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Technical Report

Purification of homogeneous forms of fungal peroxygenase

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Abbreviations: **AaP**, *Agrocybe aegerita* peroxygenase; **FPLC**, fast protein liquid chromatography; **IEF**, preparative isoelectric focusing

Extracellular peroxygenase from the agaric fungus *Agrocybe aegerita* is a versatile biocatalyst that oxygenates various substrates by means of hydrogen peroxide. The enzyme is routinely produced in suspensions of soybean meal and has until now been purified by several steps of fast protein liquid chromatography (FPLC) using different ion exchangers. The final protein fraction had a molecular mass of 46 kDa but still consisted of several incompletely separated proteins with slightly differing isoelectric points (pI 5.2, 5.6, 6.1), probably representing differently glycosylated isoforms. This made it difficult to further purify the individual protein forms. Since homogeneous protein fractions are a pre-requisite for X-ray crystallography and specific structure-function studies, an appropriate FPLC procedure was developed starting with pre-purification of crude peroxygenase on SP Sepharose followed by chromatofocusing on a Mono P column and elution with a pH gradient. Three sufficiently separated main protein peaks were eluted from the Mono P column and confirmed to be distinct forms of aromatic peroxygenase with different pI/s. All A.

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3 *aegerita* peroxygenase forms oxygenated toluene and naphthalene and no catalytic
4 differences were observed between them. We tested also two devices for
5 preparative isoelectric focusing (Rotofor, IsoPrime systems) for peroxygenase
6 separation but resolution and protein recovery were not sufficient.
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14 **1 Introduction**

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19 The agaric mushroom *Agrocybe aegerita* produces a secretory heme-thiolate
20 protein (*A. aegerita* peroxygenase, AaP) that can be regarded as a functional hybrid
21 of peroxidases and cytochrome P450 monooxygenases [1–3]. The enzyme is able
22 to oxygenate (epoxidize, hydroxylate), brominate and oxidize a wide range of
23 aromatic and aliphatic substrates and hence may be one of the most versatile
24 oxidoreductases known so far [4–6]. The fungus secretes considerable amounts of
25 this peroxygenase particularly in soybean meal suspension [1]. However, the
26 purification of AaP is laborious and requires several steps of ultrafiltration and fast
27 protein liquid chromatography (FPLC) using different ion exchangers to get rid of the
28 soy proteins and peptides. The final enzyme fractions obtained show one mass
29 band (46 kDa) on SDS-PAGE, but still consist of two to six different proteins (three
30 major forms along with a varying number of minor ones) with pI s between 4.9 and
31 5.7 (differences in pI 0.1–0.8) [1]. However, specific analyses such as X-ray
32 diffraction studies or comparative kinetic studies require the preparation of
33 homogeneous protein fractions, *i.e.*, the three major AaP forms must be purified to
34 homogeneity [7].
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54 The use of high-resolution Mono Bead resins in ion exchange chromatography (*e.g.*,
55 Mono Q, Mono S) is a well established and has been successfully applied for the
56 purification of protein isoforms and isoenzymes [8–10]. The application of FPLC
57 using a pH gradient, the so-called chromatofocusing, has been successfully used for
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3 separating antibodies and their fragments, as well as peptides and oligonucleotides.
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5 However, there are only a few articles describing the purification of extracellular
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7 fungal enzymes by chromatofocusing [11–15].
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10 Preparative isoelectric focusing (IEF) in buffered liquids is another established
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12 method for purifying similar protein forms and is usually performed using a multi-
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14 compartment electrolyzer or a Rotofor cell device [16–20]. Both methods are tricky
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16 and time consuming, and have been applied mainly for the purification of peptides,
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18 protein fragments and small proteins, polynucleotides or intracellular bacterial
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20 enzymes [20–23]. Reports on the separation and fractionation of different forms of
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22 fungal secretory proteins by these preparative IEF devices are rarely found in the
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24 literature [18].
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27 In the present work, we studied the separation of different peroxygenase proteins
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29 (AaP isoforms) by preparative IEF and chromatofocusing using pH gradients with
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31 the aim of obtaining homogeneous protein fractions for further comparative studies.
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33 Both methods are known to be sensitive to slightly varying surface charges of
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35 proteins, and hence may be the methods of choice for the successful separation of
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37 quite similar peroxygenase molecules.
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43 **2 Materials and methods**

