, 1991; Sessa and Anderson, 1981; Toiguchi et al., 1989). The column was equilibrated with 20 mM Tris-HCl (pH 7.4) containing 0.5 M NaCl, 1 mM $CaCl_2$, and 1 mM $MnCl_2$ and eluted with a gradient of 0−100% 0.5 M methyl α-D-mannopyranoside (Sigma, St. Louis, MO) in the same buffer. Fractions were analyzed for absorption at A_{280nm} and A_{395nm} and for peroxidase activity using guaiacol as the substrate. Peroxidase-containing fractions were pooled, desalted, and concentrated using an Amicon 8010 filter apparatus with a YM-10 filter and dialyzed overnight against 20 mM Tris-HCl (pH 7.4). The purity of the final sample was analyzed by HPLC and SDS−PAGE (Schägger and von Jagow, 1987), and its specific activity was determined as described below.

Incubations with MNMA. Incubations with MNMA were performed in (final concentrations) 0.1 M potassium acetate (pH 4) for SP or in 0.1 M potassium phosphate (pH 7.6) for HRP and MP8, containing 5 μM heme protein and 1 mM MNMA added as 1% (v/v) of a 0.1 M stock solution in dimethyl sulfoxide. To some incubations was added ascorbic acid at a final concentration of 3 mM. The incubation mixture was preincubated for 2 min at 70 °C for SP and at 37 °C for HRP and MP8 (unless indicated otherwise). The reaction was started by the addition of H_2O_2 (2.5 mM final concentration). The incubation was carried out for 1 min (unless indicated otherwise) and stopped by freezing the sample into liquid nitrogen. Samples were stored in liquid nitrogen until analyzed and defrozen by centrifugation in an Eppendorf centrifuge (5 min; 13000*g*; 4 °C) prior to analysis by HPLC.

HPLC. HPLC chromatography of SP samples solubilized as described above and of solutions of purified SP, HRP, and MP8 was performed on a Bakerbond wide-pore butyl RP-7116-00 column (4.6 × 250 mm) (J. T. Baker Research Products, Deventer, The Netherlands). Elution was performed at a flow of 1 mL/min using a linear gradient from 0.1% trifluoroacetic acid in 100% nano pure to 0.1% trifluoroacetic acid in 50% water plus 50% acetonitrile in 50 min. Detection was performed at 210−450 nm using a photodiode array detector (Waters 996). HPLC analysis of the incubations for N-demethylation of MNMA was performed on a reversed-phase Lichrosphere RP8 column (4.6 × 150 mm) (Alltech, Breda, The Netherlands) using a linear gradient from 0 to 80% methanol in water in 26 min at a flow of 1.0 mL/min. Detection was at 332 nm using a diode array detector (Waters 996). Products were identified and quantified using commercially available standards. The concentration of MA was quantified using a calibration curve consisting of the HPLC peak area plotted against the concentration of the compound in the injected sample.

Determination of Heme and Protein Content. For the sample containing purified SP, the heme concentration was determined by using the pyridine−chromogen method (Aron et al., 1986). The heme content of solubilized SP samples was generally low compared to the other proteins present. This hampered the heme determination by the pyridine−chromogen method. Heme determination for these samples was done using HPLC and a calibration curve made with SP from Enzymol.

Protein was measured according to the method of Bradford (Sigma) using bovine serum albumin (Boehringer Mannheim, Germany) as the standard.

Peroxidase Activity with Guaiacol. Peroxidase activity was detected on the basis of oxidation of guaiacol as described by Gillikin and Graham (1991). The assay was performed in 50 mM potassium acetate (pH 5.5) at 25 °C and contained (final concentrations) 8 mM guaiacol, 0.5 mM H_2O_2, and 2 nM peroxidase. The reaction was initiated by the addition of H_2O_2.

RESULTS

Heme Protein Content of Different Soybean Samples. Figure 2a presents the HPLC chromatogram of soybean extract with detection at 280 nm for protein content and—more importantly—at 395 nm for heme-containing proteins. The HPLC chromatogram of soybean extract contains one major heme-containing peak with retention time of 49.2 min. Table 1 summarizes the results from this and similar HPLC analyses of different types of commercially available soybean preparations. The different samples vary considerably with respect to their heme protein compositions, although especially the peak at 49.2 min and, to a minor extent, the peak at 55.1 min are present in most of the samples. The results obtained indicate either the presence of different heme-containing proteins or the presence of different degradation or glycosylated products of one major heme protein in the different commercial samples.

