Modification of halogen specificity of a vanadium-dependent bromoperoxidase

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Abstract

The halide specificity of vanadium-dependent bromoperoxidase (BPO) from the marine algae, *Corallina pilulifera*, has been changed by a single amino acid substitution. The residue R397 has been substituted by the other 19 amino acids. The mutant enzymes R397W and R397F showed significant chloroperoxidase (CPO) activity as well as BPO activity. These mutant enzymes were purified and their properties were investigated. The maximal velocities of CPO activities of the R397W and R397F enzymes were 31.2 and 39.2 units/mg, and the K_m values for Cl^- were 780 mM and 670 mM, respectively. Unlike the native enzyme, both mutant enzymes were inhibited by NaN_3. In the case of the R397W enzyme, the incorporation rate of vanadate into the active site was low, compared with the R397F and the wild-type enzyme. These results supported the existence of a specific halogen binding site within the catalytic cleft of vanadium haloperoxidases.

Keywords: haloperoxidase; chloroperoxidase; marine algae; site-directed mutagenesis; vanadate

Haloperoxidases catalyze the halogenation of organic substrates in the presence of hydrogen peroxide. A variety of halogenated compounds are found in many marine macroalgae. Haloperoxidase enzymes have been characterized from *Corallina* species (Itoh et al. 1986; Sheffield et al. 1993), *Ascophyllum nodosum* (Wever et al. 1985), and *Ulvella lens* (Ohshiro et al. 1999). These enzymes are thought to function in the biosynthesis of halogenated natural products. Haloperoxidases are named after the most electronegative halide they can use; that is, bromoperoxidase (BPO) utilizes Br^- and I^- as a substrate, whereas chloroperoxidase (CPO) acts on Cl^-, Br^-, and I^-. Among the marine macroalgae, BPO from the red alga, *Corallina pilulifera*, has been extensively characterized (Itoh et al. 1985, 1986, 1987a,b), and was found to require vanadium in the form of vanadate as an essential cofactor for the enzyme activity (Krenn et al. 1989). The cDNAs for two distinct BPO isomers have been cloned from *C. pilulifera* (Shimonishi et al. 1998). They shared about 90% homology, and one of them (*bpo1*) coding for a protein of 598 amino acids with a calculated molecular mass of 65,312 Daltons has been expressed in *Escherichia coli* and *Saccharomyces cerevisiae* (Ohshiro et al. 2002). The BPO from *Corallina officinalis* is similar to the *C. pilulifera* BPO1 with 92.1% sequence identity. The X-ray structures of both enzymes have been determined (Brindley et al. 1998; Isupov et al. 2000; Littlechild and Garcia-Rodriguez 2003) and they can be superimposed with a 0.5 Å RMS deviation for 593 matching Cα atoms. Other vanadium-dependent BPOs, for example, from the algae, *A. nodosum* (de Boer et al. 1986), *Fucus distichus* (Vreeland et al. 1998), and *Laminaria digitata* (Colin et al. 2003), and CPOs from the fungi, *Curvularia inaequalis* (van Schijndel et al. 1993; Simons et al. 1995) and *Embellisia didymospora*

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(Barnett et al. 1998) have also been described. The amino acid sequence identities between vanadium BPOs and CPOs are low; however, the amino acids involved in vanadate coordination are highly conserved. The analyses of the three-dimensional structures of the enzymes from *A. nodosum* (Weyand et al. 1999), *C. inaequalis* (Messerschmidt and Wever 1996; Macedo-Ribeiro et al. 1999), *C. officinalis* (Isupov et al. 2000), and *C. pilulifera* (Littlechild and Garcia-Rodriguez 2003) have confirmed that these residues are in a similar structural arrangement. Because the catalytic site of BPO resembles that of CPO, it is thought that subtle differences in surrounding amino acid residues must determine the halide specificity.

Several mutant haloperoxidase enzymes from *C. inaequalis* and *C. officinalis* have been constructed by site-directed mutagenesis (Hemrika et al. 1999; Carter et al. 2002) and their enzymological properties have been examined. In each case the target amino acid residues were located within the active sites, and all the mutant enzymes prepared had lower activity than the wild-type enzyme. This paper describes the identification of two mutant *C. pilulifera* BPO enzymes that have significant CPO activity. In this study, we demonstrate that a single amino acid substitution can broaden the halogen specificity of this enzyme.