44 **2.1 Enzyme assay and chemicals**

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50 Activity of peroxygenase was determined in 1-mL cuvettes by following the oxidation
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52 of veratryl alcohol into veratraldehyde ($\epsilon_{310} = 9.3 \text{ mM}^{-1} \text{ cm}^{-1}$) in 50 mM sodium
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54 citrate-phosphate buffer at pH 7 [1]. All chemicals used were of the highest purity
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56 available and were obtained from Sigma-Aldrich (Weinheim, Germany) and Merck
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58 (Darmstadt, Germany).
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2.2 Preparation of protein crude extract

All purification experiments were started from crude AaP produced by the Black poplar mushroom, *A. aegerita* strain TM A1 (strain deposited at DSMZ no. 22459). Production was carried out in a 5-L stirred-tank bioreactors (Biostat B, Braun Biotech International GmbH, Melsungen, Germany) containing a complex liquid medium based on soybean meal [1]. Culture liquid (4 L) was harvested just after the activity had reached its maximum level (about 1500 U/L). The fungal mycelium was removed by filtration (filter GF6; Schleicher & Schuell, Dassel, Germany) and the enzyme-containing liquid was concentrated 60-fold by two steps of ultrafiltration using a tangential-flow cassette system (Omega Minisette™, cut-off 10 kDa, Pall Corporation Hauppauge, NY, USA) and a 150-mL stirred cell system (10 kDa cut-off polysulphone membrane, Pall Life Sciences, Dreieich, Germany).

2.3 Protein purification by ion exchange chromatography

For subsequent protein separation, the crude AaP was pre-purified by FPLC using an ÄKTA system (GE HealthCare Europe GmbH, Freiburg, Germany) fitted with the strong cation exchanger SP Sepharose FF (column XK 26/20, GE HealthCare). Proteins were eluted with a linear gradient of 0–0.6 M NaCl in 10 mM sodium acetate buffer (pH 4.75) at a flow rate of 13 mL/min⁻¹ (modified according to [1]). AaP-containing fractions were pooled, concentrated and dialyzed against 10 mM sodium acetate (pH 5.0; 10 kDa cut-off polysulphone membrane, Pall Life Sciences). This pre-purified AaP fraction was afterwards used for all further separation studies.

In a first approach, we attempted to separate the AaP proteins by ion exchange on Mono Bead™ columns using either strong cation (Mono S, 5 mm × 50 mm, pH 4.5)

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3 or strong anion (Mono Q, 5 mm × 50 mm, pH 7.0) exchangers. Separations were
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5 carried out with sodium acetate (10 mM) as solvent and an increasing concentration
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7 of NaCl (0–0.3 M) for elution.
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10 11 12 **2.4 Protein purification by preparative IEF**

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17 IEF separation was done with an IsoPrime multi-chambered electrofocusing unit
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19 (Hoefer Scientific Instruments, San Francisco, CA, USA). The purification module
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21 consisted of eight chambers separated by seven isoelectric membranes of pre-
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23 determined pH. The polyacrylamide (5%) membranes were reinforced by glass fiber
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25 filters (GF53, Schleicher & Schuell) and contained covalently bound acrylamido
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27 buffers (*pI* select, Fluka Chemie GmbH, Buchs, Switzerland), which imparted a
28
29 definite pK_a value to each membrane. Membrane preparation was performed
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31 according to the manufacturer's instructions using the software program Doctor-pH
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33 (Hoefer Scientific Instruments) that determined the required concentration of the
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35 acrylamido buffers for adjusting the desired pH. Seven slightly differing pH values
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37 (5.00, 5.37, 5.53, 5.65, 5.73, 5.85, 6.00) were chosen, covering the whole *pI* range
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39 of possible AaP forms (previously determined by analytical IEF [1]). After
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41 assembling the membranes, a pre-run was carried out at 11 °C and 4 W constant
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43 power supply (3000 V, 20–25 mA, 3.5 mL/min) with deionized water to establish the
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45 pH gradient within the membranes. After removing the pre-run water, the protein
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47 sample was placed into the central chamber. The outer chambers were filled with
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49 the corresponding electrolytes. The anode buffer was 5 mM acetic acid and the
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51 catholyte was 5 mM histidine. Separation runs were done in the constant power
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53 mode (4 W) resulting in currents up to 25 mA with a flow rate of 2.5 mL/min. To
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55 improve protein separation and recovery, running conditions were varied in following
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3 ranges: phase 1 (0.5–2 h, 650–800 V), phase 2 (1–1.5 h, 1000–1500 V), phase 3
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5 (0.5–25 h, 1500–3000 V).
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8 Further separation experiments by preparative IEF were carried out with a Rotofor
9 unit (Bio-Rad, Hercules, CA, USA). The enzyme solution was mixed with
10 ampholytes (pH 4–7, Fluka Chemie), which then generated a linear pH gradient in
11 the electrical field of the focusing chamber. A pre-run of the system was performed
12 (5 min, 5 W) with deionized water prior to loading the protein sample. After injection
13 of the enzyme solution through the inlet of the sample chamber, the run was
14 performed at a constant power of 4 W and 450 V for 4 h. To achieve sufficient
15 separation, the concentration of electrolyte buffers (500 mM acetic acid pH 2.2;
16 100 mM histidine pH 7.6; 500 mM propionic acid pH 2.6; 100 mM histidine pH 7.6)
17 and of ampholytes (2–4%) was varied. Finally the protein fractions were collected by
18 applying a vacuum to the separation chamber. All active fractions were later
19 analyzed by analytical IEF.
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35 36 37 **2.5 Protein purification by chromatofocusing** 38 39 40

