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Indolyl-3-acetaldoxime dehydratase from the phytopathogenic fungus *Sclerotinia sclerotiorum*: Purification, characterization, and substrate specificity

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ABSTRACT

The purification and characterization of indolyl-3-acetaldoxime dehydratase produced by the plant fungal pathogen *Sclerotinia sclerotiorum* is described. The substrate specificity indicates that it is an indolyl-3-acetaldoxime dehydratase (IAD, EC 4.99.1.6), which catalyzes transformation of indolyl-3-acetaldoxime to indolyl-3-acetonitrile. The enzyme showed Michaelis–Menten kinetics and had an apparent molecular mass of 44 kDa. The amino acid sequence of IAD, determined using LC–ESI-MS/MS, identified it as the protein SS1G_01653 from *S. sclerotiorum*. IADSs was highly homologous (84% amino acid identity) to the hypothetical protein BC1G_14775 from *Botryotinia fuckeliana* B05.10. In addition, similarity to the phenylacetaldoxime dehydratases from *Gibberella zeae* (33% amino acid identity) and *Bacillus* sp. (20% amino acid identity) was noted. The specific activity of IADSs increased about 17-fold upon addition of $Na_2S_2O_4$ under anaerobic conditions, but in the absence of $Na_2S_2O_4$ no significant change was observed, whether aerobic or anaerobic conditions were used. As with other aldoxime dehydratases isolated from microbes, the role of IADSs in fungal plant pathogens is not clear, but given its substrate specificity, it appears unlikely that IADSs is a general xenobiotic detoxifying enzyme.

1. Introduction

Indolyl-3-acetaldoxime (**1**) is a metabolite of central importance to cruciferous plants (family Brassicaceae) due to its role as a biosynthetic precursor of several chemical defenses. A number of inducible (phytoalexins) and constitutive (phytoanticipins) defenses of crucifers are biosynthetically derived from tryptophan via indolyl-3-acetaldoxime (**1**) (Pedras et al., 2007; Pedras and Yaya, 2010; Bender and Celenza, 2009; Sugawara et al., 2009). For example, the phytoalexin brassinin (**4**) and the phytoanticipin glucobrassicin (**7**) derive from **1** via *S*-methyl indolyl-3-acetothiohydroxamic acid (**2**) (Pedras and Okinyo, 2008), whereas the phytoalexin camalexin (**5**) derives from **1** via indolyl-3-acetonitrile (**3**) (Glawischnig, 2007; Nafisi et al., 2007) (Fig. 1). In addition, one of the pathways leading to the plant hormone indolyl-3-acetic acid (**6**) appears to involve indolyl-3-acetaldoxime (**1**) as an intermediate (Pollmann et al., 2006). Undoubtedly, the numerous natural products deriving from indolyl-3-acetaldoxime (**1**) convey its vital contribution to the overall fitness of cruciferous species.

Investigation of the metabolism of indolyl-3-acetaldoxime (**1**) by three plant pathogenic fungi [*Leptosphaeria maculans* (Desm.) Ces. et Not. asexual stage *Phoma lingam* (Tode ex Fr.) Desm., *Rhizoctonia solani* Kuhn, and *Sclerotinia sclerotiorum* (Lib.) de Bary], and by an insect pathogenic fungus (*Beauveria bassiana*) established that these fungal species metabolized oxime **1** to indolyl-3-acetic acid (**6**) (Pedras and Montaut, 2003). Interestingly, while the plant fungi metabolized **1** via indolyl-3-acetonitrile (**3**), metabolism by the insect pathogen (*B. bassiana*) took place via tryptophol (**8**) (Fig. 2). Although the fungal enzymes responsible for those fungal biotransformations were not isolated, the puzzling similarity between the transformation carried out by plant fungal pathogens and cruciferous plants was noted (Pedras and Montaut, 2003).

The current interest on microbial aldoxime dehydratases reflects the biotechnological application of these enzymes as catalysts (Kato and Asano, 2005; Martinkova et al., 2008). To date, three aldoxime converting enzymes were registered in the IUBMB Enzyme Nomenclature database: aliphatic aldoxime dehydratase (EC 4.99.1.5, from *Pseudomonas chlororaphis*), indolyl-3-acetaldoxime dehydratase (EC 4.99.1.6, from *Gibberella fujikuroi*), and phenylacetaldoxime dehydratase (EC 4.99.1.7, from various microbial species). The aliphatic aldoxime dehydratase (EC 4.99.1.5) was shown to contain Ca^{2+} and protoheme IX requiring Fe^{2+} for activity (Oinuma et al., 2003). This dehydratase (EC 4.99.1.5) displayed a strong preference for aliphatic aldoximes, such as butyraldoxime and acetaldoxime, over aromatic aldoximes; no activity was found with benzaldoxime or pyridine-4-aldoxime. Indolyl-3-acetaldoxime dehydratase (IAD, EC 4.99.1.6) was partially purified from *G. fujikuroi* (Kumar and Mahadevan, 1963) using ammonium

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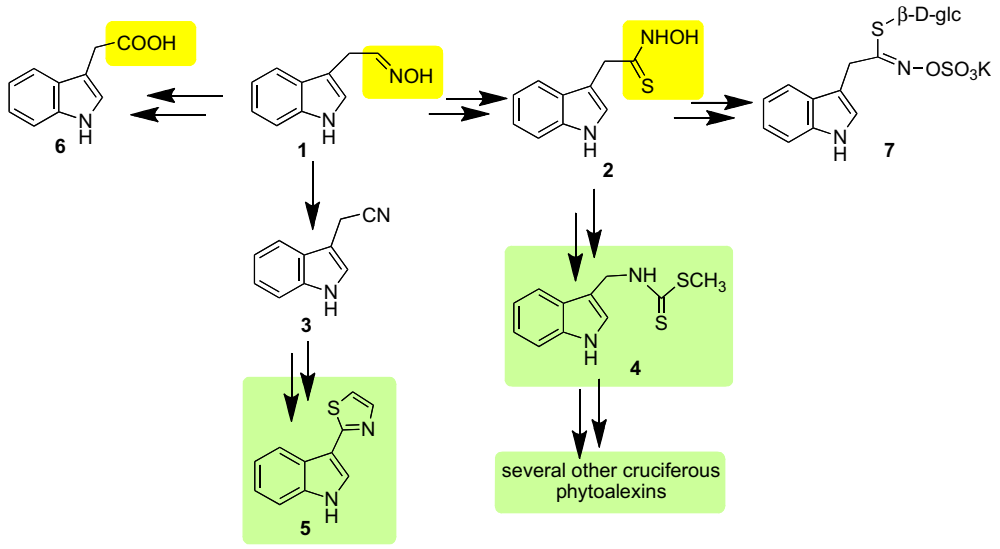


Fig. 1. *E*/*Z*-Indolyl-3-acetaldoxime (**1**) is a biosynthetic precursor of various cruciferous phytoalexins such as brassinin (**4**) and camalexin (**5**), the plant hormone indolyl-3-acetic acid (**6**) and the phytoanticipin glucobrassicin (**7**).