Results

Enzyme activity of mutant enzymes

Comparison of the amino acid residues around the catalytic sites of the algal BPO from *C. officinalis* and the fungal CPOs from *C. inaequalis* and *E. didymospora* revealed that the amino acid residue at the position 395 of BPO from *C. officinalis,* which is close to the catalytic vanadate, is arginine, whereas it is tryptophan in the case of the two fungal CPOs. It was demonstrated that there was no difference in the active sites between two BPOs from *C. officinalis* and *C. pilulifera* (Littlechild and Garcia-Rodriguez 2003). We have targeted the corresponding residue of *C. pilulifera*, R397 (Fig. 1), which has been changed to each of the other 19 amino acid residues by site directed mutagenesis.

The BPO and CPO activities of each cell-free extract of the recombinant yeast expressing the mutant BPO genes have been determined (Fig. 2). As suggested from the comparison of the active site residues between BPO and CPO described above, R397W had a significant CPO activity. In addition, R397F also showed an appreciable CPO activity, and the CPO activities of R397L, R397Q, and R397E were clearly greater than that of the wild-type enzyme. On the contrary, mutant enzymes R397G, R397A, R397P, and R397S had lost BPO activity. To study the catalytic activity of the mutant enzymes R397W and R397F in more detail, these were purified to homogeneity.

Purification and general characterization of mutant enzymes

Both mutant enzymes were purified according to the method used for the wild-type enzyme with slight modifications. The maximal BPO activities for mutant enzymes R397W (469 units/mg) and R397F (459 units/mg) were

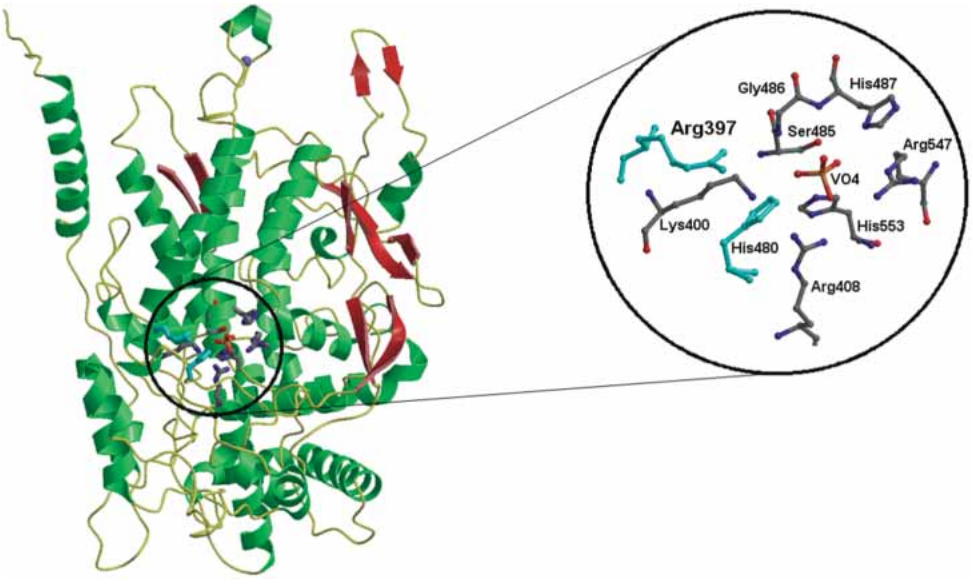

Figure 1. A diagrammatic representation of the structure and active site of the BPO subunit from *C. pilulifera* (PDB code 1UP8). The active site residues are shown as ball-and-stick models. The residues that are conserved in all vanadium BPOs and CPOs are shown in gray. The residues that vary between the different enzymes are shown in cyan. The figure was prepared using the program BOBSCRIPT (Esnouf 1997) and rendered using the program Raster3D (Merritt and Bacon 1997).