In addition to the heme protein composition, the amount of heme protein present in the various soybean preparations could be derived from these HPLC data. Because direct measurement of heme content by spectroscopic methods was hampered by the high amount of other proteins present in the samples, the heme content was derived from the HPLC data using a

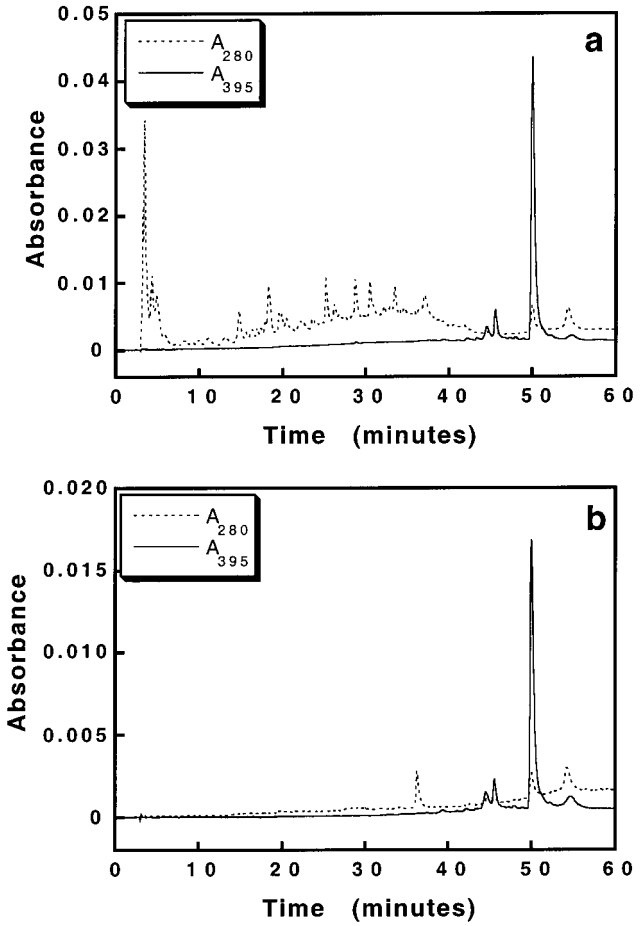

Figure 2. HPLC chromatogram of (a) soybean extract and (b) purified SP from soybean extract. Detection was at 280 and 395 nm.

calibration curve made on the basis of commercially available SP. Table 1 presents the heme contents of the different samples. Marked differences are observed. The soybean extract especially contains a relatively large amount of heme proteins, suggesting that the extraction procedure was directed at selective enrichment of soluble and/or heme proteins. Also of interest is that both soybean flakes as well as soybean pellets contain about the same amount of heme as the solubilized fraction from dried soybeans, whereas the soybean flour was relatively rich in heme content.

Altogether the data indicate large differences among the various soybean preparations. On the basis of heme content per gram of soybean preparation, the soybean extract shows the highest specific heme content, and, therefore, the best starting material for purification of SP. In addition, one heme-containing peak dominates in the soybean extract, namely, the component with HPLC retention time of 49.2 min, also dominant in the least processed sample, the dried soybeans, and present in most of the commercially available SP preparations.

Purification of SP from Soybean Extract. Figure 2b presents the HPLC chromatogram (detection at 280 and 395 nm) of SP purified from soybean extract using affinity chromatography on concavaline A−Sepharose. Comparison of this chromatogram to the one presented in Figure 2a indicates the significant increase in purity of the heme-containing protein. The specific activity of the final SP preparation was 8032 units/mg of protein as measured with guaiacol as the substrate. The purification factor of the final SP preparation was 10. On SDS−PAGE, the purified SP showed one prominent protein band of 37 kDa.