41 This method was also accomplished using the ÄKTA-FPLC system mentioned
42 above but applying a pH instead of a salt gradient. SP Sepharose-pre-purified
43 protein was separated on a chromatofocusing column containing the mixed anion
44 exchanger Mono P (5 mm × 200 mm, GE HealthCare). The column was pre-
45 equilibrated with 25 mM Bis-Tris-HCl buffer (pH 7) and the protein solution loaded
46 onto the column by a sample pump (GE Healthcare Europe). The bound AaP
47 protein was eluted with a pH gradient established by 10% Polybuffer 74 (pH 4.0,
48 GE HealthCare) at a flow rate of 1 mL/min. Active fractions were collected with an
49 auto-sampler, pooled and analyzed by analytical IEF.
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2.6 Analytical IEF and protein determination

To determine the purity of protein and to evaluate the success of separation, the different protein fractions were electrophoretically analyzed using a Mini-Cell electrophoresis system (XCell Sure Lock™ Invitrogen, Karlsruhe, Germany) and Novex pre-cast IEF gels (pH 3–7, Invitrogen). Electrophoretical separation was carried out according to the manufacturer's protocol over 2.5 h at up to 6 mA (500 V limit). Protein bands were visualized with the Colloidal Blue Staining Kit from Invitrogen.

Protein concentration was determined by the method of Bradford using the Roti®-Nanoquant Protein Assay Kit (Roth, Karlsruhe, Germany) with bovine serum albumin H4 (Roth) as the standard.

2.7 Toluene and naphthalene oxidation by different AaP fractions

The major AaP fractions obtained after Mono P separation were compared with respect to their ability to oxygenate toluene and naphthalene [3]. Reactions were performed with 0.08 nmol of each AaP fraction and 1000 μM substrate in 1.5-mL HPLC vials (total reaction volume 1 mL) in potassium phosphate buffer (10 mM, pH 6.8) under continuous supply of H₂O₂ (KDS 100, KD Scientific, Holliston, MA, USA) at rates of 200 nmol/min over 10 min [6]. In the case of toluene, the reaction mixture contained in addition 5 mM ascorbic acid to prevent further oxidation of cresols formed [4]. Reaction products were identified and quantified by HPLC and authentic standards as described previously [3].

3 Results and discussion

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3 The different forms of peroxygenase from AaP secreted during the growth in soy
4 bean media showed slightly differing pI/s and an almost identical molecular mass of
5 46 kDa [1]. Since pure proteins are a fundamental pre-requisite for protein
6 crystallography and comparative kinetic studies, we tested several high-resolution
7 methods to obtain homogeneous AaP fractions.
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13 14 15 16 **3.1 Ion exchange chromatography** 17