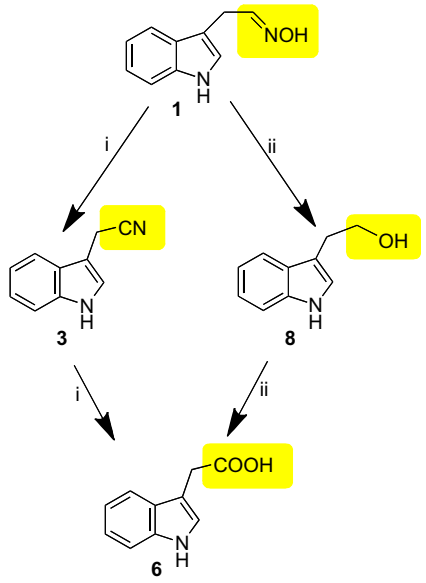


Fig. 2. Biotransformation of *E*/*Z*-indolyl-3-acetaldoximes (**1**) by: (i) the plant pathogenic fungi *Leptosphaeria maculans*, *Rhizoctonia solani*, or *Sclerotinia sclerotiorum*; (ii) the insect pathogenic fungus *Beauveria bassiana*.

sulfate fractionation followed by ion exchange chromatography (Shukla and Mahadevan, 1968, 1970). IAD activity was shown to increase in the presence of ascorbic acid, dehydroascorbic acid, pyridoxal-5′-phosphate (PLP), and ferrous ions, whereas thiols inhibited enzymatic activity. To the best of our knowledge, no further reports on IAD (EC 4.99.1.6) have appeared since 1970. The third aldoxime converting enzyme, phenylacetaldoxime dehydratase Oxd-B1 (EC 4.99.1.7), catalyzing dehydration of *Z*-phenylacetaldoxime to phenylacetonitrile, was purified from a *Bacillus* sp. (Kato et al., 2000). The enzyme was shown to be a monomer of 40 kDa containing a loosely bound protoheme I with an Fe^{2+} requirement for activity, and a His-306 catalytic residue (Kobayashi et al., 2005). Another bacterial oxime dehydratase purified from *P. chlororaphis* contained also a protoheme and required Fe^{2+} for activity, as did a dehydratase from *Rhodococcus globerous* (Xie et al., 2003). A fungal aldoxime dehydratase (Oxd) was isolated from *Gibberella zeae* (Kato and Asano, 2005) and found to have similar enzymatic properties to those of bacterial Oxd-B1. Both Oxd (fungal) and Oxd-B1 (bacterial) enzymes were classified as EC 4.99.1.7, since these enzymes showed maximum activity with phenylacetaldoxime as substrate.

Plant aldoxime dehydratases, by contrast, are not as well characterized as their microbial counterparts. Brassicaceae do not appear to have a soluble enzyme(s) able to convert indolyl-3-acetaldoxime (**1**) to indolyl-3-acetonitrile (**3**) *in vitro*, although a soluble indolyl-3-acetaldoxime converting enzyme in banana leaves was reported earlier (Mahadevan, 1963; Kumar and Mahadevan, 1963). An enzyme catalyzing the conversion indolyl-3-acetaldoxime (**1**) to indolyl-3-acetonitrile (**3**) was detected in the plasma membrane of seedlings of Chinese cabbage (*Brassica rapa* L.) (Helmlinger et al., 1985). This enzyme was partially purified by gel filtration and shown to have a molecular mass around 40 kDa, K_m 6.3 μM and a pH optimum of 6.0. A cytochrome P_{450} enzyme (CYP71A13) expressed in *Escherichia coli*, was shown to catalyze the conversion of indolyl-3-acetaldoxime (**1**) to indolyl-3-acetonitrile (**3**) in *Arabidopsis thaliana* L. (Nafisi et al., 2007). Although phenylacetaldoxime was shown to bind to the active site of CYP71A13, it was not established to be converted to the nitrile.

The characterization and identification of fungal enzymes involved in the conversion of acetaldoxime **1** will provide unique opportunities to investigate similarities between plant and fungal enzymes and the potential roles of these enzymes in the interaction of cruciferous plants with their fungal pathogens. Toward this end, the present study reports the screening of cruciferous phytopathogenic fungi for IAD activity, followed by purification, characterization, sequence identification and substrate specificity of the enzyme produced by the phytopathogenic fungus *S. sclerotiorum*, hereon designated as IADSs. Comparison of the amino acid sequence of IADSs with those of other proteins in the NCBI database shows that IADSs does not exhibit substantial sequence similarity to other characterized aldoxime dehydratases from either microbial or plant origin.

2. Results and discussion

2.1. Synthesis of potential substrates and products of IADSs

To establish the substrates used by IADSs, a series of aromatic and aliphatic oximes and the corresponding nitriles were prepared

as shown in Figs. 3–5. Aldoximes **1**, **13**, **14**, **19** and **20** were obtained from oximation of the corresponding aldehydes using hydroxylamine hydrochloride and sodium acetate in an ethanol solution. Those aldehydes not commercially available were obtained from reduction of either the corresponding esters **9a** and **10a**, or from the nitriles **3**, **17** and **18**, as follows.

3-(3-Indolyl)propanal oxime (**13**) and 4-(3-indolyl)butanal oxime (**14**) were synthesized in three steps from 3-(3-indolyl)propanoic acid (**9**) and 4-(3-indolyl)butanoic acid (**10**), respectively. First, each acid was treated with EtOH and catalytic amount of H_2SO_4 (10 mol%) at reflux temperature to yield the corresponding ethyl ester in almost quantitative yield. Next, each ester was reduced with DIBAL-H (1.5 M in toluene) to afford the aldehyde, which was directly used in the next step without further purification. Each aldehyde was allowed to react with hydroxylamine hydrochloride and sodium acetate in EtOH to afford the corresponding oxime as a mixture of *E*/*Z* isomers. Oxime **14** has not been previously reported, whereas oxime **13** was reported, but its spectroscopic data is not available (Kuchkova and Semenov, 1975). Nitriles **15** and **16** were prepared from dehydration of the corresponding oximes **13** and **14**, respectively, upon treatment with Ac_2O and pyridine in CH_2Cl_2, in reasonable yields (Fig. 3).

