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Regular paper

Oxalic acid degradation by a novel fungal oxalate oxidase from *Abortiporus biennis*

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Oxalate oxidase was identified in mycelial extracts of a basidiomycete *Abortiporus biennis* strain. Intracellular enzyme activity was detected only after prior lowering of the pH value of the fungal cultures by using oxalic or hydrochloric acids. This enzyme was purified using size exclusion chromatography (Sephadex G-25) and ion-exchange chromatography (DEAE-Sepharose). This enzyme exhibited optimum activity at pH 2 when incubated at 40°C, and the optimum temperature was established at 60°C. Among the tested organic acids, this enzyme exhibited specificity only towards oxalic acid. Molecular mass was calculated as 58 kDa. The values of K_m for oxalate and V_{max} for the enzyme reaction were 0.015 M and 30 mmol min^{-1}, respectively.

Key words: oxalate oxidase, oxalic acid, *Abortiporus biennis*

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INTRODUCTION

Abortiporus biennis is a ligninolytic basidiomycete fungus belonging to the ecological group of white rot fungi that are very efficient natural wood degraders. These fungi possess a unique system for efficient degradation of a lignin polymer which constitutes the major carbon source on Earth. The main constituents of this system are extracellular enzymes, like laccase and different peroxidases – manganese-dependent peroxidase (MnP), lignin peroxidase (LiP), and versatile peroxidase (VP) (Jarosz-Wilkołazka *et al*., 2008; Polak & Jarosz-Wilkołazka, 2012). Enzymatic degradation of lignin is supported in multiple ways by the action of low-molecular-weight compounds (Kaneko *et al*., 2005), and among them, a very important role is played by oxalic acid. This dicarboxylic acid is a predominant organic acid secreted by many fungi and has several ecological roles, e.g. facilitation of the MnP action by chelating unstable Mn^{3+} ions or buffering pH values outside the hyphae (Hofrichter, 2002). The biosynthesis of oxalic acid in fungi most likely implicates the tricarboxylic acid cycle, involving the hydrolytic cleavage of oxaloacetate to acetate and oxalate, or the glyoxylate cycle involving oxidation of glyoxylate to oxalate (Mäkelä *et al*., 2010). There is diversity in secretion of oxalic acid among varied ecological groups of wood degrading fungi. Brown rot fungi generally secrete oxalic acid in higher amounts than white rot fungi (Hastrup *et al*., 2012; Mäkelä *et al*., 2010). This can be due to the fact that white rot fungi are able to decompose oxalic acid *via* the action of oxalate decarboxylase (EC 4.1.1.2), which converts oxalate to formic acid and carbon dioxide (Mäkelä *et al*., 2002). The degradation of oxalate *via* action of oxalate oxidase (EC 1.2.3.4), described in our study, is atypical for fungi and was found predominantly in higher plants. The best characterised oxalate oxidase originates from cereal plants (Dunwell, 2000). Currently, only three oxalate oxidases of basidiomycete fungi have been described - an enzyme from *Tilletia contraversa* (Vaisey *et al*., 1961), the best characterised so far enzyme from *Ceriporiopsis subvermispora* (Aguilar *et al*., 1999), and an enzyme produced by *Abortiporus biennis* (Grąz *et al*., 2009). The enzyme from *C. subvermispora* was also expressed in *Pichia pastoris* and characterised as Mn(II)-containing bicupin protein sharing homology with bicupin microbial oxalate decarboxylase (Moussatche *et al*., 2011). Oxalate oxidase decomposes oxalic acid to carbon dioxide and hydrogen peroxide. Hydrogen peroxide is in turn the key component of the catalytic cycle of all peroxidases and can also be generated by fungal feedback-type enzymes, like glucose-1-oxidase (EC 1.1.3.4), aryl alcohol oxidases (EC 1.1.3.7), and pyranose-2-oxidase (EC 1.1.3.10) (Leonowicz *et al*., 1999). Oxalic acid also plays a significant role in biological and geochemical processes in the soil (Gadd, 2007). For example, oxalate has a large effect on the availability of phosphorous and calcium, and is associated with weathering of soil minerals and precipitation of insoluble metal oxalates (Jarosz-Wilkołazka & Grąz, 2006, Jarosz-Wilkołazka *et al*., 2006, Grąz *et al*., 2009). Oxalic acid is a known toxic compound associated with plant pathogenic fungal strains, such as *Sclerotinia sclerotiorum* (Hegedus & Rimmer, 2005). The level of oxalic acid is also an important factor in many technologies applied in the industry, such as the paper industry or brewing processes (Dunwell *et al*., 2000, Sjöde *et al*., 2008).

