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Analysis of microperoxidases using liquid chromatography, post-column substrate conversion and fluorescence detection

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Abstract

A liquid chromatographic method with on-line activity determination for microperoxidases has been developed. After enzymatic digestion of a cytochrome, possibly under formation of microperoxidases, the product mixture is separated by reversed-phase liquid chromatography. The products first pass a diode-array detector, and are then subjected to a reaction with 4-(*N*-methylhydrazino)-7-nitro-2,1,3-benzooxadiazole (MNBDH) and hydrogen peroxide. In a reaction coil, microperoxidases catalyze the reaction under formation of the fluorescent 4-(*N*-methylamino)-7-nitro-2,1,3-benzooxadiazole (MNBDA). Quantification of the microperoxidases is performed using a fluorescence detector at an excitation wavelength of 470 nm and an emission wavelength of 545 nm, respectively. For this LC-based detection system, limits of detection are 3×10^{-8} mol/L, limits of quantification are 9×10^{-8} mol/L, and a linear range from 9×10^{-8} mol/L to 3×10^{-6} mol/L is obtained for the microperoxidases MP-9 and MP-11. A highly active microperoxidase MP-6 was found in the reaction of cytochrome *c* from bovine heart with protease from streptomyces griseus.

Keywords: Microperoxidases; HPLC; Post-column derivatization; Fluorescence detection; MS detection; Digestion

1. Introduction

Horseradish peroxidase (POD) is one of the most widely used enzymes in analytical chemistry [1,2]. POD catalyzes the oxidation of an organic substrate by hydrogen peroxide. The POD-catalyzed reaction can be detected in different ways. Frequently, organic substrates are oxidized under formation of colored or fluorescent products, thus enabling the use of optical detection techniques. The large number of POD applications includes the use as a marker enzyme in immunoassays [3,4] and as a catalyst in various biosensors [5,6]. Another relevant field is environmental analysis of hydrogen peroxide and primary hydroperoxides [7,8]. The most important drawbacks of POD for analytical applications are its limited compatibility with organic solvents and its high molecular mass.

Microperoxidases have been known since decades as products of the digestion of cytochrome *c* with proteolytic enzymes [9–11]. Microperoxidases contain the heme group of cytochrome *c* plus a peptide chain with at least five amino acids, and their name results from their catalytic properties, which are similar to those of POD. They are named according to the number of amino acids, which are still attached to the heme group. For example, MP-8 is a microperoxidase with an octapeptide chain. The molecular masses of the most commonly used microperoxidases, MP-8, MP-9 and MP-11, are in the range between 1500 and 2000 Da. Due to their lower molecular masses, they can be coupled to other biomolecules (e.g., for immunochemical applications) in a more defined way than the large enzyme POD with its multiple functional groups. Furthermore, compatibility with organic solvents is improved compared with POD [12].

However, surprisingly few analytical applications of microperoxidase have been described up to now. These comprise various biosensors [13–15] and chemiluminescence detection schemes with [16–20] and without [21,22] initial liquid chromatographic or capillary electrophoretic separation. The chemiluminescence assay takes advantage of the catalytic effect of microperoxidases on the oxidation of luminol or related compounds under generation of an intense emission signal. For the determination of fatty acid hydroperoxides, Schmitz et al. [23] used laser-induced fluorescence detection

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after oxidation of *p*-hydroxyphenylacetic acid to a fluorescent dimer in the presence of microperoxidase-11 as a catalyst. Recently, our group described 4-(*N*-methylhydrazino)-7-nitro-2,1,3-benzooxadiazole (MNBDH) as a new substrate for MP-11 [24]. Its oxidation to the fluorescent 4-(*N*-methylamino)-7-nitro-2,1,3-benzooxadiazole (MNBDA) by hydrogen peroxide in the presence of microperoxidase-11 was used as one part of a dual substrate enzyme assay.

These examples show that attractive analytical applications of microperoxidases are already available. On the other hand, their further use is hampered by their high price, their limited availability and the lack of a method to rapidly test the activity of newly synthesized microperoxidases. Therefore, a chromatographic system should be developed, being able to provide on-line information of the character and the activity of a microperoxidase or a mixture of different microperoxidases, which are formed in a proteolytic digest. The development of such a system is described within this manuscript.