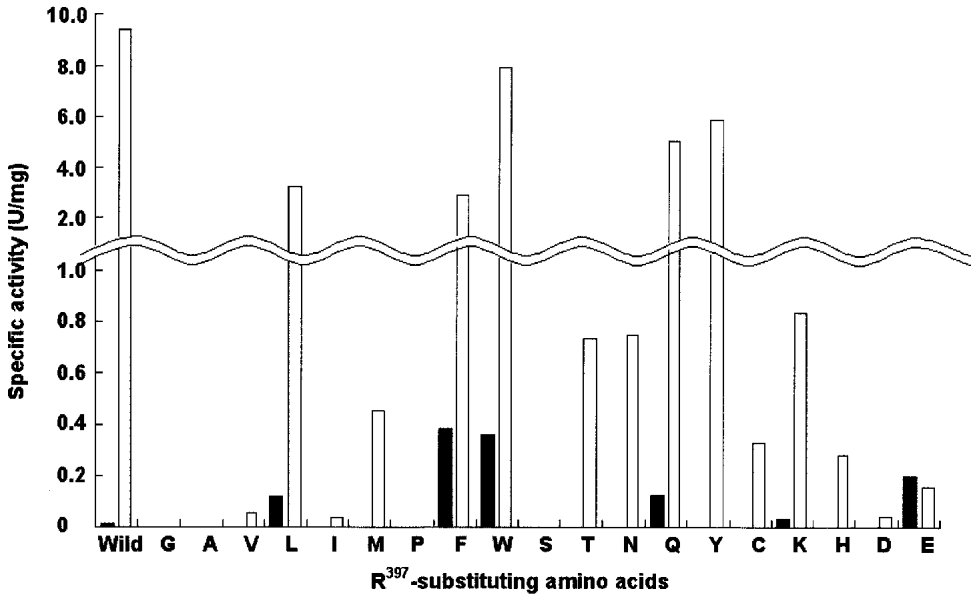

Figure 2. A histogram representation of the BPO (open bar) and CPO (closed bar) activities of the mutant enzymes in the cell-free extracts after preincubation with 10 mM Na_3VO_4 at 30°C for 2 h as described in Materials and Methods.

almost the same as that of the wild-type enzyme (483 units/mg). On the contrary, the maximal CPO activities of R397W and R397F were 31.2 and 39.2 units/mg, respectively, which were 47 and 59 times higher than that of the wild-type enzyme (0.67 units/mg).

The kinetic studies were performed with the mutant enzymes using KBr, KCl, and hydrogen peroxide as substrates. The results are summarized in Table 1. The K_m values of R397W and R397F for KCl were determined to be 780 mM and 670 mM, respectively (Fig. 3). For the wild-type enzyme, the K_m value for KCl could not be determined because the activity was so low as shown in Figure 3. The addition of 1 M sodium sulfate to the assay mixture had little effect on the rate of the enzyme reaction (data not shown). The K_m values for KBr and hydrogen peroxide were similar for the wild-type and the mutant enzymes. Other enzymatic properties, such as the optimal temperature and pH, heat, and pH stabilities, and the effect of inhibitors on the activities, were also similar for each enzyme except for the inhibitory effect of NaN_3. The activity of the wild-type enzyme was not inhibited by 1 mM NaN_3, while both mutant enzymes lost about 80% of their activities in the presence of 1 mM NaN_3 under the same experimental conditions. It has been demonstrated that the mutant proteins, R397W and R397F, were folded into the same oligomeric state as the wild-type enzyme due to the similar elution profile after gel filtration. Because the other mutant enzymes constructed in this study were not purified, we cannot exclude the possibility that they could have modifications in their folding.

Table 1. *Kinetic parameters of the wild-type and the mutant BPO enzymes*

Substrate	Enzyme	K_m (mM)	V_{max} (U/mg)	$kcat/K_m$ (M^{-}–sec^{-1})
H_2O_2[a]	Wild type	0.1	432	3.8×10^6
	R397W	0.1	469	3.8×10^6
	R397F	0.1	459	6.0×10^6
KBr	Wild type	8.4	432	5.4×10^4
	R397W	7.1	469	7.0×10^4
	R397F	4.3	459	1.1×10^5
KCl	Wild type	N.D.	0.7	N.D
	R397W	780	31.2	4.3×10^1
	R397F	670	39.2	6.2×10^1

[a] In the presence of KBr.
N.D. = not determined.