In this study, we examined factors influencing the induction of the oxalate oxidase activity in cultures of white rot fungus *Abortiporus biennis* and characterised selected properties of the novel fungal oxalate oxidase (OXOAb).

MATERIALS AND METHODS

The organism and growth conditions. *Abortiporus biennis* was obtained from the Fungal Collection of the Biochemistry Department, Maria Curie-Skłodowska University, Lublin, Poland. Stock cultures were maintained on 2% malt extract agar (MEA) slants at 4°C. The *inoculum* was pre-cultured on 2% MEA for one

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Abbreviations: OXO, oxalate oxidase; OXOAb, oxalate oxidase from *Abortiporus biennis*

week at 25°C. The experiments were performed in Erlenmeyer flasks using liquid medium containing glucose (10 g L^{-1}) and potato extract (4 g L^{-1}). The inoculated flasks were incubated at 25°C with a rotation mode (160 rpm). After 7 days of cultivation, OXOAb production by the *A. biennis* strain was induced by addition of oxalic acid, sodium oxalate, or hydrochloric acid (to the final concentration of 10 mM) to the growth media. After 24 hours, the mycelium was harvested and homogenised. The mycelial extract was centrifuged, assayed for the OXOAb activity, and frozen for further analysis.

Oxalate oxidase activity (OXOAb) assay (Aguilar *et al.*, 1999). The standard assay of this enzyme was based on the measurement of enzymatically generated hydrogen peroxide. The reaction mixture contained 0.3 mL of 20 mM oxalic acid in 0.05 M succinate buffer, pH 3.5, and 0.2 mL of the enzyme. After incubation of the reaction mixture for 15 min at 40°C, 0.45 mL of 0.2 mM phenol red solution and 0.05 mL of horseradish peroxidase (6.25 U) in 0.05 M succinate buffer, pH 3.5, were added. Following incubation at 30°C for 15 min, 0.1 mL of 5 M NaOH was added and the absorbance at 610 nm was measured. A standard curve was used to calculate the amount of H_2O_2 (prepared in 0.05 M succinate buffer) generated during the OXOAb reaction. One enzyme unit was defined as the amount of enzyme required to produce 1 mmol of H_2O_2 per minute, under standard assay conditions.

To determine the optimum pH value for OXOAb activity, the enzyme was incubated with oxalic acid in 0.05 M succinate buffer of pH 2, 2.5, 3, 3.5, and 4. The optimum temperature for OXOAb activity was determined using succinate buffer pH 3.5 and incubation at 30°C, 40°C, 50°C, 60°C, 70°C, and 80°C. To determine the substrate specificity of OXOAb, the standard activity assay was performed using citric, malic, malonic, glycolic, glioxalic, succinic, and acetic acids at 2 mM and 20 mM concentrations at pH 3.5. The K_m and V_{max} values were determined using standard conditions of the reaction.

Determination of protein concentration. Protein concentration was determined using the Bradford method (1976) and bovine serum albumin was used as a standard.

Determination of hydrogen peroxide concentration. The chemiluminescence method based on the Co (II)-catalysed oxidation of luminol by H_2O_2 was applied according to Perez and Rubio (2006).

Determination of organic acid concentration. The concentration of organic acids in the cultures of *A. biennis* was monitored by capillary electrophoresis using an Agilent 7100 Capillary Electrophoresis System equipped with a UV-Vis detector. The separation was carried out using a fused silica capillary 50 μm ID with a 50 cm length to the detection window. The voltage applied was –25 kV and the capillary temperature was maintained at 15°C. Samples were injected hydrodynamically for 5 s (50 mbar) and organic acid was detected by indirect UV-detection at a wavelength of 350 nm (bandwidth 20 nm) and a reference wavelength of 230 nm (bandwidth 10 nm). The buffer solution was freshly prepared every day by dissolving phthalic acid (5 mM), cetyltrimethylammonium bromide (CTAB, 0.26 mM), and methanol (0.5% v/v) in MiliQ water (Chen *et al.*, 1997). Peak identification was done by spiking with commercially available formic and oxalic acids.