2. Experimental

2.1. Chemicals

All chemicals were purchased from Aldrich (Steinheim, Germany), Merck (Darmstadt, Germany) and Fluka (Neu-Ulm, Germany) in the highest quality available. Acetonitrile and water for HPLC were from Biosolve (Valkenswaard, The Netherlands), gradient grade. For LC–MS measurements, acetonitrile and water were LC–MS grade (Biosolve, Valkenswaard, The Netherlands). The microperoxidases MP-11 and MP-9 (both from equine heart cytochrome *c*), trypsin and protease type XIV from streptomyces griseus (Pronase E) as well as cytochrome *c* from bovine heart were purchased from Sigma (Deisenhofen, Germany). The synthesis of MNBDH was performed as described in literature [25].

2.2. Instrumentation

The optimization of the experimental parameters was performed using a microplate reader from BMG LabTechnologies (Offenburg, Germany) with FLUOstar software version 2.10-0 and FLUOstar Galaxy software version 4.30-0. For the measurements, the following filters were used: 470 nm (bandwidth ±15 nm) for excitation and 545 nm (bandwidth ±10 nm) for emission. Corning (Costar No. 3915, black) 96-wells microtitration plates used for these measurements were purchased from Diagonal (Münster, Germany).

Liquid chromatographic separation and detection were performed with the following system (all components from Shimadzu, Duisburg, Germany): two LC-10AS pumps, degasser GT-154, SPD-M10Avp diode-array detector, RF-10AXL fluorescence detector, SIL-10A autosampler, software Class LC-10 version 1.6. and CBM-10A controller unit. The injection volume was 10 μL. A Prontosil 120-5-phenyl column (Bischoff Chromatography, Leonberg, Germany) was used; particle size 5 μm, pore size 120 Å; column dimensions 250 mm × 3 mm.

For HPLC separation, the following binary gradient consisting of buffer A (NH_4Ac/HAc, both 10 mM, pH 7) and 5% A in acetonitrile (B) with a flow rate of 0.3 mL/min was used:

Time (min)	c_B (%)
0.03	10
12.5	30
25	30
26	10
28	10

The post-column derivatization set-up is shown in Fig. 1. After separation and UV/vis-detection (wavelength 405 nm), the analytes were mixed with the substrate solution consisting of MNBDH (5×10^{-4} M in acetonitrile) and H_2O_2 (0.167 M in water), which were delivered by a Sp230iw syringe pump (World Precision Instruments, Berlin, Germany) equipped with two gas tight 1 mL SGE syringes (Supelco, Bellefonte, PA, USA). The reagents were added with a flow rate of 0.5 μL/min. The subsequent reaction loop was a 25 m knitted Teflon tubing coil with an inner diameter of 0.3 mm. The reaction products were detected with the fluorescence detector at an excitation wavelength of 470 nm and an emission wavelength of 545 nm, respectively.

Calibrations were performed for MP-9 and MP-11 solutions for concentrations between the limits of detection (LOD) and the end of the linear range, at which the LODs were determined according to S/N = 3 and the limits of quantification (LOQ) according to S/N = 10.

For LC–MS investigations, an Agilent Technologies (Waldbronn, Germany) HP1100 liquid chromatograph for binary

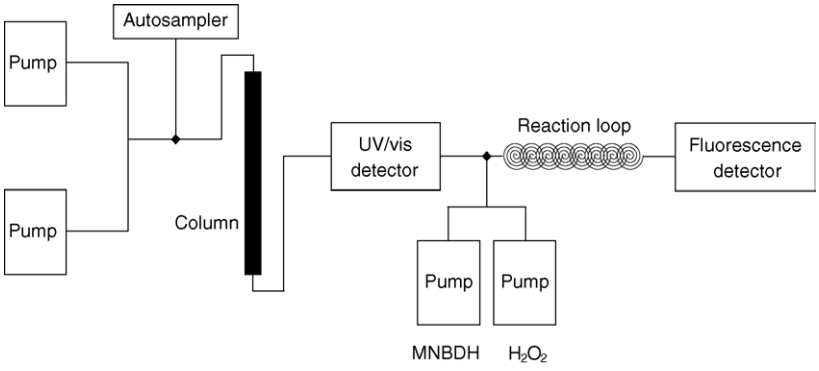

Fig. 1. Set-up for the separation, post-column reaction and detection of microperoxidases.

gradient elution (pump model G1312A), including an autosampler (G1313A) and a DAD (G1315B), coupled to an esquire 3000*plus* ion trap mass spectrometer from Bruker Daltonics (Bremen, Germany) was used.