Efficiency of vanadate incorporation into the catalytic site

A preincubation of the recombinant enzymes with vanadate was necessary to achieve their maximal activities. In the case of the wild-type enzyme, 1 mM Na_3VO_4 was preincubated with the enzyme for 2 h at 30°C (Ohshiro et al. 2002). In the case of R397W, we found that the enzyme activity in the cell-free extract was low even after preincubation with 1 mM Na_3VO_4. Therefore, the enzyme solution was preincubated with 10 mM Na_3VO_4 for 2 h at 30°C before the activity assay. The time courses of enzyme activities were compared with the purified wild-type and mutant enzymes

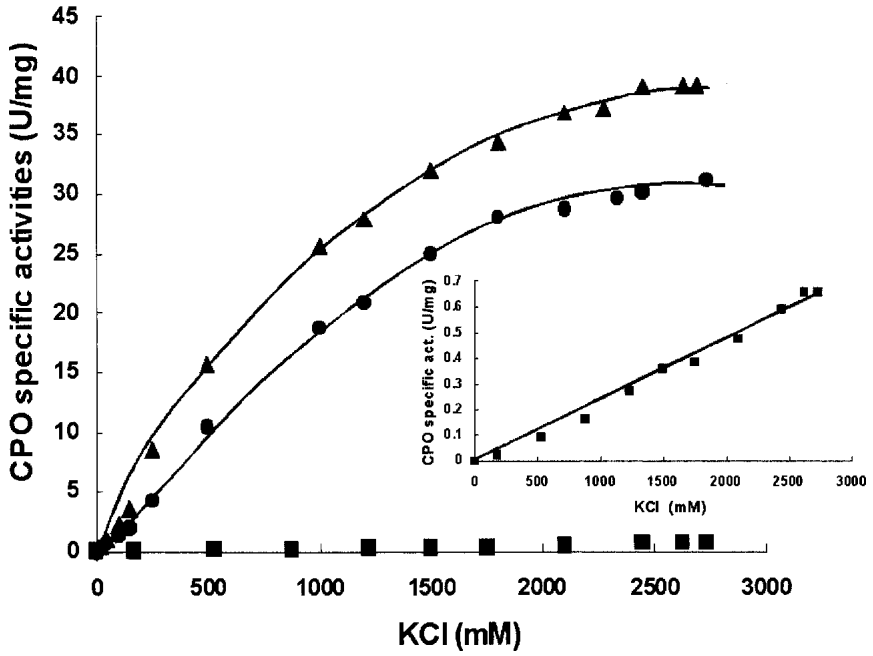

Figure 3. The effect of the KCl concentration on the CPO activities of the wild-type enzyme (filled squares), and mutant enzymes R397W (filled circles), and R397F (filled triangles). The reaction mixture contained 100 mM MES buffer (pH 6.5), 60 μM MCD, 2 mM H_2O_2, the indicated concentrations of KCl and the enzyme was preincubated with vanadate.

preincubated with 1 mM Na_3VO_4. As a result, the wild-type and R397F enzymes reached the full activities within 1 h, whereas it took approximately 24 h for the R397W enzyme to achieve maximum activity. These results showed that the incorporation rates of vanadate into the active sites of the wild-type and R397F enzymes were much higher than that of the R397W enzyme.

Discussion

This paper describes the first example of mutant vanadium-dependent BPO enzymes showing significant CPO activity. All of the vanadium-dependent haloperoxidases reported so far have similar structures around their vanadate binding sites and most of the corresponding amino acid residues are conserved. From the protein structure data of BPO from *C. officinalis* (PDB code 1QHB) and from *C. pilulifera* (PDB code 1UP8), the residues shown in Figure 1 surrounding the active site are in identical positions. Among these nine residues, it was proposed that H553 was covalently linked to vanadate, and K400, R408, H487, and R547 (*C. pilulifera* numbering) formed hydrogen bonds with vanadate oxygens (Littlechild and Garcia-Rodriguez 2003). These five residues and S485 and G486 were conserved in all the haloperoxidases whose amino acid sequences have been determined. The two residues, R397 and H480, vary between the vanadium haloperoxidases from different species. In this study, we have demonstrated that the single amino acid substitution at R397 in BPO from *C. pilulifera* has changed the substrate specificity for the halogen substrate. However, the CPO activities of the mutant enzymes in the present study were smaller than that of haloperoxidase from the fungi, *C. inaequalis* ($kcat/K_m = 3.4 \times 10^4$ M^{-1} sec^{-1}; van Schijndel et al. 1993), which was originally designated as CPO. Additional mutations should be performed to enhance CPO activity of the BPO from *C. pilulifera*.