Enzyme purification. Gel filtration on Sephadex G-25. The crude extract obtained after homogenization and centrifugation of induced mycelium of *A. biennis* was loaded onto a Sephadex G-25 column (2.5 × 30 cm) previously equilibrated with 20 mM phosphate buffer pH 6.5. The column was eluted with 20 mM phosphate buffer, pH 6.5, at a flow rate of 0.5 mL/min and 5-mL fractions were collected and monitored for protein and OXOAb activity.

Ion exchange chromatography on DEAE-Sepharose. Protein fractions with OXOAb activity collected after gel filtration were loaded onto a DEAE-Sepharose column equilibrated with 20 mM phosphate buffer, pH 6.7. The bound proteins were eluted with a linear gradient of NaCl (0–75%) in 20 mM phosphate buffer, pH 6.7. The 3-mL fractions were collected at the flow rate of 1 mL/min and tested for protein and OXOAb activity. The active fractions were pooled, desalted using ultrafiltration, and stored at –20°C.

Polyacrylamide gel electrophoresis. SDS-PAGE was performed according to the method of Laemmli (1970), using 12% running gel and 5% stacking gel of polyacrylamide. The proteins were silver stained. The molecular weight was calculated using SynGene GeneTools. For the determination of the activity, the SDS was omitted and 9% running gel was prepared. The staining procedure comprised incubation with oxalic acid as a substrate and subsequent incubation with 2,6-dimethoxyphenol and horseradish peroxidase.

RESULTS

Degradation of oxalic acid in *A. biennis* cultures

In the tested *A. biennis* cultures, oxalate oxidase (OXOAb) activity was observed only after exogenous addition of oxalic acid as an inductor to the growth media. To confirm the way of oxalic acid degradation by *A. biennis*, the presence of formic acid in the growth media as a product of decarboxylation of oxalate, and the presence of hydrogen peroxide as a product of oxalic acid oxidation by oxalate oxidase, were tested. As shown in Fig. 1, H_2O_2 was detected in the growth media of *A. biennis* as the main product of oxalic acid degradation after 24 hours of induction. This suggests an oxidative activity of *A. biennis* towards oxalic acid catalysed by oxalate oxidase. Only

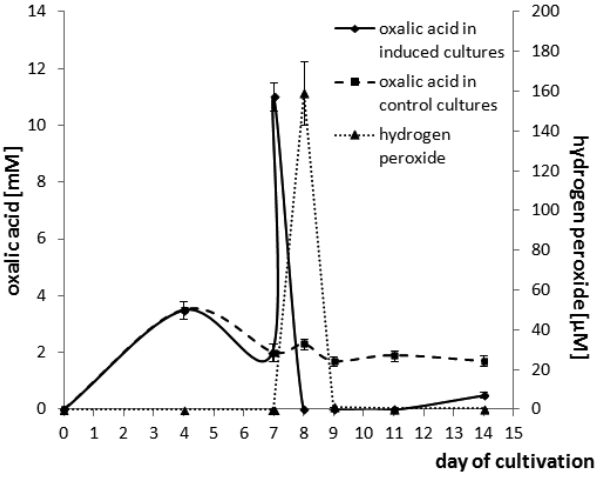

Figure 1. Oxalic acid degradation and hydrogen peroxide appearance after exogenous addition of oxalic acid on the 7th day of *A. biennis* growth.

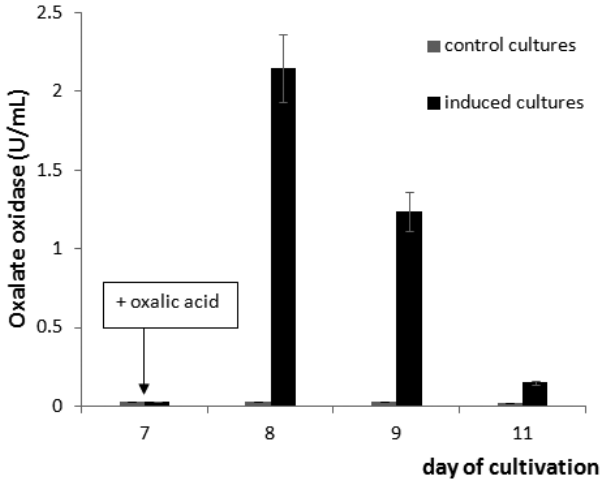

Figure 2. Intracellular oxalate oxidase activity in the control and induced with oxalic acid (final concentration 10 mM) cultures of *A. biennis*.
The arrow indicates the day of the inductor addition.