The LC separation of the analytes was carried out under the same conditions as described above.

All MS measurements were performed by means of electrospray ionization in the positive-ion mode. Mass spectra were recorded in the full scan mode, scanning from $m/z = 500$ to 3000. The ion count cumulative target for the ion trap mass analyzer was 100,000, with a maximum accumulation time of 200 ms. The electrospray voltage was 3750 V. Other ion source parameters were 145 V on the transfer capillary exit, 40 V at the skimmer, 40 psi nebulizer gas and 10.0 L/min of drying gas with a temperature of 365 °C. In case of MS/MS experiments, auto-fragmentation was performed with one precursor selection (excluding the mass range $m/z = 500$–685) and a fragmentation amplitude of 1.20 V.

The detected masses of the different microperoxidases were confirmed by means of comparison with the information on the expasy peptide database [26].

2.3. Procedures

2.3.1. pH optimization

In order to find the best suitable pH for the post-column reaction, the conversion of MNBDH by means of MP-11 and H_2O_2 was performed on micro-titration plates in 0.5 pH steps in the pH range from 2 to 7.5. For every pH, 100 μL of a blank and of five MP-11 concentrations (1×10^{-7} M to 1×10^{-9} M) were pipetted in fourfold onto a micro-titration plate. Subsequently, 50 μL of a H_2O_2 (1.67×10^{-3} M)/MNBDH (5×10^{-4} M) mixture (volume ratio 8.3:1) were added to each well. After incubation at room temperature, fluorescence was measured at excitation and emission wavelengths of 470 and 545 nm, respectively.

2.3.2. Opimization of acetonitrile content

The influence of the acetonitrile content on the MP-11 reaction was investigated as well by performing the conversion of MNBDH by means of MP-11 and H_2O_2 on micro-titration plates. In this case, the reaction was executed at pH 7, and the acetonitrile amount was varied. For each acetonitrile concentration (2, 10, 20, 30, 40 and 50%), the reaction was carried out as described above, and the micro-titration plate was read out in the same way.

2.3.3. Digestion

For the digestion, each of the four above-mentioned cytochromes was dissolved to a 2×10^{-4} M solution in a NH_4HCO_3 buffer (15 mM, pH 8). For the trypsin solution, 0.145 mg was dissolved in 1 mL of the same buffer and diluted by a factor 100 just prior to use (4×10^{-8} M). Equal amounts of protein solution and enzyme solution were added to each other and this mixture was placed in a stove for an overnight digestion at 37 °C. The digestion with the unspecific pronase E was performed in the same way, but with the difference that 2 mg of the enzyme were dissolved in 10 mL of bis-tris-propane buffer (10 mM, pH 7). The digests were analyzed both by means of the HPLC post-column method described above and by LC–MS, respectively.

3. Results and discussion

Initial work regarding the substrate MNBDH resulted in the finding that, despite the attractive spectroscopic properties of the substance, its applicability for the detection of enzymatic conversions is limited [24]. The reason is its slow conversion by horseradish peroxidase (POD), which allows the use of MNBDH as a substrate only in those cases when POD is present in excess concentrations. Therefore, the determination of hydrogen peroxide, glucose or glucose oxidase leads to favorable analytical figures of merit [27], while the attempt to detect POD itself does not. More recently, it was found that, actually, MNBDH may rapidly be converted by microperoxidases [24]. The respective reaction is presented in Fig. 2. In combination with liquid chromatographic separation and post-column conversion, this reaction should thus allow the detection of different microperoxidases within a single chromatographic run. Aim of this work was the development of a respective system and its application to the detection of microperoxidases in cytochrome *c* digests.