Table 1. Composition of Heme-Containing Proteins in Various Commercial Soybean Preparations As Determined by HPLC Analysis with Detection at 395 nm[a]

sample (obtained from)	heme content, nmol of heme/ g of sample	retention time of peaks at 395 nm, min	% of total peak area at 395 nm
soybean extract (ADUMIN)	1291	43.8	2.8
		44.9	8.0
		49.2	80.7
		55.1	4.5
		Σ others	4.0
soybean flakes (QUEST)	2	28.2	18.8
		49.2	28.8
		55.1	52.4
		Σ others	0.0
soybean pellets (ADUMIN)	6	3.0	72.0
		49.2	25.6
		55.1	2.4
		Σ others	0.0
soybean flour (Cargill)	75	33.3	53.6
		55.1	46.4
		Σ others	0.0
dried soybeans (Mervo Products)	8	33.3	24.0
		49.2	76.0
		Σ others	0.0

[a] Only those peaks with an area >2% of the total intensity are specified.

Influence of pH and Temperature. The pH optimum was determined for the conversion of MNMA to MA by purified SP and for the peroxidase activity of SP assayed with guaiacol. In both cases SP has a pH optimum at 4. At pH 4 the temperature dependence was also investigated. Reaction rates increased with increasing temperature up to an optimum at 70 °C. Blank incubations without H_2O_2 or without SP showed no product formation at all pH values and temperatures tested. On the basis of these results all subsequent incubations with SP were performed at 70 °C and pH 4.

N-Demethylation of MNMA As Catalyzed by Different Peroxidases. Figure 3 presents the HPLC chromatograms of an incubation of MNMA with purified SP and, for comparison, with similar concentrations of HRP and MP8. All assays were performed at the pH optimum of the respective enzymes (Dunford, 1991; Osman et al., 1996). The temperature used was 37 °C for HRP because of its thermoinstability (McEldoon and Dordick, 1996) and 70 °C for MP8 and SP. Formation of MA is observed in all cases. Formation of *N*-methylanthranilate (Figure 3, arrow 2) or of anthranilate (Figure 3, arrow 1) is not observed.

The results obtained with the different peroxidase samples vary not only with respect to the amount of MA formed but also with respect to the amount and nature of the side products observed. From control experiments we can state that this difference in secondary product composition was not linked to the different pH and temperature conditions used for each enzyme. Comparison of the chromatograms in Figure 3 reveals that SP proved to be the most efficient biocatalyst for the N-demethylation of MNMA to MA. Measurement of the MNMA demethylation by SP at 37 °C results in only