Indolyl-3-acetaldoxime (**1**) was obtained from reduction of indolyl-3-acetonitrile (**3**) with DIBAL-H followed by oximation, as detailed in Section 4 and previously described for the tetradeuterated compound (Pedras and Okinyo, 2006). Naphthylacetaldoximes **19** and **20** were prepared from the corresponding naphthylacetonitriles **17** and **18** using DIBAL-H in toluene (Fig. 4). Aliphatic oximes **22**, **24**, and **26**, were obtained from the corresponding aldehydes, after oximation was carried out in good yields. Replacement of toluene with CH_2Cl_2, increased the yields slightly (Fig. 5).

2.2. Screening for IAD activity in plant fungal pathogens and optimization of culture conditions

In preliminary experiments, because *L. maculans*, *R. solani*, and *S. sclerotiorum* were found to metabolize *E*/*Z*-indolyl-3-acetaldoximes to the corresponding acetonitrile **3** (Pedras and Montaut, 2003), crude cell-free extracts were prepared and screened for IAD activity as described in Section 4. While crude cell-free mycelial extracts of *R. solani* showed no dehydratase activity, crude cell-free mycelial extracts of both *L. maculans* (isolate BJ 125, virulent

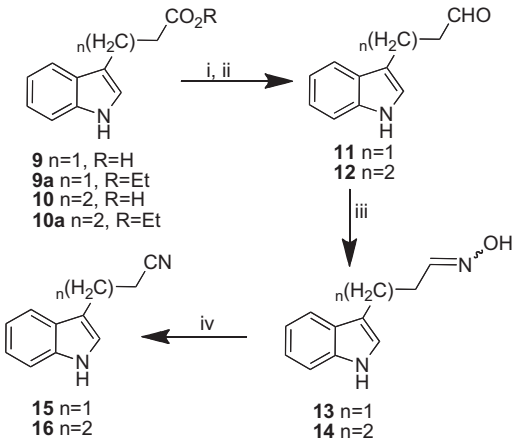


Fig. 3. Syntheses of *E*/*Z*-3-(3-indolyl)propanal oximes (**13**) and *E*/*Z*-4-(3-indolyl)butanal oximes (**14**); reagents and conditions: (i) H_2SO_4 (10 mol%), 95% EtOH, reflux, 2 h; (ii) DIBAL-H (1.5 M in toluene), −78 °C, 30 min, 2 M HCl; (iii) $NH_2OH{\cdot}HCl$, AcONa, 95% EtOH, H_2O, 0 °C, 3 h; (iv) Ac_2O, pyridine, CH_2Cl_2, 45 °C, 12 h.

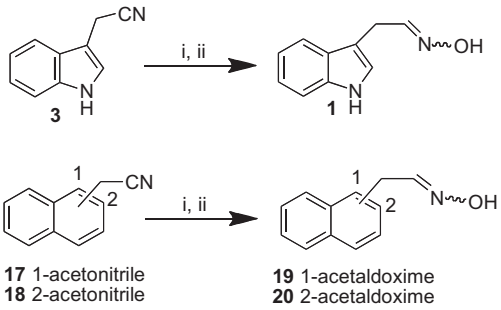


Fig. 4. Syntheses of *E*/*Z*-indolyl-3-acetaldoximes (**1**), *E*/*Z*-naphthyl-1-acetaldoximes (**19**), and *E*/*Z*-naphthyl-2-acetaldoximes (**20**); reagents and conditions: (i) DIBAL-H (1.5 M in toluene), CH_2Cl_2, −78 °C, 20 min; (ii) $NH_2OH{\cdot}HCl$, AcONa, 95% EtOH, H_2O, 0 °C.

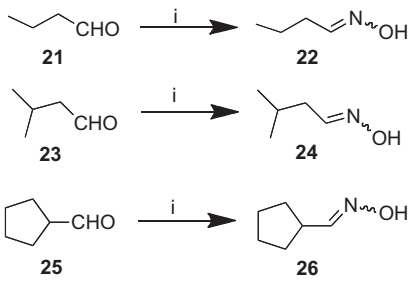


Fig. 5. Syntheses of *E*/*Z*-indolyl-3-acetaldoximes, *E*/*Z*-butanal oximes (**22**), *E*/*Z*-2-methylbutanal oximes (**24**) and *E*/*Z*-cyclopentylcarboxaldehyde oximes (**26**); reagents and conditions: (i) $NH_2OH{\cdot}HCl$, AcONa, 95% EtOH, H_2O, 0 °C.

on canola) and *S. sclerotiorum* displayed reasonable IAD activity ranges (1–4 nmol/min/mg). Absence of IAD activity in crude cell-free mycelial extracts of *R. solani* may be due to the small amount of enzyme produced under the tested culture conditions. This hypothesis is consistent with the much slower rates of biotransformation of indolyl-3-acetaldoxime (**1**) in cultures of *R. solani* than in cultures of *L. maculans* or *S. sclerotiorum* (Pedras and Montaut, 2003).

S. sclerotiorum was chosen to isolate the protein(s) with IAD activity because the availability of its genome sequence database would offer the opportunity to find the complete sequence of IADSs and then compare it with other acetaldoxime dehydratases available in public databases. Hence, to optimize culture conditions to obtain maximum IAD activity, sclerotium-initiated cultures of *S. sclerotiorum* were incubated under different light and temperature conditions. Cell-free extracts obtained from mycelia of 5-day-old cultures incubated under constant light yielded the best IAD activity. In addition, cell-free extracts obtained from mycelia of cultures incubated at 20, 23 and 28 °C showed that the highest IADSs activity was obtained at 23 ± 2 °C. At temperatures $\geqslant$28 °C, sclerotia of *S. sclerotiorum* did not germinate.

2.3. Purification of IADSs

IAD activity was detected in crude cell-free extract of mycelia of *S. sclerotiorum* using a mixture of *E*/*Z*-indolyl-3-acetaldoximes (**1**) as substrate. To determine the likely cellular localization of IADSs, crude cell-free protein extracts were fractionated into soluble, membrane and cell wall fractions, as described in Section 4 (Table 1). The highest total and specific activities were found in the soluble fraction, which indicated that the enzyme was most likely present in the cytoplasm. This soluble fraction was used for purification of the enzyme.

Table 1
Fractionation of proteins from mycelia of *Sclerotinia sclerotiorum* for cellular localization of IADSs.