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20 The first chromatographic separation step was performed on the strong cation
21 exchanger SP Sepharose FF and resulted in the removal of most yellowish-colored
22 components of the soy bean medium (proteins/peptides and phenolics) [24, 25]. The
23 elution profile showed one heme-related peak (420 nm), and fractions possessing a
24 veratryl alcohol oxidizing activity (at pH 7), which consisted of three proteins with
25 different pI (Figs. 1A and B). These latter fractions were collected.
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33 ((Fig. 1))
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35 The pre-purified and pooled enzyme fraction (Fig. 1) was used for further separation
36 by ion exchange chromatography. The studies were focused on Mono Beads™
37 (Mono S, Mono Q), which are known to guarantee high resolution of protein
38 separation [26]. However, the separation effect was not fully sufficient and just one
39 active major fraction eluted from the Mono S column (Fig. 1C). In a previous study,
40 two active enzyme fractions were obtained (designated as AaP I and II) by this
41 purification step [1], and the major fraction (AaP II) has been used in most
42 biochemical studies on AaP published so far [3–6]. The second ion chromatographic
43 method was based on the strong anion exchanger Mono Q. Although the resolution
44 was slightly better than that of the Mono S column, only two of the three main AaP
45 forms were partly separated, and no pI-pure AaP protein was obtained (Fig. 1D).
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60 The overall insufficient purification of peroxygenases by ion exchange
chromatography may be caused by similar surface charges of the proteins, which

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3 hampered their complete separation. Similar findings were reported for other fungal
4 secretory proteins. Thus, two laccase isoforms of the well-investigated white-rot
5 fungus *Ceriporiopsis subvermispota* could not be fully separated by ion exchange
6 chromatography though their *pI* values differed to a greater extent than those of
7 AaP. These laccase isoforms, and similarly an acidic manganese peroxidase form of
8 the white-rot fungus *Nematoloma frowardii* b19 (nowadays referred to as *Phlebia* sp.
9 *Nfb19*), were purified to homogeneity by preparative IEF [18, 27, 28].
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20 **3.2 Preparative IEF**

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25 In consequence, our next attempt to separate the different AaP forms was the
26 application of preparative IEF. Initially, a focusing technique using a multi-
27 compartment electrolyzer (IsoPrime, Hoefer, Amersham Pharmacia Biotech,
28 Freiburg, Germany) was tested. However, almost the entire pre-purified protein
29 bound irreversibly to the chamber membranes, which markedly swelled during the
30 focusing process. This led to a high loss of AaP activity (up to 95%), so that this
31 technique could not be used for separation of larger amounts of AaP. It has also been
32 reported that the purification of a bacterial α -amylase by a multi-compartment
33 electrolyzer failed due to severe problems of protein precipitation near the *pI* value
34 of the enzyme [23]. In fact, reports describing a successful separation of proteins or
35 peptides by the multi-compartment electrolyzer refer mostly to intracellular non-
36 glycosylated proteins, oligopeptides or amino acids [29–31], and to our best
37 knowledge, protocols concerning the isolation of extracellular fungal enzymes by
38 this technique are lacking.
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55 In most cases, the isolation of fungal proteins and their isoforms by IEF was done
56 using thick IEF gels and a Multiphor II apparatus (Pharmacia, Sweden), which is no
57 longer commercially available (personal communication, GE Healthcare) or by a
58 Rotofor unit (Bio-Rad) [8, 17, 18]. Unfortunately, the latter procedure was also not
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3 suitable for fractionating the complex mixture of AaP proteins. The almost equal
4 distribution of active enzyme fractions, without forming distinct activity maxima, and
5 the accumulation of the AaP forms II and III (pI 5.6 and 6.1) with increasing pH
6 (fractions 8-12 – which becomes evident in the corresponding IEF gel) proved that
7 no distinct AaP form can be obtained by this method (Figs. 2A and B). Related
8 findings were reported for peroxidase isoforms of the grape plant (*Vitis vinifera*) and
9 for other heme proteins like horseradish peroxidase or lactoperoxidase [17, 23]. The
10 low buffering capacity in the region around their pI/s was responsible for the
11 equilibrium not being reached and for the failure of the focusing/separation process
12 [32]. While the purification of single forms of fungal enzymes by preparative IEF has
13 been described in a few reports [16, 33–35], the applicability of this procedure for
14 the separation of chloroperoxidase or peroxygenases has not been demonstrated so
15 far.