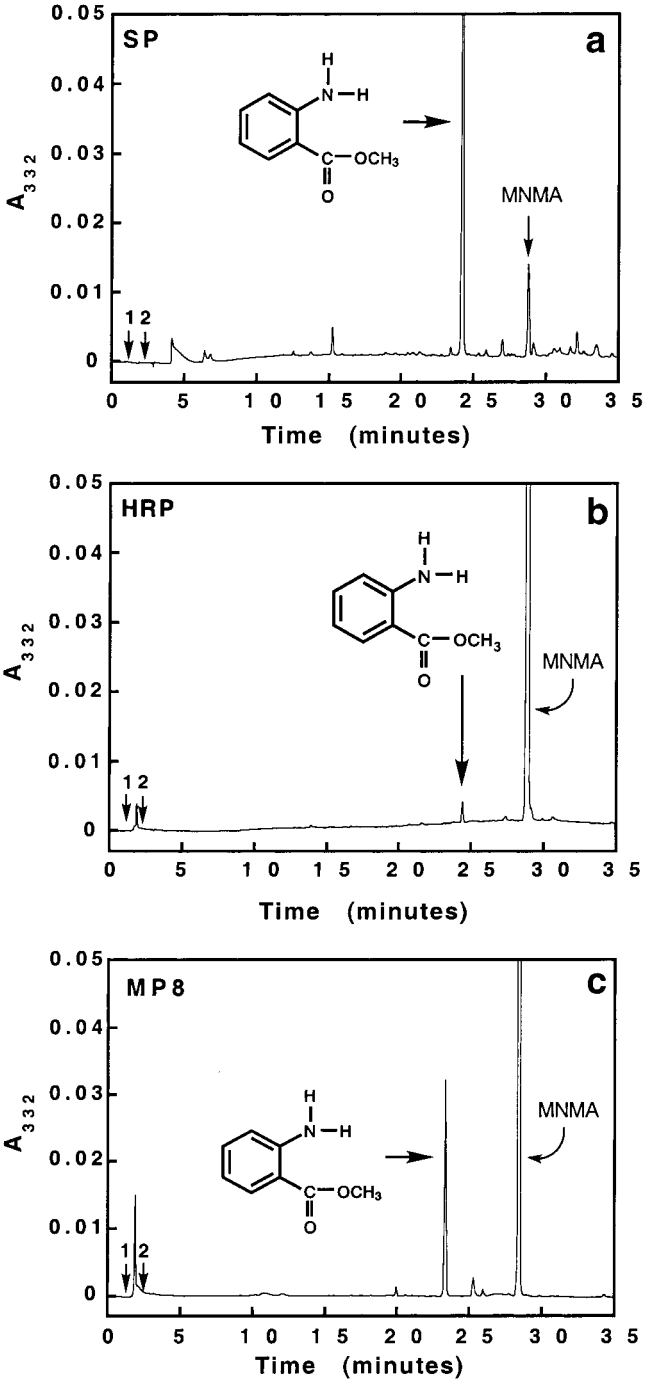

Figure 3. HPLC chromatograms of the incubation of MNMA with H_2O_2 and (a) SP purified from soybean extract, (b) HRP, and (c) MP8. The concentration of the enzymes was 5 μM. The incubations were performed during 1 min at 70 °C and pH 4 for SP, at 37 °C and pH 7.6 for HRP, and at 70 °C and pH 7.6 for MP8. The substrate MNMA is eluted after 29 min, whereas the product MA is eluted after 24 min. Arrows 1 and 2 indicate where anthranilate and *N*-methylanthranilate, respectively, should elute.

17% of the amount of MA formed at 70 °C (data not shown), whereas measurement of the MNMA demethylation by MP8 at 37 °C results in 63% of the amount of MA formed at 70 °C (data not shown). This illustrates that the relatively high potential of SP as compared to MP8 and especially HRP is related to the ability of the enzyme to function at relatively high temperature. Another important observation is that upon conversion

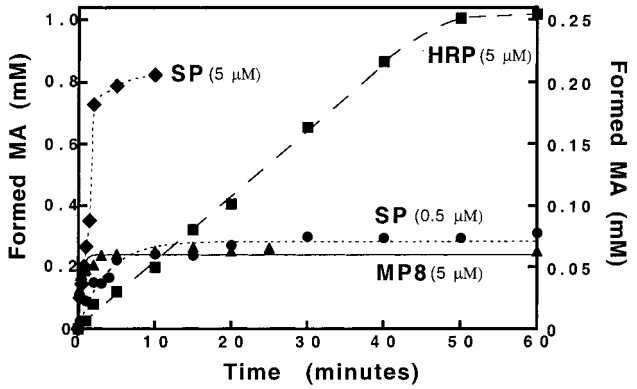

Figure 4. Operational stability of purified SP (···) (left *Y*-axis), HRP (- - -) (right *Y*-axis), and MP8 (—) (right *Y*-axis) in the conversion of MNMA (SP at 70 °C and pH 4; HRP and MP8 at 37 °C and pH 7.6). The enzyme concentration was 5 μM in all cases. As the MNMA conversion by 5 μM SP was hampered by substrate limitation, the operational stability of SP was determined also with 0.5 μM SP (●) (right *Y*-axis).

of MNMA to MA by SP the amount of side products compared to the amount of MA formed seems low (Figure 4a). Upon complete conversion of MNMA the yield of SP-catalyzed MA production amounted to 82% within 10 min under the conditions specified.

Operational Stability. Figure 4 presents the formation of MA from MNMA as catalyzed by the different peroxidases in time. Due to its relatively high activity SP had to be analyzed for operational stability at a concentration 10 times lower than MP8 and HRP. However, measurement of SP operational stability at different concentrations of the enzyme (1, 0.5, and 0.2 μM) showed no significant influence of SP concentration on its operational stability.