Protein fraction	Volume of fraction (ml)	Protein (mg)	Total activity (nmol/min)	Specific activity (nmol/min/mg)
Soluble	20	100	279	2.78
Cell wall	19	158	55.5	0.35
Membrane	14	88.3	2.7	0.03

An enzyme with IAD activity was purified 39-fold in 2% yield from the crude cell-free extract of mycelia of *S. sclerotiorum* using a mixture of *E*/*Z*-indolyl-3-acetaldoximes (**1**) as substrate. To the best of our knowledge, this is the first report of the purification and characterization of an enzyme that dehydrates aldoximes from *S. sclerotiorum*. Crude protein extracts were prepared from mycelial cultures grown under conditions reported in Section 4. In preliminary purification attempts, loss of activity of IADSs was related to absence of detergent and glycerol in buffers. For this reason, Triton X-100 (0.015%) and glycerol (2–3%) were added to all purification buffers. In this manner, enzymatic activities were stabilized, but an activity decrease continued to occur, albeit slower, even within one week of storage at −20 °C. Therefore, after each purification step, the enzyme fractions were stored up to a maximum of 24 h at −20 °C and immediately used for the next step of purification. The chromatographic purification of IADSs involved three purification steps: (1) ion-exchange on DEAE Sephacel, (2) hydroxyapatite chromatography, and (3) gel filtration on Superdex G-75, as detailed in Section 4. Table 2 summarizes the yields and purification factors obtained in each purification step. Fractions with IADSs activity obtained in the last chromatographic separation were pooled, concentrated and used for biochemical analysis. Aldoxime dehydratases from other sources such as indolyl-3-acetaldoxime dehydratase from *G. fujikuroi* (Shukla and Mahadevan, 1970), phenylacetaldoxime dehydratase from *Bacillus* sp. (Kato et al., 2000), and recombinant aldoxime dehydratase from *Rhodococcus* sp. N-771 (Kato et al., 2004) were reported to lose activity during purification.

Analysis by SDS–PAGE (stained with Coommassie brilliant blue) of cell-free extracts and active fractions obtained in each chromatographic step was carried out. SDS–PAGE of the active fraction obtained from Superdex 75 chromatography indicated a single band, with an apparent molecular mass of 44 kDa (Fig. 6). In addition, Superdex 75 chromatography of the purified IADSs suggested that it was a native monomer since it was eluted at a position corresponding to a molecular mass similar to that determined by SDS–PAGE.

2.4. Identification of purified IADSs tryptic peptides by LC–ESI-MS/MS

To identify the purified protein IADSs, the band obtained from SDS–PAGE was analyzed by LC–ESI-MS/MS. The identity of the enzyme was established by the algorithms of Mascot distiller. IADSs

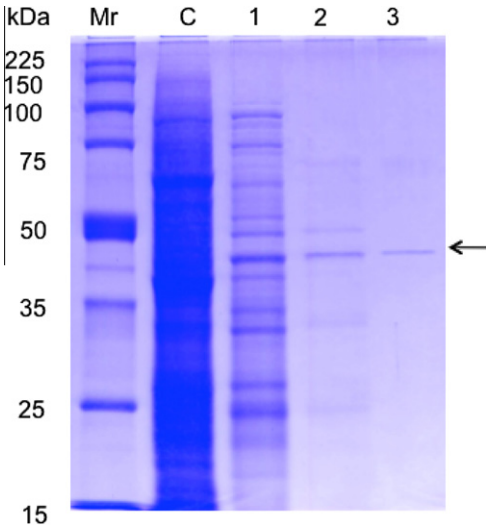


Fig. 6. SDS–PAGE of fractions and purified enzyme: M_r, marker proteins (Bio-Rad); lane C, crude homogenate (40 μg); lane 1, DEAE-Sephacel pooled fractions (15 μg); lane 2, hydroxyapatite chromatography (2 μg); lane 3, purified IADSs after Superdex 75 (1 μg).

was identified as the hypothetical protein SS1G_01653 from *S. sclerotiorum* (Accession No. XP_001597459 in the BLAST data bank). Searches to determine the peptidic sequence similarity using BLAST revealed a strong similarity (84% amino acid identity) to the hypothetical protein BC1G_14775 (Accession No. XP_001547065 in the BLAST data bank) from *Botryotinia fuckeliana* (*Botrytis cinerea*) B05.10 (Fig. 7). In addition, some similarities to aldoxime dehydratases such as phenylacetaldoxime dehydratase from *G. zeae* (33% amino acid identity) and *Bacillus* sp. (Oxd-B1) (20% amino acid identity) were found. The amino acid sequence of IADSs was aligned with sequences of characterized aldoxime dehydratases to determine molecular properties and position of conserved amino acid residues. Based on multiple sequence alignments of the aldoxime dehydratases IADSs and homologous enzymes Oxd from *G. zeae* and Oxd-B1 from *Bacillus* sp., a conserved His was observed only at position His-307 (Fig. 7). However, comparison of IADSs to Oxd from *G. zeae* indicated that four His residues were conserved at positions His-23, His-60, His-253 and His-307 (Kato and Asano, 2005). Similarly, comparison of IADSs to Oxd-B1 from *Bacillus* sp. established that three His residues were conserved at positions His-295, His-303, and His-307 (Kato and Asano, 2005). Furthermore, additional common features of aldoxime dehydratases found in IADSs, Oxd, and OxB-1 sequences included the conserved Tyr–Trp–Gly (YWG) motifs, beginning at residue Tyr-138 for IADSs. Histidine residues are important for the catalytic function of aldoxime dehydratases. A combination of site-directed mutagenesis, spectroscopic measurements and X-ray crystallography of the aliphatic aldoxime dehydratase from

Table 2
Purification of IADSs obtained from mycelia of *Sclerotinia sclerotiorum*.

Purification step	Yield[a]		Specific activity (nmol/min/mg)	Recovery[b] (%)	Purification factor[c] (fold)
	Total protein (mg)	Total activity (nmol/min)			
Crude cell-free extract	19.8 mg	88.9	4.50	100	1
DEAE Sephacel	0.72	14.7	20.4	17	4
Hydroxyapatite	0.040	6.1	153	7	34
Superdex 75	0.008	1.4	175	2	39

[a] Mycelia obtained from 100 ml of sclerotium-initiated cultures.
[b] Recoveries are expressed as percentage of initial activity.
[c] Purification factors are calculated on the basis of specific activities of crude cell-free extract and active fractions.