We have prepared another mutant enzyme, H480F; however, no enzymatic activity was detected for this protein (data not shown). It has been stated that the biochemical difference between BPO and CPO in the oxidation of halide ions could be attributed to this His residue in BPO from *C. officinalis* (Isupov et al. 2000) because it is located at the position of the Phe residue, which was suggested to bind the chloride ion in the active site of CPO from *C. inaequalis* (Messerschmidt and Wever 1996). Our data show that the loss of the activity of the mutant enzyme H480F does not agree with the above proposal; however, we cannot exclude the possibility that this mutant enzyme is misfolded.

It appears that the halogen specificity of the vanadium peroxidases is determined by the affinity of its active site to a particular halide. The results obtained from the mutant enzymes described in this study indicate that the amino acid residues phenylalanine and tryptophan at position 397 favor chloride binding. The location of residue 397 is close to the vanadate binding site in *C. pilulifera* BPO. It would appear that aromatic amino acid residues favor chloride binding as seen by two tryptophan residues being involved in chloride binding of haloalkane dehalogenase from *Xanthobacter au-*

totrophilicus (Verschueren et al. 1993), and a phenylalanine residue located at the chlorine binding site of various amylases (Machius et al. 1995). In the case of BPO from *C. pilulifera*, the substituted tryptophan or phenylalanine residues at position 397 could participate in chloride binding. In the native BPO enzyme the active site cavity provides the correct electrostatic environment to favor bromide binding to arginine at this site. The corresponding amino acid residue is Trp in BPO from the brown alga, *A. nodosum* as well as the vanadium CPO. It was reported that this BPO showed CPO activity (Soedjak and Butler 1990), and the value of K_m for KCl was 344 mM, which is the same order of magnitude as those of mutant enzymes in the present study. Therefore, the Trp residue would be preferable for the chloride binding as described above. However, the specific CPO activity of the enzyme from *A. nodosum* (0.49 U/mg) was much less than those of the mutant enzymes. In the case of the *Corallina* BPO mutants, which utilize chloride as a substrate, other parts of the protein molecule might play an important role in the progress of the enzyme reaction.

The lack of activity of the R397G, R397A, R397P, and R397S mutants is proposed to be due to the small size of their amino acids side chains. Although the electrostatic potential in the active site favors the halogen binding in these mutants, its binding site is not well defined. Residues such as R397Q, R397L, and R397E will aid solvent exclusion and restrict the position of the halide ion to the correct site for halogenation. The reason why the R397Y mutant enzyme shows no CPO activity, in contrast to the other two aromatic mutant enzymes R397W and R397F, could be due to its deprotonation in the environment of the active site. The bulky tryptophan residue of the R397W mutant enzyme also appears to hinder the incorporation of vanadate as it is located at the entrance of vanadate binding pocket.

Regarding the inhibition of the mutant enzymes by azide, it would appear that R397 ensures specific binding of the charged bromide ion and disfavors binding of azide. In the two mutant enzymes R397W and R397F, however, hydrophobic interaction would provide binding for both chloride and bromide. Azide ions also have high affinity for this site and compete for binding, thus inhibiting the halogenation reaction.

The single amino acid substitution of R397 increases the affinity of the BPO enzyme for chloride ions thereby broadening its halide specificity. The structural analysis of the mutant enzymes is in progress, and these results will further our understanding of halogen binding and specificity in the vanadium-dependent haloperoxidases.

Materials and methods

Plasmid and microorganism

The plasmid pTNT30, a yeast-*E. coli* shuttle vector, contained the *bpo1* gene of *C. pilulifera* (Ohshiro et al. 2002). *S. cerevisiae* strain BJ1991 was used as the host of the expression plasmid. Transformation of the strain BJ1991 was performed according to the method previously reported (Hemrika et al. 1999; Ohshiro et al. 2002).

Site-directed mutagenesis

Site-directed mutagenesis was carried out with the QuickChange site-directed mutagenesis kit (Stratagene) using two oligonucleotides per each amino acid replacement according to the manufacturer's instructions. The mutagenic primers were designed to alter the codon of R397 to those of the other 19 amino acids and the codon of H480 to that of phenylalanine. The DNA sequences of the mutant enzymes were confirmed using an ABI 373 or an ABI PRISM 3100 automated DNA sequencer.