To describe operational stability in a quantitative way, eq 1, describing the kinetics of inactivation as

$$d[P]/dt = v = V_{max}\, e^{(-k_i t)} \qquad (1)$$

reported by Spee et al. (1995), can be applied. Using this equation to analyze our data provides k_i values of 0.5 $\pm$ 0.1, 0.02 $\pm$ 0.004, and 2.6 $\pm$ 0.4 min^{-1} for SP, HRP, and MP8, respectively. This inactivation constant, k_i, gives information about the stability of the different peroxidases: a high k_i means low operational stability. The operational stability of SP is 5-fold higher than that observed for MP8, but at least 25-fold lower than that of HRP. Furthermore, the operational stability of SP in soybean extract (before purification) was comparable to that for purified SP (data not shown).

Addition of Ascorbate. Previous studies reported the mechanism of peroxidase-catalyzed heteroatom dealkylation to be either cytochrome P450-like or peroxidase-like (Kedderis and Hollenberg, 1983; Walker-Griffin et al., 1978). To investigate the mechanism of the SP-catalyzed N-demethylation of MNMA, experiments were performed in the presence of ascorbate, known to effectively block peroxidase-type of reaction chemistry but not MP8-catalyzed cytochrome P450 type conversion of aniline to *p*-aminophenol (Osman et al., 1996). Peroxidase-type of reaction chemistry is defined as H_2O_2-dependent initial one-electron oxidation of the substrate (Dunford et al., 1976).

Upon addition of 3 mM ascorbate to the incubations of the present study, the production of MA by SP as well

as the peroxidase activity assayed with guaiacol appeared to be fully inhibited.

DISCUSSION

The present work describes the possibilities and limitations for using commercially available SP preparations and other peroxidases for the production of the food flavor MA from MNMA.

SP was isolated from soybean extract, purified, and characterized. The peroxidase-catalyzed conversion of guaiacol and of MNMA to MA at low pH is in line with early reports of peroxidase-catalyzed conversions observed at even lower pH values, which supported the notion that SP is resistant to acidic conditions and holds onto its heme more tightly than does HRP (McEldoon et al., 1995). Comparison of the pH optimum observed in the present study to pH optima reported in the literature (McEldoon et al., 1995; Schmitz et al., 1997; Sessa and Anderson, 1981; Toiguchi et al., 1989) suggests that the actual pH optimum observed for SP may vary between 2 and 6 depending to some extent on the substrate and assay conditions used. Alternatively, this might be due to the fact that SP used in the different studies was obtained from different soybean sources, resulting in purification of different isoenzymes. The temperature optimum of 70 °C for the conversion of MNMA to MA by SP and for the conversion of guaiacol by SP is in line with results reported by others (McEldoon et al., 1995; McEldoon and Dordick, 1996; Toiguchi et al., 1989), who found temperature optima of 40−60 °C for other assays and SP inactivation to occur above 80 °C. The observation in the present study that the pH and temperature effects on demethylation of MNMA are similar to the pH and temperature effects on peroxidase activity assayed with guaiacol corroborates the conclusion that the demethylation proceeds by a peroxidase-type of reaction mechanism.

Because MNMA from citrus leaves is a relatively cheap source and MA is more expensive than MNMA, the investigated reaction provides a route for the natural production of the Concord grape flavor. The observation that ascorbate fully inhibits the SP-catalyzed N-demethylation of MNMA also corroborates the hypothesis that the N-dealkylation proceeds by a peroxidase-type of reaction mechanism and not by a cytochrome P450-type of reaction mechanism (Hollenberg et al., 1985; Kedderis and Hollenberg, 1983; Miwa et al., 1983; Nakamura et al., 1992; Pandey et al., 1989). The observation of peroxidase-catalyzed N-demethylation is in line with literature data, which report peroxidase-catalyzed N-dealkylations of other N-alkylated substrates (Kedderis and Hollenberg, 1983; Walker-Griffin et al., 1978).

In line with other peroxidases, and especially MP8, SP showed a limited operational stability. This limited operational stability of MP8 is known from previous studies (Osman et al., 1996) and has been ascribed to its open active site, providing possibilities for μ-oxo dimer formation as well as for intermolecular inactivating reactions occurring once the heme catalyst becomes activated to its high-valent-iron-oxo-porphyrin form upon reaction with H_2O_2 (Balch et al., 1984; Bonnet and McDonagh, 1973; Brown et al., 1978; Kaim and Schwederski, 1994). The fact that SP, with its full polypeptide chain, shows a limited operational stability ($k_i = 0.5 \pm 0.1$ min^{-1}), only 5-fold higher than that observed for MP8 ($k_i = 2.6 \pm 0.4$ min^{-1}), suggests that the nature of the inactivation mechanism could be intramolecular. Because HRP ($k_i = 0.02 \pm 0.004$ min^{-1}) showed an operational stability at least 25 times higher than that observed for SP and 130 times higher than that observed for MP8, the present results with SP indicate that the presence of a large protein chain around the porphyrin cofactor in a peroxidase is, by itself, insufficient to explain the observed differences in operational stability.