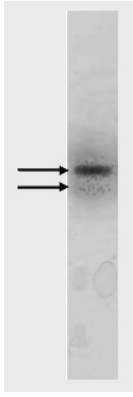

Figure 3. PAGE stained for oxalate oxidase activity.

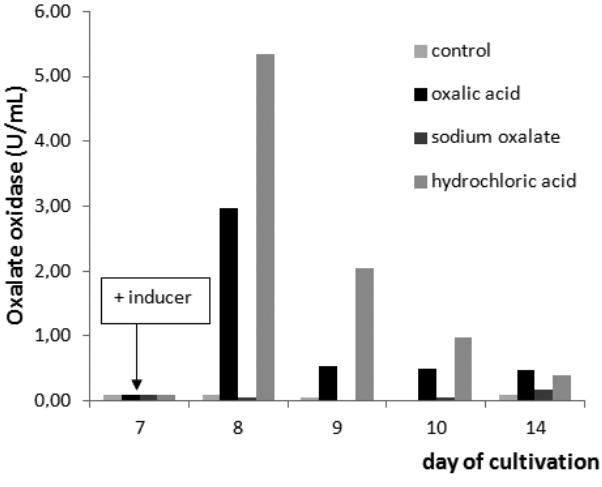

Figure 4. The effect of *A. biennis* culture supplementation with inducers (oxalic acid, sodium oxalate or hydrochloric acid) on OXOAb activity in mycelia.
The arrow indicates the day of the inductor addition. Control was set up without any inducer addition.

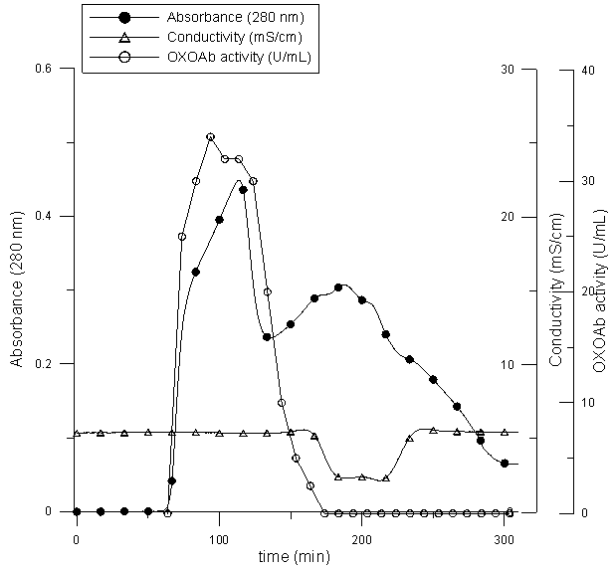

Figure 5. Elution profile of OXOAb activity obtained after size exclusion chromatography on Sephadex G-25.

intracellular activity of OXOAb was detected after induction of *A. biennis* cultures with oxalic acid (Fig. 2). No extracellular oxalate oxidase activity was detected in the growth media. Native intracellular OXOAb was visualised as two protein bands in a polyacrylamide gel, as presented in Fig. 3.

Factors influencing oxalate oxidase production by *A. biennis*

To specify if the expression of oxalate oxidase in *A. biennis* mycelium was induced by oxalic acid – the substrate of the enzyme, or by lowering the pH value of the growth media, a solution of oxalic acid, sodium oxalate, and HCl were added separately to the cultures of *A. biennis* as inductors. As shown in Fig. 4, the activity of *A. biennis* oxalate oxidase was detected in the mycelium of cultures induced by oxalic acid and HCl, but was not detected in cultures induced by addition of sodium oxalate. This indicates that the lowering of the pH value is sufficient for induction of oxalate oxidase activity during the growth of *A. biennis*.