3.1. Optimization of the reaction of microperoxidase with MNBDH/H_2O_2

The MNBDH/H_2O_2-system was optimized with respect to all those parameters being significant for the development of an enzyme-catalyzed post-column reaction. Regarding the optimization, MP-11 was selected as a model microperoxidase, and all reactions were carried out using micro-titration plates and a microplate fluorescence reader. The optimization of the pH is presented in Fig. 3. It is obvious that the highest signals are obtained between pH 6 and 7. Thus, the buffer system NH_4Ac/HAc (both 10 mM, pH 7) was chosen as the mobile phase in LC. The reaction time has only a minor influence: Already at a reaction time of 30 s, a strong signal is observed, and the increase of signal intensity with time (up to 6 min) is small.

For the LC separations, reversed-phase systems are most favorable with respect to ease of use. However, most enzymatic reactions experience strong adverse effects in the presence of increasing organic solvent concentration. Therefore, representative for the entire group of microperoxidases, the MP11-reaction

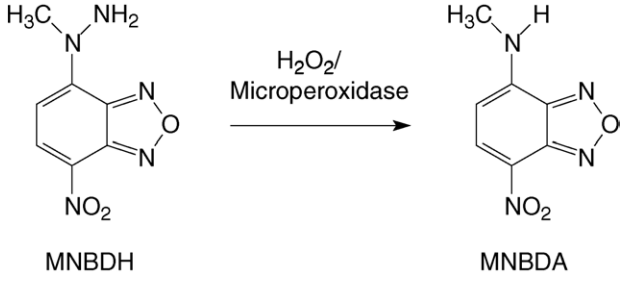

Fig. 2. Conversion of the non-fluorescent MNBDH to the strongly fluorescent MNBDA under microperoxidase catalysis.

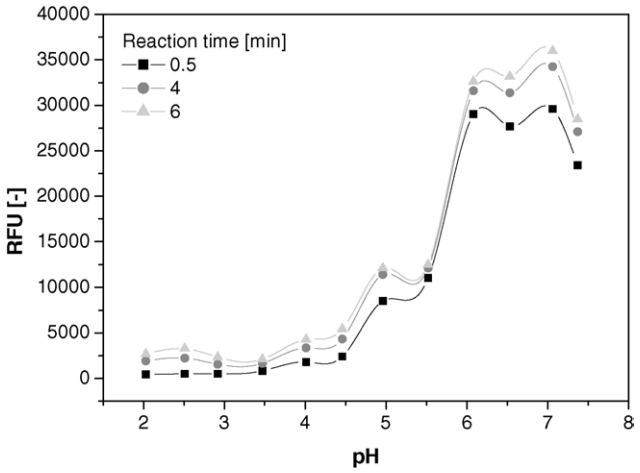

Fig. 3. Dependency of the MP-11-catalyzed oxidation of MNBDH by hydrogen peroxide on the pH at different reaction times ($c_{MP\text{-}11}$ = 10 nmol/L).

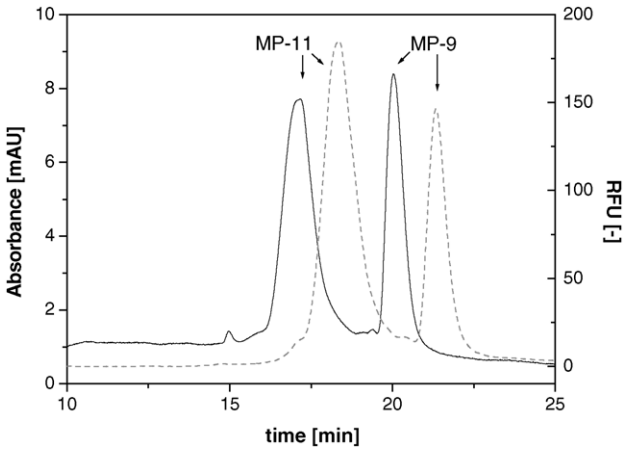

Fig. 5. Separation of MP-11 and MP-9 standards (3 μmol/L each) with UV/vis (solid line) and fluorescence (dotted line) detection.