Despite its relatively low operational stability, SP proved to be the most efficient biocatalyst for the production of MA with high yield and purity. This potential of SP to catalyze the N-demethylation of MNMA to MA more efficiently than HRP and MP8 was especially observed at high temperature and low pH values, at which SP appeared to be optimally active.

ABBREVIATIONS USED

SP, soybean peroxidase; MP8, microperoxidase 8; HRP, horseradish peroxidase; MNMA, methyl *N*-methylanthranilate; MA, methylanthranilate.

LITERATURE CITED

Aron, J.; Baldwin, D. A.; Marques, H. M.; Pratt, J. M.; Adams, P. A. Hemes and hemoproteins. 1. Preparation and analysis of the heme-containing octapeptide (microperoxidase-8) and identification of the monomeric form in aqueous solution. *J. Inorg. Biochem.* **1986**, *27*, 227−243.

Balch, A. L.; Chan, Y. W.; Cheng, R. J.; La Mar, G. N.; Latos-Grazynski, L.; Renner, M. W. Oxygenation patterns for iron(II) porphyrins. Peroxo and ferryl ($Fe^{IV}O$) intermediates detected by ^1H nuclear magnetic resonance spectroscopy during the oxygenation of (tetramesitylporphyrin)iron(II). *J. Am. Chem. Soc.* **1984**, *106*, 7779−7785.

Bonnet, R.; McDonagh, A. F. The meso-reactivity of porphyrins and related compounds. Part VI. Oxidative cleavage of the haem system. The four isomeric biliverdins of the IX series. *J. Chem. Soc., Perkin. Trans. 1* **1973**, 881−888.

Brown, S. B.; Hatzikonstantinou, H.; Herries, D. G. The role of peroxide in haem degradation. A study of the oxidation of ferrihaems by hydrogen peroxide. *Biochem. J.* **1978**, *174*, 901−907.

Chance, B. The kinetics and stoichiometry of the transition from the primary to the secondary peroxidase peroxide complexes. *Arch. Biochem.* **1952**, *41*, 416−424.

Cunningham, I. D.; Bachelor, J. L.; Pratt, J. M. Kinetic study of the H_2O_2 oxidation of phenols, naphtols and anilines catalyzed by the heme octapeptide microperoxidase-8. *J. Chem. Soc., Perkin Trans. 2* **1991**, 1839−1843.

Dawson, J. H. Probing structure−function relations in heme-containing oxygenases and peroxidases. *Science* **1988**, *240*, 433−439.

Diehn, S. H.; Burkhart, W.; Graham, J. S. Purification and partial amino acid sequence of a wound-inducible developmentally regulated anionic peroxidase from soybean leaves. *Biochem. Biophys. Res. Commun.* **1993**, *195*, 928−934.

Dolphin, D.; Forman, A.; Borg, D. C.; Fajer, J.; Felton, R. H. Compounds I of catalase and horseradish peroxidase: π-cation radicals. *Proc. Natl. Acad. Sci. U.S.A.* **1971**, *68*, 614−618.

Dunford, H. B. Horseradish peroxidase: structure and kinetic properties. In *Peroxidases in Chemsitry and Biology*; Everse, J., Everse, K. E., Grisham, M. B., Eds.; CRC Press: Boca Raton, FL, 1991; Vol. II, pp 1−24.

Dunford, H. B.; Stillman, J. S. On the function and mechanism of action of peroxidases. *Coord. Chem. Rev.* **1976**, *19*, 187−251.

Gijzen, M.; Van Huystee, R.; Buzell, R. I. Soybean seed coat peroxidase. A comparison of high-activity and low-activity genotypes. *Plant Physiol.* **1993**, *103*, 1061−1066.