Purification of oxalate oxidase from *A. biennis*

The mycelium of *A. biennis* from the culture induced with oxalic acid was homogenised and the OXO-specific activity was detected in the supernatant after centrifugation. The supernatant (the crude extract of OXOAb) was loaded onto a Sephadex G-25 column. After chromatography, the OXOAb activity was detected in the first peak (Fig. 5). The next step of the purification procedure was performed on a DEAE-Sepharose column and the elution profile is presented in Fig. 6. Fractions with the high activities of OXOAb were collected and desalted. The enzyme was finally purified 11.1 fold, with 1.7% recovery, when compared to the crude extract (Table 1). After size exclusion and ion-exchange chromatography, the collected fractions exhibiting OXOAb activities were analysed by SDS-PAGE, which revealed a band of protein with a molecular size calculated as 58 kDa (Fig. 7).

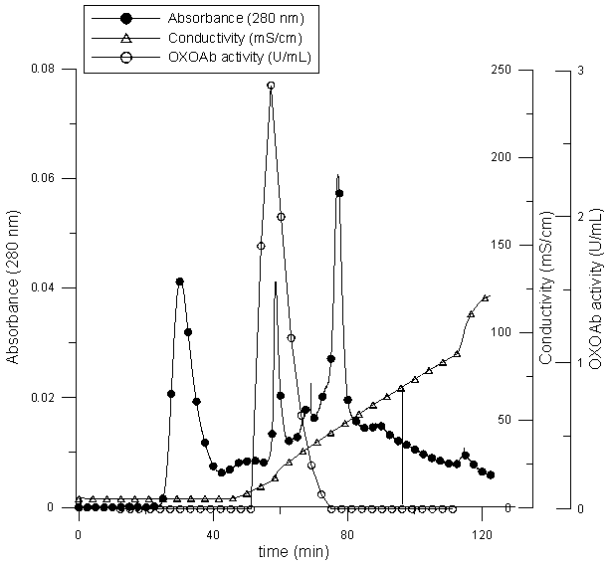

Figure 6. Elution profile of OXOAb activity obtained after ion exchange chromatography on DEAE-Sepharose.

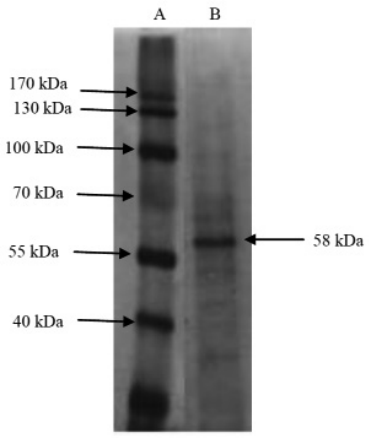

Figure 7. SDS-PAGE of OXOAb active fractions obtained after the purification procedure; lane A, protein marker; lane B, enzyme fraction obtained after chromatography on DEAE-Sepharose.

Properties of OXOAb

The optimum pH value for OXOAb activity was assayed in the pH range between 2 and 4. OXOAb displayed a maximum activity at pH 2, when incubated at 40°C. This activity decreased with the increasing pH and was almost gone at pH 4 (Fig. 8). The optimum temperature for oxidation of oxalic acid by OXOAb

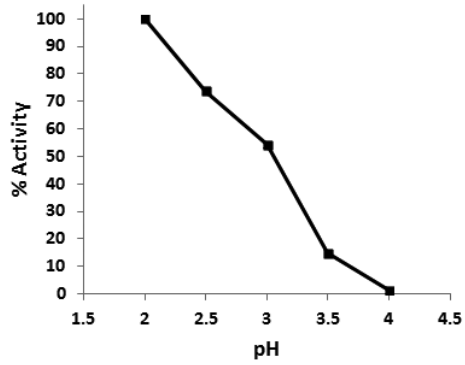

Figure 8. The effect of pH values on OXOAb activity. The activity of OXOAb measured at pH 2 was taken as 100%.