16 ((Fig. 2))

3.3 Chromatofocusing

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18 Since the separation methods sensitive to a low variation of the protein's surface
19 charge seemed to be the only remaining possibility for the purification of the AaP
20 forms, we tested chromatofocusing on a Mono P column in combination with a pH
21 gradient elution. The AaP proteins eluted from the column one by one according to
22 their pI/s with decreasing pH (from 7 to 4) of the mobile phase. Figure 3 shows the
23 respective elution profile and reveals the separation of six veratryl alcohol oxidizing
24 fractions (three major and three minor ones), corresponding to the earlier finding of
25 six AaP forms with different pI/s [1]. Thus, we finally obtained the three major AaP
26 forms (AaP I–III) with a recovery of up to 79% overall, as well as specific activities of
27 77, 94 and 103 U/mg (Table 1). These three proteins formed single bands with pI/s
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3 of 5.2, 5.6 and 6.1 in the analytical IEF gel, proving the success of the purification
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5 procedure (Fig. 3B).

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7 ((Fig. 3))((Table 1))
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10 11 **3.4 Substrate oxidation by major AaP forms**

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16 Toluene and naphthalene were used as substrates to prove the true peroxygenase
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18 nature of the AaP proteins obtained after Mono P separation. Results are
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20 summarized in Table 2 and show no significant differences between the different
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22 AaP forms, either in regard to the product pattern or to the percentage of individual
23
24 AaP forms, either in regard to the product pattern or to the percentage of individual
25
26 oxidation products. All AaP fractions oxygenated toluene at both the side chain and
27
28 the aromatic ring yielding benzyl alcohol, benzaldehyde and benzoic acid, and *o*-
29
30 cresol, *p*-cresol and methylhydroquinone, respectively. The ratio of side chain vs.
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32 aromatic hydroxylation was about 2:1 in all cases. Naphthalene was regioselectively
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34 converted into 1-naphthol and 2-naphthol at a ratio of 12:1. Differences in this ratio
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36 compared to an earlier study [3] can be explained by the use of a syringe pump for
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38 continuous peroxide supply instead of the repeated addition of H₂O₂. The findings
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40 suggest that the different AaP fractions represent quite similar enzyme proteins that
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42 may differ just in their glycosylation pattern [7]. Similar observations have already
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44 been made for the different isoforms of chloroperoxidase [36, 37].
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47 ((Table 2))
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50 51 **4 Concluding remarks**

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56 Our results suggest that preparative IEF techniques are unsuited to the separation
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58 of fungal peroxygenases with similar molecular mass and *pI*. On the other hand, we
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60 were able to demonstrate that pre-purification on SP Sepharose followed by