was investigated in the presence of acetonitrile, as shown in Fig. 4. Obviously, the signal intensity decreases with increasing acetonitrile concentration. However, the signal intensity at 10% acetonitrile is higher than the intensity in the absence of acetonitrile. A possible reason for this phenomenon is the known dependency of the fluorescence intensity of benzooxadiazole derivatives on the solvent composition. Typically, mixed aqueous-organic solutions or even purely organic solvents are more advantageous than water [28]. It is therefore assumed that the optimum at 10% acetonitrile is the best compromise between enzymatic conversion and spectroscopic properties. In practice, the acetonitrile content should not be higher than 30% to still obtain a reasonable signal.

These findings were then used to develop a suitable LC method for the separation of microperoxidases MP-11 and MP-9. The phenyl-modified RP-column in combination with the NH_4Ac/HAc buffer (A) and the 5% buffer in acetonitrile (B) mixture turned out to be appropriate for the separation. Applying the gradient mentioned above, which did not reach more than 30% of organic solvent, a baseline separation of the two microperoxidases within 22 min could be achieved. All conditions performed were in good accordance with a favorable post-column reaction.

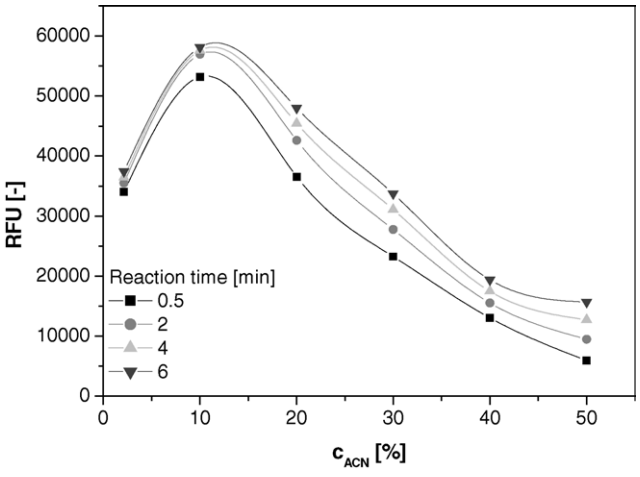

Fig. 4. Dependency of the MP-11-catalyzed oxidation of MNBDH by hydrogen peroxide on the concentration of acetonitrile (ACN) at different reaction times ($c_{MP\text{-}11}$ = 10 nmol/L).

3.2. HPLC-separation and post-column derivatization of microperoxidase standards

The set-up of the separation and post-column detection system is presented in Fig. 1. A binary gradient was delivered with a high-pressure gradient system. An UV/vis detector was used to monitor the column effluent prior to addition of the reagents. For stability reasons, the solutions of MNBDH and hydrogen peroxide were stored in separate syringes and delivered with one syringe pump. The two solutions were mixed in a mixing tee prior to the addition to the LC eluent. A reaction loop of poly(tetrafluoroethylene) with a length of 25 m (inner diameter: 0.3 mm) was used to achieve a high turnover of the microperoxidase-catalyzed reaction. Finally, a fluorescence detector was used to monitor the concentration of the formed MNBDA. This set-up caused a delay of 1 min between the UV/vis and fluorescence detector traces using a flow rate of 0.3 mL/min.

Standards of MP-11 and MP-9 (each of 3 μmol/L) were analyzed using the post-column system. As presented in Fig. 5, a good separation between the two microperoxidases was obtained. In respect of the LC-based detection system, limits of detection were 3×10^{-8} mol/L, limits of quantification were 9×10^{-8} mol/L, and the linear range was from 9×10^{-8} mol/L to 3×10^{-6} mol/L for both microperoxidases. The RSD for multiple determination at the concentration of 3×10^{-7} mol/L was 3.6% for MP-9 and 7.3% for MP-11 ($n = 3$). The figure shows that fluorescence detection is superior to UV/vis detection owing to more stable baselines and higher signal intensities. This is due to the inherently better limits of detection of fluorescence versus UV/vis detection as well as due to the selectivity of the post-column detection system, combining both the advantages of the selective LC separation and the selective enzymatic reaction,