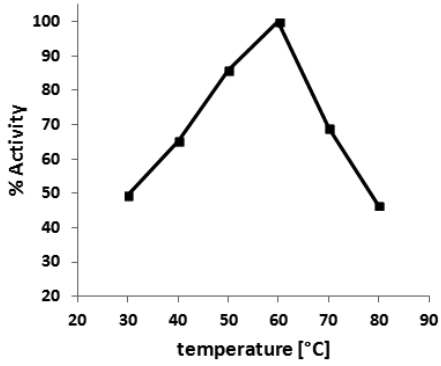

Figure 9. The effect of the temperature range (30–80°C) on OXOAb activity.
The activity of OXOAb measured at 60°C was taken as 100%.

is presented in Fig. 9. When OXOAb was assayed at temperatures in the range from 30°C to 80°C, the highest activity was found at 60°C. To determine substrate specificity, several organic acids, including citric, malic, malonic, glycolic, glioxalic, succinic, and acetic acid, were used as pSossible substrates for OXOAb. None of the tested carboxylic acids were oxidised by the OXOAb. The effect of the oxalic acid concentration on the initial velocity of the enzyme reaction was tested. The V_{max} of the enzyme was achieved at 0.03 mol min^{-1}. The K_m value for oxalic acid was found to be 0.015 M.

DISCUSSION

In this study, we tested the liquid cultures of *A. biennis* to confirm the unique ability of this fungus to degrade oxalic acid by oxidation. In our earlier study, we

Table 1. Summary of the purification procedure of OXOAb.

Fraction	Volume (mL)	Protein (mg)	Activity (U/mL)	Total activity (U)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Crude extract	60	706.2	28	1680	2.4	1	100
Gel filtration (Sephadex G-25)	20	105	34	680	6.5	2.7	40.5
Ion exchange chromatography (DEAE-Sepharose)	10	1.1	2.9	28.7	26.3	11.1	1.7

described enhanced oxalate oxidase activity in *A. biennis* in the presence of metal oxides (Grąz *et al*., 2009). Decarboxylation of oxalic acid by oxalate decarboxylase in basidiomycete fungi is typical and well described (Kesarwani *et al*., 2000; Mäkelä *et al*., 2002; Mäkelä *et al*., 2010). The oxidative way of oxalic acid decomposition is atypical for basidiomycete fungi and has been so far described only in *Ceriporiopsis subvermispora* (Aguilar *et al*., 1999) and *Tilletia contraversa* (Vaisey *et al*., 1961). Oxalic acid can be degraded by oxalate decarboxylase (EC 4.1.1.2) occurring in fungi, by oxalyl-CoA decarboxylase (EC 4.1.1.8) detected in bacteria, and by oxalate oxidase (EC 1.2.3.4) observed in plants (Svedružić *et al*., 2005). This rigid division of oxalate decomposition activities is now unsettled because of the latest reports concerning fungal oxalate oxidase from *C. subvermispora* (Aguilar *et al*., 1999) and oxalate oxidase from *A. biennis,* as well as plant oxalyl-CoA decarboxylase found in *Arabidopsis* (Foster *et al*., 2012). Oxalate oxidase and oxalate decarboxylase are members of the cupin superfamily of proteins. All so far described oxalate oxidases from plants (also known as germins) are classified as monocupins (Dunwell, 2000; Svedružić *et al*., 2005). The best characterised and purified fungal oxalate oxidase from *C. subvermispora* revealed the oxalate decarboxylase side reaction and was unexpectedly classified as a bicupin protein, the same as all known fungal oxalate decarboxylases (Escutia *et al*., 2005). The specific activity of purified *C. subvermispora* enzyme expressed in *P. pastoris* showed direct correlation with manganese ions content. Under methanol-induced expression, oxalate oxidase was secreted into the growth media as a soluble and functional enzyme, which is in contrast to the native intracellular enzyme from *C. subvermispora*. The pH optimum was similar and amounted to pH 3.5 for the native protein, and 4.0 for an enzyme expressed in *P. pastoris*. It was suggested that the recombinant protein was inhibited by succinate and by citrate in a competitive and uncompetitive manner, respectively (Moussatche *et al*., 2011). It was also demonstrated that bacterial oxalate decarboxylase from *B. subtilis* can be converted into an oxidase by mutation in the active site of the enzyme (Burrell *et al*., 2007). In our study, we have found oxalate oxidase activity (OXOAb) in *A. biennis* mycelium after induction of this enzyme with oxalic acid or HCl. To detect the OXOAb activity in *A. biennis*, only reduction of the pH value of the growth media was required. Known fungal oxalate decarboxylases have an inducible character, like an oxalate decarboxylase from basidiomycete *Flammulina velutipes* induced by oxalic acid (Azam *et al*., 2002), or an enzyme from *Trametes versicolor* induced by both, oxalic acid and inorganic acids (Zhu & Hong, 2010). Mäkelä and co-workers (2009) found a rather constitutive expression of an oxalate decarboxylase in *Dichomitus squalens* after addition of oxalic acid stimulating oxalate decarboxylase activity, but no increase in the transcript amount was observed, which suggests activation rather than transcriptional upregulation of the enzyme. The rapid degradation rate of oxalic acid by *A. biennis* observed in this study could also suggest the activation of the already existing enzyme rather than the induction process, but this hypothesis requires further studies.