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3 chromatofocusing can be a powerful tool to attain this goal. To show that the
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5 separated AaP proteins are in fact differently glycosylated isoforms of one and the
6
7 same protein (and do not represent different isoenzymes originating from different
8
9 genes), future studies will focus on the determination of individual catalytic constants
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11 (K_m and k_{cat}) for the different peroxygenase forms and also follow specific molecular
12
13 approaches to correlate AaP gene transcription and enzyme secretion (e.g., by real-
14
15 time PCR). The separation method described here has already been successfully
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17 applied to prepare larger quantities of homogenous AaP forms. This was achieved
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19 by repeated injection of pre-purified protein and repeated running of the separation
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21 program until sufficient amounts of the three major proteins were collected (up to
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23 5 mg). Meanwhile the purified proteins have successfully been crystallized and are
24
25 currently being studied with respect to their crystal structure.
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37 *Industriechemikalien”).*
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- 48
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- 50
- 51 • German Environmental Foundation))
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55 *The authors have declared no conflict of interest.*
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11 **Figure 1.** (A) FPLC elution profile of the first purification step (pre-purification) of
12 AaP using the strong cation exchanger SP Sepharose FF. The pooled AaP fraction
13 obtained was the starting material for all further separation studies. (B) Analytical
14 IEF gel of the pre-purified AaP proteins (2), protein marker (1). (C) Partial separation
15 of AaP proteins on the strong cation exchanger Mono S, and (D) on the strong anion
16 exchanger Mono Q. Solid line, absorbance at 420 nm (heme-thiolate of AaP); black
17 circles, AaP activity assayed with veratryl alcohol at pH 7.0; dashed lines, NaCl
18 gradient.
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30 **Figure 2.** Preparative IEF of AaP using a Rotofor unit. (A) Gray bars, AaP fractions
31 recovered from the focusing cell and assayed with veratryl alcohol (pH 7.0); black
32 circles, pH gradient. Fractions with the highest AaP activity are highlighted in gray
33 (fraction 5–12) and were analyzed in an IEF gel (B). M, protein marker.
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39 **Figure 3.** Separation of different AaP forms by chromatofocusing. (A) Elution profile
40 recorded after chromatofocusing on the mixed anion exchanger Mono P; separated
41 AaP forms are highlighted in gray. Solid line, absorbance at 420 nm; black
42 circles, AaP activity assayed with veratryl alcohol (pH 7.0); thin dashed
43 line, concentration buffer B (%); thick dashed line, actual pH gradient. (B) Separated
44 AaP forms visualized in an IEF gel; AaP I (pI 6.1), AaP II (pI 5.6), AaP III (pI 5.2).
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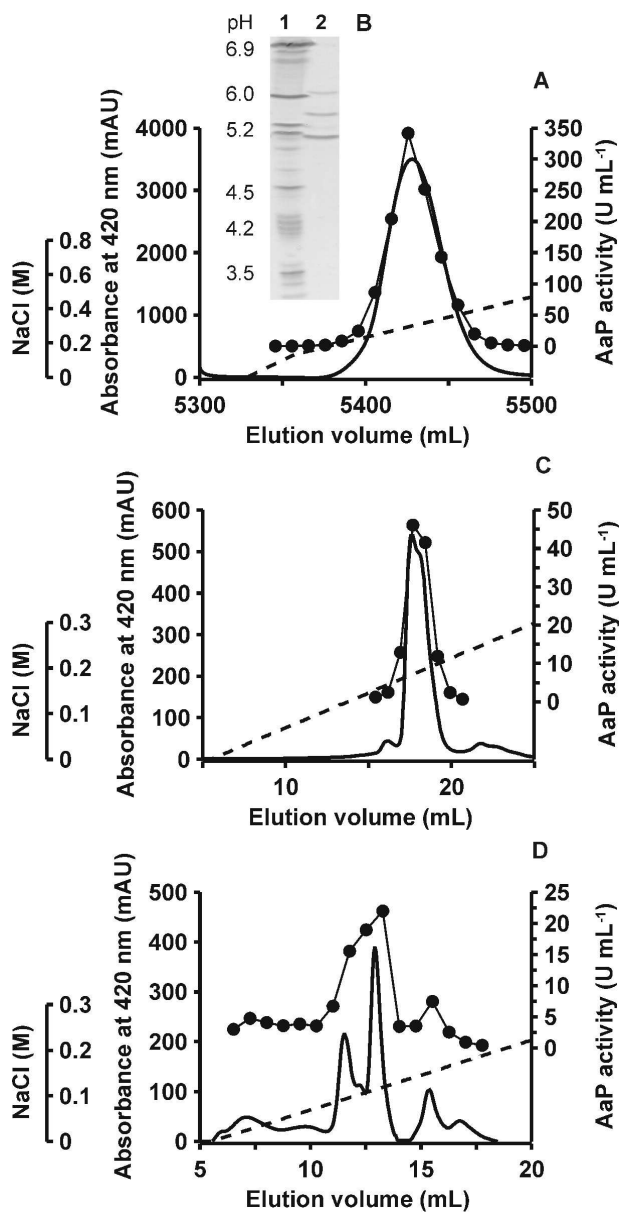
Table 1. Purification of the three main isoforms of AaP (I–III) by FPLC

Purification step	Protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification (x-fold)
Culture liquid	60	141	2	100	
Ultrafiltration	55	140	2	99	1
SP Sepharose	35	135	4	96	2
Mono P, AaP I	0.56	43	77	30	33
Mono P, AaP II	0.35	33	94	23	40
Mono P, AaP III	0.36	37	103	26	44

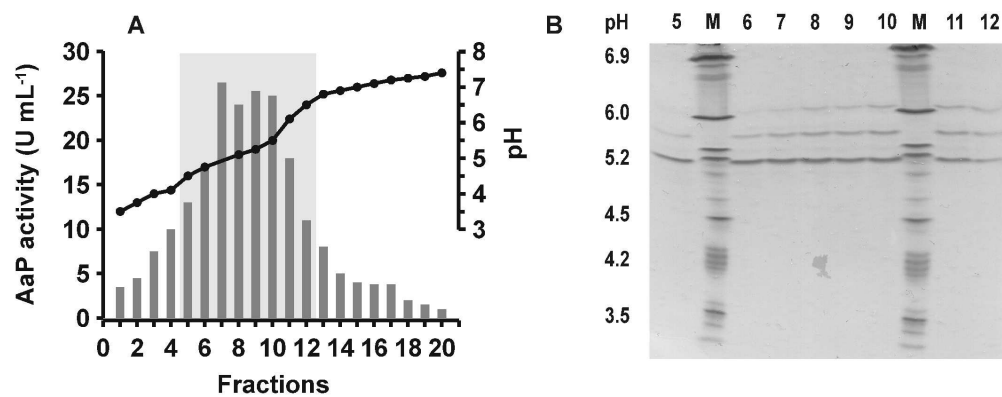
Table 2. Oxidation of toluene and naphthalene by the three major AaP forms obtained after Mono P separation