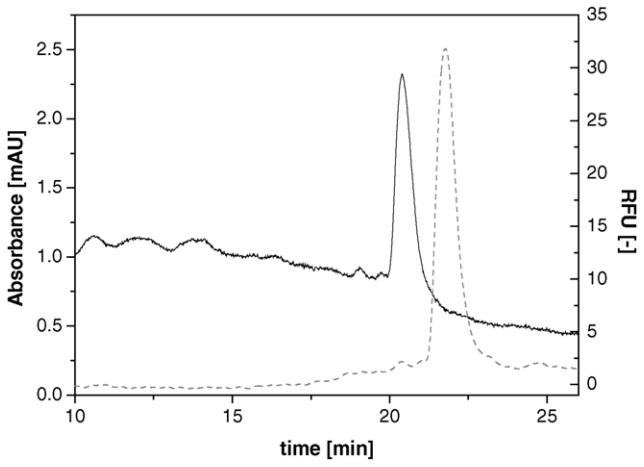

Fig. 6. Chromatogram of a digest of cytochrome *c* from bovine heart (1 μmol/L) with trypsin. Only MP-9 is detected by means of UV/vis (solid) and fluorescence (dotted) spectroscopy.

respectively. The limits of detection are inferior for the LC-based system compared with the micro-titration plate system because of shorter reaction times, the presence of organic solvents, lower amounts of the analyte in the detection system and the dilution of the analytes during separation. However, the limits of detection are still better than required for the identification and quantification of microperoxidases from cytochrome *c* digests, as demonstrated on the following.

3.3. Tryptic digestion of cytochrome c from bovine heart and analysis of products

Digestions of cytochrome *c* were carried out using various proteolytic enzymes. The detailed procedures are described in the Experimental Section. For the digest of cytochrome *c* from bovine heart with trypsin, only MP-9 was detected (Fig. 6). This was also predicted due to the very specific cleavage properties of trypsin.

The identity of MP-9 was confirmed by electrospray ionization mass spectrometry. In Fig. 7, the ESI mass spectra of the respective digest (1) and of commercially available MP-9 (2) are compared. In both spectra, the peak of $m/z = 1635.1$ is the $[M + H]^+$ of MP-9, which has only a very low abundance (see insert). The $[M + 2H]^{2+}$ peak is the base peak in these mass spectra with an m/z of 817.6, whereas the $[M + H + Na]^{2+}$ peak of $m/z = 828.6$ has a lower intensity again. Although the comparison of both spectra shows a higher signal-to-noise ratio for (2), which is due to a higher MP-9 concentration, it is visible that the noise itself is very similar for both samples.

3.4. Digestion of cytochrome c from bovine heart with a protease from streptomyces griseus and analysis of products

The digest of cytochrome *c* from bovine heart with a protease from streptomyces griseus was investigated as well. In Fig. 8, the