All known oxalate degrading enzymes possess optimum activity at low pH values. The OXOAb was active only at acidic pH values below pH 4, with the highest activity at pH 2. A similar optimum pH value (pH 2.6) was described for OXO from *Tilletia contraversa* (Vaisey *et al*., 1961). The oxalate oxidase from *C. subvermispora* showed its maximum activity at pH 3.5, similar to known plant enzymes which exhibited their pH optima in the acidic range varying between pH 3.2, reported for maize roots (Velutić & Šulković, 2000), pH 3.5 for *Amaranthus spinosus* leaves (Goyal *et al*., 1999) and wheat seedlings (Hu & Guo, 2009), to pH 5 for *Sorghum* leaves (Pundir & Nath, 1984).

In our study, the optimum temperature for OXOAb activity was 60°C. OXOAb retained 20% of its initial activity after 15 minutes of pre-incubation at 60°C, and only 5% of its initial activity after pre-incubation at 80°C (data not shown). This indicates a protective character of the substrate on the active site of the enzyme. The optimum activity for fungal oxalate oxidase from *C. subvermispora* was obtained at 40°C (Aguilar *et al*., 1999). Plant enzymes showed their optimum temperatures at 40°C for OXO from *A. spinosus* (Goyal *et al*., 1999), and 37°C for the enzyme from barley roots (Kotsira & Clonis, 1997). Plant enzymes reveal higher thermal stability, e.g. strawberry fruit oxalate oxidase retained 76% of its initial activity after incubation at 80°C for 30 minutes (Dahiya *et al*., 2010).

Among the tested organic acids, OXOAb oxidised only oxalic acid. The K_m value for oxalate was found to be 1.5×10^{-2} M and the V_{max} value was 0.03 mol min^{-1}. These parameters are higher than those found for oxalate oxidase from the *C. subvermispora* fungus which has K_m for oxalate of 1×10^{-4} M and was inhibited at an oxalic acid concentration above 2.5 mM. Known oxalate oxidases from plants revealed different values of K_m for oxalate degradation, e.g. it was 1.67×10^{-3} M for a strawberry fruit enzyme (Dahiya *et al*., 2010), 2.1×10^{-4} M for an enzyme from wheat seedlings (Hu and Guo, 2009), and 2.16×10^{-3} M for OXO from *Amaranthus* leaves (Goyal *et al*., 1999). Oxalate oxidases isolated from plants are multimeric proteins (Svedružić *et al*., 2005). The molecular weight of fungal oxalate oxidase from *C. subvermispora* was 400 kDa and was suggested to be a homohexameric enzyme. The molecular weight of 58 kDa observed for OXOAb in this study (SDS-PAGE) corresponds to the molecular weight of a monomer of oxalate oxidase from *C. subvermispora* determined as 65.5 kDa (Aguilar *et al*., 1999).

CONCLUSIONS

Searching for novel enzymes with potentially divergent properties from those described so far seems to be well justified because oxalate-degrading enzymes have a great potential in various biotechnological and diagnostic applications. Oxalate oxidase from *Abortiporus biennis* represents a unique catalytic activity in basidiomycete fungi. In our opinion, development of knowledge in the field of plant and microorganism enzymatic regulation of the level of oxalate in the environment could contribute to progress in the field of bioremediation, geomycology, and agriculture.

Acknowledgements

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