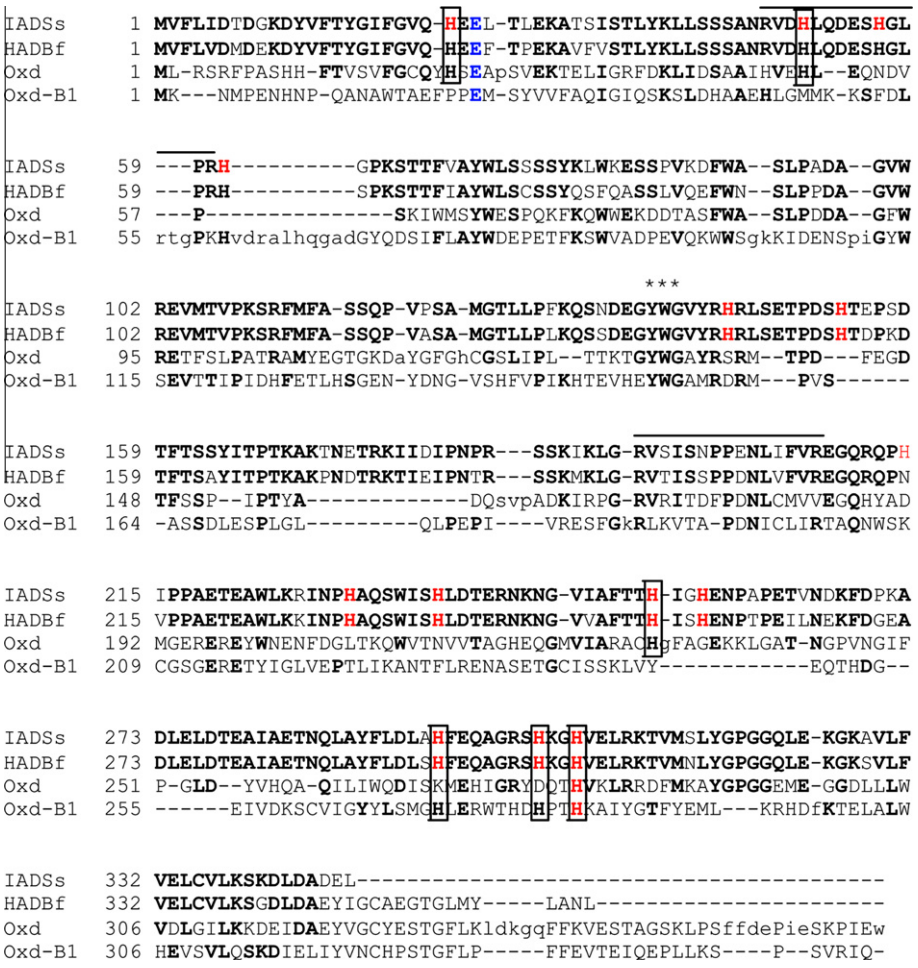


Fig. 7. Alignment of amino acid sequences of IADSs (purified in this work), HADBf (hypothetical protein from *Botryotinia fuckeliana* B05.10, Accession No. XP_001547065), Oxd (phenylacetaldoxime dehydratase from *Gibberella zeae*, Accession No. XP_386658) and Oxd-B1 (phenylacetaldoxime dehydratase from *Bacillus* sp., Accession No. BAA90461). Identical residues to IADSs are shown in bold. Rectangles indicate the putative conserved histidine residues and asterisks indicate conserved YWG motif. Overlines indicate peptide sequences obtained by LC–MS/MS after proteolytic cleavage of IADSs by trypsin.

P. chlororaphis B23 (OxdA) demonstrated that histidine residues were involved in binding of the prosthetic group (heme) to the enzyme, thus playing a crucial role in catalysis (Konishi et al., 2004; Oinuma et al., 2004, 2005; Sawai et al., 2009).

Analyses of the complete sequence of IADSs using Compute pI/Mw tool software predicted a pI of 5.5 and a molecular mass of ca. 41 kDa, which is in reasonable agreement with the molecular mass obtained by gel filtration and SDS–PAGE (44 kDa).

2.5. Characterization and substrate specificity of IADSs and effect of additives

The pH and temperature dependence of IADSs activity indicated that the maximum activity of the enzyme was observed in the pH range of 7.2–7.5 (Fig. 8A) at 25 °C (Fig. 8B), similar to those previously reported for aldoxime dehydratases Oxd-B1 and Oxd (Kato et al., 2000, 2004; Kato and Asano, 2006; Xie et al., 2003).

The substrate saturation curves of IADSs were determined in the presence of increasing concentrations of indole-3-acetaldoxime. The results showed that the enzyme follows Michaelis–Menten kinetics. The K_m was found to be 0.29 ± 0.04 mM, V_{max} 0.22 ± 0.01 U/mg (3.67 ± 0.17 mkatals/kg) and k_{cat} 10 s^{-1}, where U = μmol of product formed per min (Fig. S1). These parameters significantly differ from homologous enzymes Oxd from *G. zeae* (K_m 1.46 mM and V_{max} 19.3 U/mg) (Kato and Asano, 2005) and Oxd-B1 from *Bacillus* sp. (K_m 2.40 mM and V_{max} 5.42 U/mg) (Kato et al., 2000). In all cases the kinetic parameters were determined under aerobic conditions.

Due to the stability and a low yield of IADSs, purification of the enzyme using three purification steps yielded insufficient quantities to test the substrate specificity of IADSs. Therefore, a faster method using a HiTrap DEAE FF chromatography was developed for partial purification of IADSs, as described in Section 4. In this manner, partial purification of IADSs and substrate specificity assays could be performed in the same day. Crude protein extract (ca. 7 mg), prepared from the mycelia grown under standard conditions, was subjected to HiTrap DEAE FF chromatography, the fractions with IADSs activity were pooled and used immediately for testing the enzyme specificity. The substrate specificity of the purified IADSs was tested using various synthetic compounds containing indolyl, naphthyl, phenyl and alkyl moieties. As summarized in Table 3, enzymatic activity toward *E*/*Z*-indolyl-3-acetaldoximes (**1**), *E*/*Z*-3-(3-indolyl)propanal oximes (**13**), *E*/*Z*-4-(3-indolyl)butanal oximes (**14**), *E*/*Z*-4-hydroxyphenyl acetaldoximes (**30**) and *E*/*Z*-4-methoxyphenylacetaldoximes (**31**) was determined. *E*/*Z*-Indolyl-3-acetaldoximes (**1**) were the best substrates for IADSs, which displayed a 2-fold lower activity with 3-(3-indolyl)propanal oxime (**13**), and an 8-fold lower activity with 4-(3-indolyl)butanal oxime (**14**). In addition, the enzyme displayed no activity toward *E*/*Z*-indole-3-carboxaldehyde oxime (**27**), naphthyl oximes **28** and **29**, *E*/*Z*-phenylacetaldoximes (**32**) and aliphatic aldoximes **22**, **24** and **26**. The significant differences in substrate