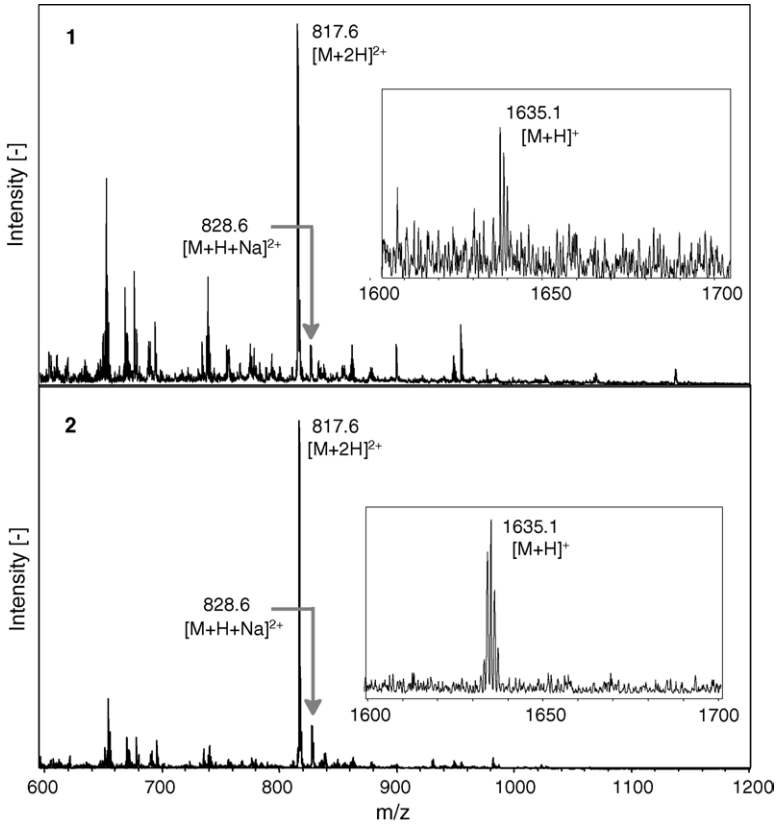

Fig. 7. (1) ESI-MS spectrum of a digest of cytochrome *c* from bovine heart (1 μmol/L) with trypsin. (2) ESI-MS spectrum of commercially available MP-9 (1 μmol/L). The peak at $m/z = 1635.1$ is caused by MP-9 (both spectra), thus confirming the data of the LC method (compare with Fig. 6).

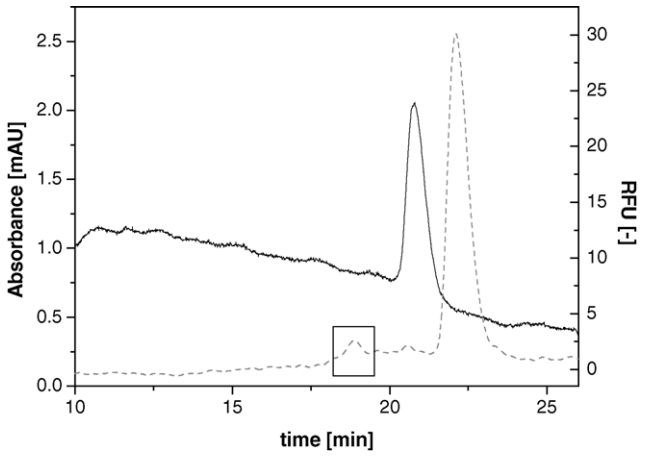

Fig. 8. Chromatogram of a digest of cytochrome *c* from bovine heart (1 μmol/L) with protease from streptomyces griseus. Only one large peak is detected with UV/vis (solid) and fluorescence (dotted) detection at a retention time similar to, but still different from MP-9. Another small peak is detected (marked with a frame) only in the fluorescence trace.

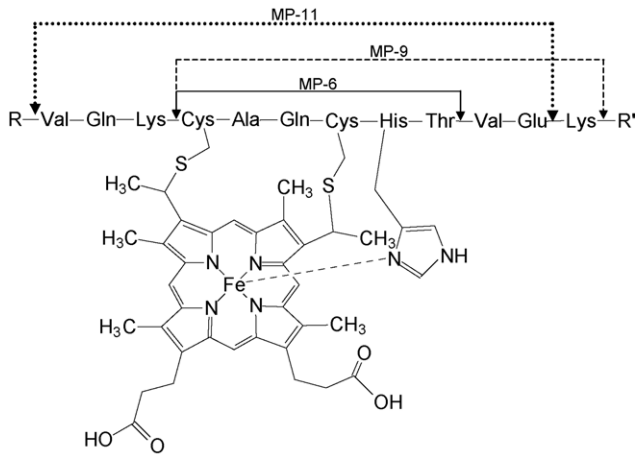

Fig. 10. Part of the primary structure of cytochrome *c* from bovine heart. Dotted lines indicate the cleavage sites to obtain MP-11, dashed lines those for MP-9 and the solid lines those for MP-6.

chromatogram of the digest with UV/vis (solid) and fluorescence (dotted) detection is presented. An intense signal is obtained with both detectors. This has a similar, but not the same retention time as MP-9. It is therefore assumed that the unknown peak represents another type of microperoxidase. The peroxidase activity of the unknown substance was estimated based on the calibration data of MP-11. Assuming that the enzymatic digest would result in a quantitative conversion of the cytochrome *c* to the unknown microperoxidase, the peak area is 2.5 times larger than it could be expected for the same concentration of MP-11. However, a smaller peak (framed in Fig. 8) was observed only in the fluorescence trace at the retention time of MP-11, thus indicating that the conversion rate to the unknown microperoxidase is at least slightly lower than 100%. This means that the activity of the unknown microperoxidase is at least 2.5 times larger than that of MP-11.