Substrate	Products	% ^{a)} of products formed by		
		AaP I	AaP II	AaP III
Toluene	Benzyl alcohol	22.5	22.1	22.0
	Benzaldehyde	25.6	25.9	26.8
	Benzoic acid	16.1	18.1	18.2
	<i>o</i> -Cresol	1.4	1.3	1.1
	<i>p</i> -Cresol	2.4	2.0	1.8
	Methylhydroquinone	32.0	30.6	30.0
	Total	100	100	99.9
Naphthalene	1-Naphthol	94.0	93.5	94.5
	2-Naphthol	6.0	7.0	6.0
	Total	102.4	99.8	99.4

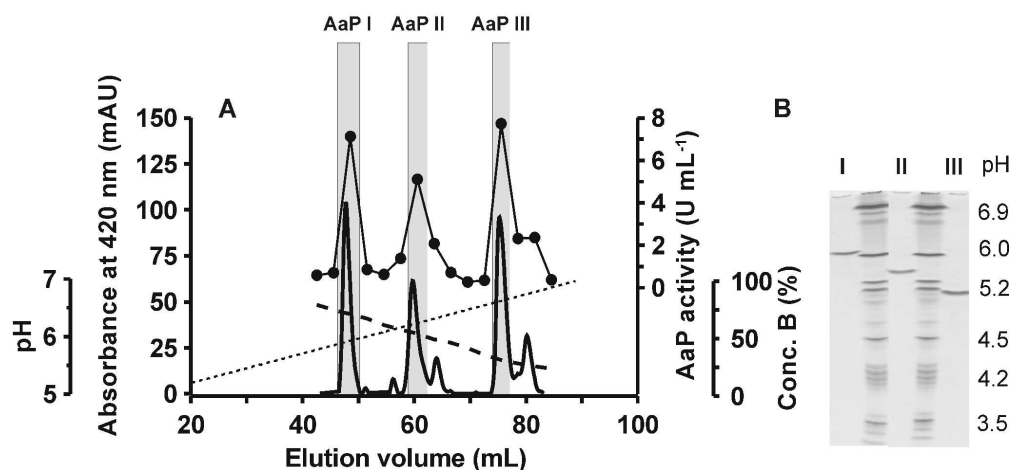
a) Percentages are given as mean values of three different measurements with SD <5%.



(A) FPLC elution profile of the first purification step (pre-purification) of *Agroclybe aegerita* peroxygenase (AaP) using the strong cation exchanger SP Sepharose FF. The pooled AaP fraction obtained was the starting material for all further separation studies. (B) Analytical IEF gel of the pre-purified AaP proteins (2), protein marker (1). (C) Partial separation of AaP proteins on the strong cation exchanger Mono S and (D) on the strong anion exchanger Mono Q. Solid line - absorbance at 420 nm (heme-thiolate of AaP), black circles - AaP activity assayed with veratryl alcohol at pH 7.0, dashed lines - NaCl gradient.



Preparative isoelectric focusing of *A. aegerita* peroxygenase using a Rotofor unit. **(A)** Grey bars – AaP fractions recovered from the focusing cell and assayed with veratryl alcohol (pH 7.0), black circles – pH gradient. Fractions with the highest AaP activity are highlighted in grey (fraction 5-12) and were analyzed in an IEF gel **(B)**, protein marker (M).



Separation of different AaP forms by chromatofocusing. **(A)** Elution profile recorded after chromatofocusing on the mixed anion exchanger Mono P, separated AaP forms are highlighted in grey. Solid line - absorbance at 420 nm, black circles - AaP activity assayed with veratryl alcohol (pH 7.0), thin dashed line - concentration buffer B (%), thick dashed line - actual pH gradient; **(B)** separated AaP forms visualized in an IEF gel - AaP I (pI 6.1), AaP II (pI 5.6), AaP III (5.2).