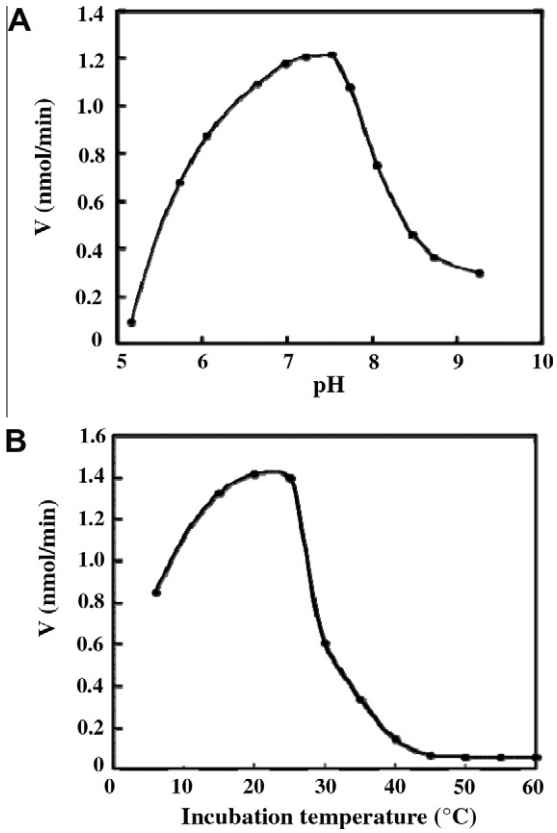


Fig. 8. Effects of pH (A) and temperature (B) on the IADSs activity.

specificity observed for the indolyl, naphthyl, and phenyl oximes suggest that the aldoxime functional group is not sufficient for substrate recognition; there is a shape and size requirement in the binding pocket of IADSs. Clearly, *E*/*Z*-indolyl-3-acetaldoximes are preferred relative to homologues containing additional CH_2 groups (**13** and **14**). As well, the lack of transformation of naphthyl oximes vs. substituted phenyl oximes **30** and **31**, but not **32**, may be due to the larger size of the naphthyl group and smaller size of the unsubstituted phenyl group. This result also indicates that the presence of an aromatic group is not sufficient for the enzymatic transformation of oximes to occur.

Previous studies indicated that the calculated heme content of a few aldoxime dehydratases from different sources was less then 1 mol of heme per mol of enzyme and that the heme was lost during purification of these enzymes (Kato et al., 2000; Oinuma et al., 2003; Xie et al., 2003). In the case of IADSs, due to insufficient amounts, it was not possible to get either heme spectra or a reliable heme stain band for the purified enzyme. Nonetheless, the activity of IADSs was examined in presence of several additives, as shown in Table 4. No significant inhibition or activation of the enzyme occurred in the presence of DTT, $FeCl_3$, NaN_3, PLP, and FAD; however, addition of 1 mM of $Na_2S_2O_4$ or $FeCl_2$ resulted in a slight increase of IADSs activity (Table 4). Previous work reported that the specific activity of aldoxime dehydratases increased substantially in the presence of $Na_2S_2O_4$ under reducing conditions (anaerobic) (Oinuma et al., 2003; Xie et al., 2003; Kato et al., 2004; Kobayashi et al., 2005). In the case of IADSs, no substantial enhancement of specific activity was observed upon addition of $Na_2S_2O_4$ under aerobic conditions. However, upon addition of $Na_2S_2O_4$ under anaerobic conditions, the specific activity of IADSs increased about 17-fold (Table 4), but in the absence of $Na_2S_2O_4$ no significant change was observed, whether aerobic or anaerobic conditions were used. By analogy with the aliphatic aldoxime dehydratase (Oinuma et al., 2003) and Oxd-B1 (Kobayashi et al., 2005), these findings suggest that IADSs contains the Fe^{2+}/Fe^{3+} cofactor that does not catalyze dehydration of indolyl-3-acetaldoxime in the oxidized form (aerobic conditions), that is the enzyme is inactive in the presence of Fe^{3+}. Upon reduction of the cofactor to Fe^{2+} with $Na_2S_2O_4$, under anaerobic conditions, IADSs becomes active, indicating that the oxidation reaction is reversible. Therefore, it is concluded that the enzyme purification steps can be performed under aerobic conditions since cofactor oxidation is reversible.

3. Conclusion

Substrate specificity studies indicated that *E*/*Z*-indolyl-3-acetaldoximes (**1**) are the best substrates for IADSs (the configuration of the oxime did not affect enzymatic activity at the concentrations of substrate used). Hence, by contrast to the aldoxime dehydratases Oxd from *G. zeae* and Oxd-B1 from *Bacillus* sp., designated as phenylacetaldoxime dehydratases (EC 4.99.1.7), IADSs is proposed to be designated as indolyl-3-acetaldoxime dehydratase (EC 4.99.1.6). In addition, the kinetic parameters for IADSs using *E*/*Z*-indolyl-3-acetaldoximes as substrate differed from those reported for Oxd and Oxd-B1. Since our results strongly suggest that IADSs has distinct substrate specificity and properties, perhaps the low sequence similarity between IADSs and either Oxd or Oxd-B1 (33% and 20% amino acid identity, respectively) is not surprising, although examples of highly homologous enzymes with different substrate specificities are known.

In conclusion, IADSs has a new primary structure and the substrate specificity expected for an indolyl-3-acetaldoxime dehydratase; this appears to be the first report of the purification, characterization and sequencing of such an enzyme. Nonetheless, as with other aldoxime dehydratases isolated from microbes, the role of IADSs in plant pathogens is not clear, particularly considering that *E*/*Z*-indolyl-3-acetaldoximes (**1**), in contrast to phytoalexins, do not usually accumulate in plant cells. As well, given its substrate specificity, it appears unlikely that IADSs is a general xenobiotic detoxifying enzyme, although indolyl-3-acetaldoxime (**1**) is somewhat inhibitory to *S. sclerotiorum* (ca. 40%, Pedras and Montaut, 2003), whereas indolyl-3-acetonitrile (**3**) is not. Toward this end, future work will address the characterization of additional indolyl-3-acetaldoxime dehydratases from other cruciferous fungal pathogens and investigation of their properties, functions, co-enzymes and chemical mechanism.