Identification of the unknown peak was performed by means of electrospray MS again, as presented in Fig. 9. With similar abundance, the $[M + H]^+$ ($m/z = 1277.8$) and the $[M + 2H]^{2+}$ ($m/z = 639.6$) confirm that the unknown substance is the microperoxidase MP-6. In Fig. 10, the structure of MP-6 is presented in comparison to those microperoxidases MP-9 and MP-11, which were used in this study. Although MP-6 is known from the biochemical literature [2], there is no analytical use up to now. Owing to its significantly increased activity and its even lower molecular mass in comparison with MP-9 and MP-11, the MP-6 could be an excellent candidate for analytical applications. In future work, larger amounts of MP-6 shall be isolated, and it shall be investigated if MP-6 exhibits the increased activity

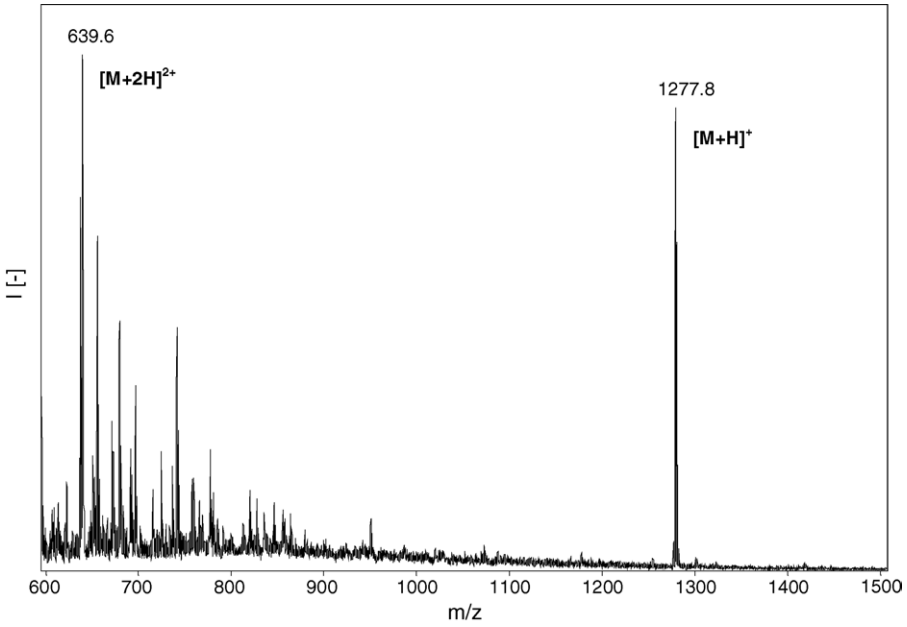

Fig. 9. ESI-MS spectrum of a digest of cytochrome *c* from bovine heart (1 μmol/L) with protease from streptomyces griseus. The peak at $m/z = 1277.8$ is caused by MP-6.

not only in case of the MNBDH/MNBDA-substrate system, but also with other substrates, e.g. *o*-phenylenediamine. Due to its lower molecular mass, it could become an attractive analytical tool in solution as well as immobilized on solid supports.

4. Conclusions

A new HPLC/post-column reaction/fluorescence detection system has been developed and applied to the determination of microperoxidases in proteolytic digests of cytochrome *c* from bovine heart. This set-up is well suited for the activity measurement of microperoxidases. Whereas the digestion of cytochrome *c* with trypsin yielded the expected MP-9, which was detected by means of fluorescence and confirmed by ESI-MS measurements, the digestion with the unspecific protease from streptomyces griseus resulted in a microperoxidase, which was characterized as MP-6, a literature-known but not yet analytically used microperoxidase. Due to its high catalytic activity, investigations regarding possible analytical application of MP-6 are planned for future work.

Acknowledgements

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