Gillikin, J. W.; Graham, J. S. Purification and developmental analysis of the major anionic peroxidase from the seed coat of *Glycine max*. *Plant Physiol.* **1991**, *96*, 214−220.

Griffin, B. W. Chloroperoxidase: A review. In *Peroxidases in Chemistry and Biology*; Everse, J., Everse, K. E., Grisham, M. B., Eds.; CRC Press: Boca Raton, FL, 1991.

Groves, J. T.; Haushalter, R. C.; Nakamura, M.; Nemo, T. E.; Evans, B. J. High-valent iron-porphyrin complexes related to peroxidase and cytochrome P450. *J. Am. Chem. Soc.* **1981**, *103*, 2884−2886.

Haim, N.; Nemec, J.; Roman, J.; Sinha, B. K. Peroxidase-catalyzed metabolism of etoposide (VP-16−213) and covalent binding of reactive intermediates to cellular macromolecules. *Cancer Res.* **1987**, *47*, 5835−5840.

Hoffman, B. M.; Roberts, J. E.; Brown, T. G.; Kang, C. H.; Margoliash, E. Electron−nuclear double resonance of the hydrogen peroxide compound of cytochrome *c* peroxidase: Identification of the free radical site with a methionyl cluster. *Proc. Natl. Acad. Sci. U.S.A.* **1979**, *76*, 6132−6136.

Hollenberg, P. F.; Miwa, G. T.; Walsh, G. S.; Dwyer, L. A.; Rickert, D. E.; Kedderis, G. L. Mechanisms of N-demethylation reactions catalyzed by cytochrome P450 and peroxidases. *Drug Metab. Dispos.* **1985**, *13*, 272−275.

Jones, P.; Suggett, A. The catalase hydrogen peroxide system. Kinetics of catalytic action at high substrate concentrations. *Biochem. J.* **1968a**, *110*, 617−620.

Jones, P.; Suggett, A. The catalase hydrogen peroxide system. A theoretical appraisal of the mechanism of catalase action. *Biochem. J.* **1968b**, *110*, 621−629.

Kaim, W.; Schwederski, B. *Bioinorganic Chemistry: Inorganic Elements in the Chemistry of Life*; Wily: New York, 1994.

Kedderis, G. L.; Hollenberg, P. F. Characterization of the N-demethylation reactions catalyzed by horseradish peroxidase. *J. Biol Chem.* **1983**, *258*, 8129−8138.

Kraehenbuhl, J. P.; Galardy, R. E.; Jamieson, J. D. Preparation and characterization of an immuno-electron microscope tracer consisting of a heme octapeptide coupled to Fab. *J. Exp. Med.* **1974**, *139*, 208−223.

Low, D. W.; Winkler, J. R.; Gray, H. B. Photoinduced oxidation of microperoxidase-8: Generation of ferryl and cation-radical porphyrins. *J. Am. Chem. Soc.* **1996**, *118*, 117−120.

McEldoon, J. P.; Dordick, J. S. Unusual thermal stability of soybean peroxidase. *Biotechnol. Prog.* **1996**, *12*, 555−558.

McEldoon, J. P.; Pokora, A. R.; Dordick J. S. Lignin peroxidase-type activity of soybean peroxidase. *Enzymol. Microb. Technol.* **1995**, *17*, 359−365.

Meunier, G.; Meunier, B. Evidences for an efficient demethylation of methoxyellipticine derivatives catalyzed by a peroxidase. *J. Am. Chem. Soc.* **1985**, *107*, 2558−2560.

Miwa, G. T.; Walsh, J. S.; Kedderis, G. L.; Hollenberg, P. F. The use of intramolecular isotope effects to distinguish between deprotonation and hydrogen atom abstraction mechanisms in cytochrome P450 and peroxidase catalyzed N-demethylation reactions. *J. Biol. Chem.* **1983**, *258*, 14445−14449.

Nakamura, S.; Mashino, T.; Hirobe, M. ^{18}O Incorporation from $H_2{}^{18}O_2$ in the oxidation of *N*-methylcarbazole and sulfides catalyzed by microperoxidase-11. *Tetrahedron Lett.* **1992**, *33*, 5409−5412.