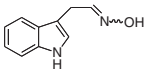
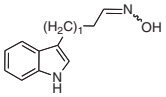
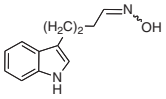
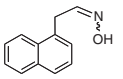
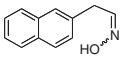
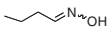
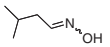
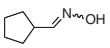
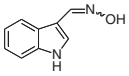
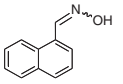
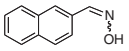
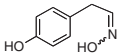
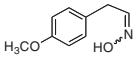
4. Experimental

4.1. Chemicals and instrumentation

All chemicals were purchased from Sigma–Aldrich Canada Ltd., Oakville, ON; solvents were HPLC grade and used as such; chromatography media and buffers used in protein purification were purchased from GE Healthcare (Quebec, Canada). Organic solvents were removed under reduced pressure in a rotary evaporator. Flash column chromatography (FCC) was carried out using silica gel grade 60, mesh size 230–400 Å. Preparative thin layer chromatography (prep TLC) was carried out on silica gel plates, Kieselgel 60 F_{254} and compounds were visualized under UV light and/or by staining. Yields refer to chromatographically and spectroscopically (^1H and ^{13}C NMR) homogeneous material. Air sensitive reagents were transferred by syringe.

Nuclear magnetic resonance (NMR) spectra were recorded on Bruker 500 MHz Avance spectrometers, for ^1H, 500.3 MHz and for ^{13}C, 125.8 MHz; chemical shifts (δ) are reported in parts per million (ppm) relative to TMS; spectra were calibrated using the solvent peaks; spin coupling constants (J) are reported to the nearest

Table 3
Relative activity of purified IADSs from *Sclerotinia sclerotiorum* using different aldoximes as potential substrates.

Aldoxime name (#)	Chemical structure	t_R (min)[a]	Relative activity[b] (%)
E/*Z*-Indolyl-3-acetaldoximes (**1**)[c]		3.3/3.6	100
E/*Z*-3-(3-Indolyl)propanal oximes (**13**)[c]		4.8	51 ± 9
E/*Z*-4-(3-Indolyl)butanal oximes (**14**)[c]		6.3	12 ± 3
E/*Z*-*E*/*Z*-Naphthyl-1-acetaldehyde oximes (**19**)		7.8/8.7	ND
E/*Z*-Naphthyl-2-acetaldehyde oximes (**20**)		8.1/9.0	ND
E/*Z*-Butanal oximes (**22**)[d]		7.1/7.5	ND
E/*Z*-3-Methylbutanal oximes (**24**)[d]		8.1/8.4	ND
E/*Z*-Cyclopentanecarboxaldehyde oximes (**26**)[d]		11.5/12.0	ND
E/*Z*-Indolyl-3-carboxaldehyde oximes (**27**)		2.9	ND
E/*Z*-Naphthyl-1-carboxaldehyde oximes (**28**)		8.3	ND
E/*Z*-Naphthyl-2-carboxaldehyde oximes (**29**)		8.5	ND
E/*Z*-4-Hydroxyphenylacetaldehyde oximes (**30**)[c]		1.8	30 ± 5
E/*Z*-4-Methoxyphenylacetaldoximes (**31**)[c]		3.5/3.9	19 ± 4
E/*Z*-Phenylacetaldoximes (**32**)[c]		7.8	ND

[a] Retention times of mixture of *E*/*Z* aldoximes detected by HPLC or GLC (under the conditions described in Section 4).
[b] Activities are expressed as percentage of activity relative to the activity obtained with indolyl-3-acetaldoximes (1.5 mM) (100% of activity for IADSs is equivalent to 14 nmol/min/mg); results are expressed as means and standard deviations of four independent experiments; ND = not detected.
[c] The nitriles (reaction products) were detected and quantified by HPLC-DAD using calibration curves built with authentic samples, as described in Section 4.
[d] The aldoximes and their potential products were detected by GLC-MS, as described in Section 4.

Table 4
Effect of various additives at 1.0 mM on the activity of IADSs obtained from mycelia of *Sclerotinia sclerotiorum*.

Additives	Relative activity (%)[a]
None – aerobic	100 ± 5
None – anaerobic	180 ± 30
$Na_2S_2O_4$ – aerobic	148 ± 22
$Na_2S_2O_4$ – anaerobic	2340 ± 470
DTT – aerobic	100 ± 5
FAD- aerobic	91 ± 8
NaN_3 – aerobic	86 ± 13
$FeCl_2$ – aerobic	233 ± 13
$FeCl_3$ – aerobic	88 ± 5
PLP – aerobic	113 ± 12

[a] Activities are expressed as percentage of the activity of IADSs obtained in the absence of $Na_2S_2O_4$; results are the means ± SD of four independent experiments. The enzyme activities were measured using protein extracts obtained from HiTrap DEAE FF chromatography, as reported in Section 4. The relative specific activity of IADSs for 100% is equivalent to 18 nmol/min/mg.

0.5 Hz. High resolution (HR) electron impact (EI) mass spectra (MS), were obtained on a VG 70 SE mass spectrometer, employing a solids probe.

HPLC analysis was carried out with Agilent high performance liquid chromatographs equipped with quaternary pump, automatic injector, and diode array detector (DAD, wavelength range 190–600 nm), degasser, and a ZORBAX SB-C18 column (3.5 μm particle size, 3.0 i.d. × 100 mm), having an in-line filter. Mobile phase: H_2O–CH_3CN 65:35 to 53:47 linear gradient for 10 min, at a flow rate 0.4 ml/min.

GC–MS analysis was carried out using a Fisons GC 8060 coupled to a VG-70SE magnetic sector mass spectrometer operated at a mass resolution of 1000, using electron-impact ionization performed at an electron energy of 70 eV. Chromatographic separation was achieved in a GS-HP-20M (Carbowax 20M) capillary column (20 m × 0.32 mm). The column temperature program was started at 40 °C for 5 min, was increased to 200 °C 10 °C/min and then kept at 200 °C for 5 min; the injection volume was 1.0 μl, using He as carrier, head pressure of 4 psi, and flow rate 1 ml/min. The samples were introduced using the direct injection mode, utilizing on column injector. The injection port temperature was 25 °C and a scan range at 25–250 amu was used at a scan speed of 0.5 s.

*4.2. Synthesis of acetaldoximes **1**, **13**, **14**, **19**, **20**, **22**, **24**, **26** and nitriles **15** and **16***

Indolyl-3-carboxaldehyde oxime (**27**), 4-hydroxyphenylacetal doxime (**30**), 4-methoxyphenylacetaldoxime (**31**) and phenylacetaldoxime

(**32**) were synthesized as previously described (Pedras and Montaut, 2003).