Cultivation of the recombinant yeast

The recombinant yeast was cultivated as described previously (Ohshiro et al. 2002). The preculture was carried out with the medium containing 0.67% Yeast nitrogen base w/o amino acids (Difco), 2% glucose, 40 μg/mL of L-tryptophan, and 50 μg/mL of L-leucine. The precultured broth was inoculated into the production medium containing 2% yeast extract, 2% peptone, and 4% D-galactose. Cultivation was carried out at 30°C for 2 days. To prepare the cell-free extracts of each mutant strain, the cells were suspended in 50 mM Tris-SO_4 buffer (pH 7.4), and disrupted with an ultraoscillator (Sonifier 450; Branson Instruments) with 0.5-mm glass beads for 1 h on ice. The supernatants after the removal of cell debris by the centrifugation were used directly for the enzyme assays.

Purification of the mutant enzymes

The mutant enzymes, R397W and R397F, were purified based upon the method described previously (Ohshiro et al. 2002). All purification procedures were carried out at 4°C or on ice unless otherwise stated. The buffer A, 50 mM Tris-SO_4 buffer (pH 7.4) was used throughout. The suspended cells in this buffer were disrupted with 0.5-mm glass beads through a Dyno-Mill homogeneizer (Willy A. Bachofen). Ammonium sulfate fractionation was carried out with the supernatant (cell-free extract) after the centrifugation as described previously (Ohshiro et al. 2002). The dialyzed enzyme solutions were applied to DEAE-Sepharose columns (5.8 × 30 cm), which had been equilibrated with the same buffer. The columns were washed with the same buffer followed by 0.2 M KCl in buffer A. The mutant enzymes R397W and R397F were eluted with 0.2 M and 0.35 M KCl in buffer A, respectively. The active fractions were combined, concentrated by ultrafiltration, and dialyzed against buffer A. The dialyzed enzyme solutions were applied onto Q-Sepharose columns (1.7 × 15 cm), which had been equilibrated with buffer A. The columns were washed with 0.3 M (R397W) or 0.35 M KCl (R397F) in buffer A, and the bound proteins were eluted with 0.4 M KCl in buffer A. For R397W enzyme, the concentrated enzyme solution was once again applied onto a Q-Sepharose column (1.7 × 11 cm), which had been equilibrated with buffer A. After the column was washed with 0.3 and 0.35 M KCl in buffer A, the enzyme was eluted with 0.4 M KCl in buffer A. For R397F enzyme, the concentrated enzyme solution was applied onto a Sepharose CL-4B column (1.8 × 94 cm), which had been equilibrated with buffer A. The active fractions were combined and concentrated by ultrafiltration. The wild-type en-

zyme was purified according to the same method as previously described (Ohshiro et al. 2002). The gel filtration step was performed for all enzymes to ensure the homogeneity of the preparation.

Enzyme assays and other analytical methods

The enzyme solution was preincubated with 10 mM Na_3VO_4 at 30°C for 2 h before measuring the enzyme activity. BPO activity was determined spectrophotometrically by the halogenation of monochlorodimedone (MCD, $\varepsilon = 19.9\ mM^{-1}\ cm^{-1}$ at 290 nm) as described previously (Yamada et al. 1985). The reaction mixture contained 100 mM MES buffer (pH 6.5), 100 KBr, 60 μM MCD, 2 mM H_2O_2, 1 mM Na_3VO_4, and the enzyme. The assay temperature was 30°C and activity followed by decrease in absorbance at 290 nm (Ohshiro et al. 2002). When measuring chloroperoxidase activity, 1.5 M KCl (99.5%, Wako Chemicals) was added to the reaction mixture instead of KBr. The protein concentration was determined by the method of Bradford (1976) using a Bio-Rad protein assay reagent with bovine serum albumin as a standard. SDS-PAGE was carried out by the method described by Laemmli (1970) employing a 12.5% gel for separation. Protein bands were visualized by staining with Coomassie Brilliant Blue G-250 dissolved in 50% methanol–10% acetic acid and destained in 30% methanol–10% acetic acid.

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