Ortiz de Montellano, P. R. Catalytic sites of hemoprotein peroxidases. *Annu. Rev. Pharmacol. Toxicol.* **1992**, *32*, 89−107.

Osman, A. M.; Koerts, J.; Boersma, M. G.; Boeren, S.; Veeger, C.; Rietjens, I. M. C. M. Microperoxidase/H_2O_2-catalyzed aromatic hydroxylation proceeds by a cytochrome P450-type oxygen-transfer reaction mechanism. *Eur. J. Biochem.* **1996**, *240*, 232−238.

Palcic, M. M.; Rutter, R.; Araiso, T.; Hager, L. P.; Dunford, H. B. Spectrum of chloroperoxidase compound I. *Biochem. Biophys. Res. Commun.* **1980**, *94*, 1123−1127.

Pandey, R. N.; Armstrong, A. P.; Hollenberg, P. F. Oxidative N-demethylation of *N*,*N*-dimethylaniline by purified isozymes of cytochrome P450. *Biochem. Pharmacol.* **1989**, *38*, 2181−2186.

Patterson, W. R.; Poulos, T. L. Crystal structure of recombinant pea cytosolic ascorbate peroxidase. *Biochemistry* **1995**, *34*, 4331−4341.

Rodriguez-Lopez, J. N.; Smith, A. T.; Thorneley, R. N. F. Recombinant horseradish peroxidase isoenzyme C: the effect of distal haem cavity mutations (His42→Leu and Arg38→Leu) on compound I formation and substrate binding. *J. Biol. Inorg. Chem.* **1996a**, *1*, 136−142.

Rodriguez-Lopez, J. N.; Smith, A. T.; Thorneley, R. N. F. Role of arginine 38 in horseradish peroxidase. A critical residue for substrate binding and catalysis. *J. Biol. Chem.* **1996b**, *271*, 4023−4030.

Ross, D.; Larsson, R.; Norbeck, K., Ryhage, R.; Moldeus, P. Characterization and mechanism of formation of reactive products formed during peroxidase-catalyzed oxidation of *p*-phenetidine. *Mol. Pharmacol.* **1985**, *27*, 277−286.

Schägger, H.; von Jagow, G. Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range of 1 to 100 kD. *Anal. Biochem.* **1987**, *26*, 368−379.

Schmitz, N.; Gijzen, M.; van Huystee, R. Characterization of anionic soybean (*Glycine max*) seed coat peroxidase. *Can. J. Bot.* **1997**, *75*, 1336−1341.

Schuller, D. J.; Ban, R.; van Huystee, R. B.; McPherson, A.; Poulos, T. L. The crystal structure of peanut peroxidase. *Structure* **1996**, *4*, 311−321.

Sessa, J. S.; Anderson, R. L. Soybean peroxidase: Purification and some properties. *J. Agric. Food Chem.* **1981**, *29*, 960−965.

Thomas, J. A.; Morris, D. R.; Hager, L. P. Chloroperoxidase. VIII Formation of peroxide and halide complexes and their relation to the mechanism of the halogenation reaction. *J. Biol. Chem.* **1970**, *245*, 3135−3145.

Toiguchi, S.; Hayashi, K.; Adachi, Y.; Motoki, M.; Haraguchi, K. Purification and characterisation of soybean oxidase. *Nippon Shokuhin Kogyo Gakkaishi (J. Jpn. Soc. Food Sci. Technol.)* **1989**, *36*, 597−602.

Van Haandel, M. J. H.; Primus, J. L.; Teunis, C.; Boersma, M. G.; Osman, A.; Veeger, C.; Rietjens, I. M. C. M. Reversible formation of high-valent-iron-oxo-porphyrin intermediates in heme-based catalysis: revisiting the kinetic model for horseradish peroxidase. *Inorg. Chim. Acta* **1998**, *275−276*, 98−105.

Walker-Griffin, B.; Ting, P. L. Mechanism of N-demethylation of aminopyrine by hydrogen peroxide catalyzed by horseradish peroxidase, metmyoglobin and protohemin. *Biochemistry* **1978**, *17*, 2206−2209.

Received for review August 27, 1999. Revised manuscript received February 17, 2000. Accepted February 24, 2000. We gratefully acknowledge financial support from IOP (Grant IKA94045).

JF9909656