*4.2.1. Indolyl-3-acetaldoxime (**1**)*

Diisobutylaluminum hydride (1.5 M solution in toluene, 0.85 ml, 1.28 mmol) was added dropwise to a stirred solution of indolyl-3-acetonitrile (**3**) (100 mg, 0.64 mmol) in dry toluene (14 ml) at −78 °C under Ar atmosphere (Pedras and Okinyo, 2006). The reaction mixture was stirred for 20 min at the same temperature, poured into ice cold 5% HCl (20 ml) and extracted with EtOAc. The organic layer was washed with $NaHCO_3$ saturated solution, and NaCl saturated solution, dried (Na_2SO_4), and concentrated under reduced pressure to give the crude indolyl-3-acetaldehyde. A solution of $NH_2OH{\cdot}HCl$ (231 mg, 3.33 mmol) and NaOAc (273 mg, 3.33 mmol) in H_2O (2 ml) was added to a solution of crude indolyl-3-acetaldehyde (152 mg, EtOH 95%, 8.5 ml) at room temperature. After 3 h the reaction mixture was concentrated to dryness, the resulting residue was diluted with H_2O (10 ml), extracted (EtOAc), and the extract fractionated FCC (silica gel, CH_2Cl_2–CH_3OH, 99:1, v/v) to afford indolyl-3-acetaldoxime (38 mg, 0.22 mmol, 35% overall yield) as a white solid.

*Indolyl-3-acetaldoxime (**1**).* HPLC t_R = 3.3 and 3.6 min, *E*/*Z* isomers. ^{1}H NMR (CD_3OD) *E*/*Z* ratio (1:0.3): δ 7.53 (*d*, *J* = 8 Hz, 0.3H), 7.52 (*d*, *J* = 8 Hz, 1H), 7.48 (overlapping *t*, *J* = 6.5 Hz, 0.3H, –*CH*═NOH), 7.34 (*d*, *J* = 8 Hz, 1.3H), 7.1 (overlapping *t*, *J* = 7 Hz, 1.3H), 7.09 (*s*, 1H, –NH–*CH*–), 7.05 (*s*, 0.3H, –NH–*CH*–), 7.00 (*t*, *J* = 7 Hz, 1.3H), 6.79 (*t*, *J* = 5.5 Hz, 1H, –*CH*═NOH), 3.8 (*d*, *J* = 5 Hz, 2H), 3.59 (*d*, *J* = 6 Hz, 0.6H). ^{13}C NMR (CD_3OD): δ 152.21, 151.58, 138.35, 138.31, 128.73, 123.80, 122.65, 119.89, 119.46, 119.33, 112.40, 111.18, 110.94, 26.95, 22.43. HR-EIMS *m*/*z* measured 174.0793 (174.0793 calc. for $C_{10}H_{10}N_2O$). EIMS *m*/*z* (relative abundance) 174 $[M]^+$ (57), 157 (20), 130 (100), 117 (14), 103 (12). FTIR ν_{max}: 3390, 3241, 1456, 1417, 1090, 927, 823, 741 cm^{-1}.

*4.2.2. 3-(3-Indolyl)propanal oxime (**13**)*

To a stirred solution of 3-(3-indolyl)propanoic acid (**9**) (150 mg, 0.79 mmol) in 95% EtOH (5 ml), H_2SO_4 (10 mol%) was added dropwise at 0 °C, the reaction mixture was allowed to warm to room temperature and heated until reflux began, this being maintained for 2 h. EtOH was removed under reduced pressure, the residue was dissolved in EtOAc (15 ml), the mixture was washed with satd. $NaHCO_3$ solution and concentrated. The product was purified using FCC (EtOAc–hexane; 15:85, v/v) to afford ethyl 3-(3-indolyl)propanoate (**9a**) (165 mg, 98%) as a white solid. To a stirred solution of ethyl 3-(3-indolyl)propanoate (**9a**) (100 mg, 0.469 mmol) in anhydrous toluene (6 ml) under argon atmosphere at −78 °C, DIBAL-H (406 μl, 0.610 mmol) was added slowly and the reaction was stirred for about 30 min at the same temperature. The reaction mixture was quenched with cold 2 M HCl and extracted with EtOAc. The combined organic extract was washed with brine, dried (Na_2SO_4), and concentrated. The crude residue 3-(3-indolyl)propanal (**11**) was directly used for the next step without further purification. A solution of $HONH_2{\cdot}HCl$ (48.9 mg, 0.70 mmol) and NaOAc (57.8 mg, 0.70 mmol) in H_2O (500 μl) was added to a cooled solution (0 °C) of crude 3-(3-indolyl)propanal (**11**) in 95% EtOH (4 ml). The reaction mixture was allowed to stir at 0 °C for 5 min, and then at room temperature for 3 h. The reaction mixture was concentrated and the residue taken in H_2O (5 ml), was extracted with EtOAc. The combined organic extract was dried over Na_2SO_4 and was concentrated under reduced pressure. Separation by FCC (EtOAc–hexane, 20:80, v/v) yielded 3-(3-indolyl)propanal oxime (**13**) (59 mg, 67% yield, over two steps) as a white solid, m.p.: 121–124 °C.

*3-(3-Indolyl)propanal oxime (**13**).* HPLC t_R = 4.8 min. ^{1}H NMR (CD_3CN) *E*/*Z* ratio (1:0.1): δ 9.05 (*br s*, 1H), 8.63 (*s*, 0.97H, major), 8.28 (*s*, 0.09H), 7.57 (*d*, *J* = 8 Hz, 1H), 7.56 (overlapping *t*, 0.09H, –C*H*═NOH), 7.38 (*d*, *J* = 8 Hz, 1H), 7.13 (*t*, *J* = 7.5 Hz, 1H), 7.07 (*s*, 1H), 7.05 (*t*, *J* = 7.5 Hz, 1H), 6.71 (*t*, *J* = 5.5 Hz, 0.97H, –C*H*═NOH), 2.91 (*t*, *J* = 7.5, 2H), 2.68 (*m*, 1.8H), 2.52 (*m*, 0.2H). ^{13}C NMR ($CDCl_3$): δ 152.78, 152.20, 137.65, 128.35, 123.08, 122.04, 122.56, 119.81, 119.47, 115.55, 112.37, 31.0, 26.17, 23.1, 22.39. HR-EIMS: calc. for $C_{11}H_{12}ON_2$ $[M^+]$ *m*/*z* 188.0950, found 188.0951. FTIR ν_{max}: 3415, 3197, 3059, 2904, 1659, 1619, 1457, 1423, 1309, 1094, 933, 819, 747 cm^{-1}.

*4.2.3. 3-(3-Indolyl)butanal oxime (**14**)*

Ethyl 3-(3-indolyl)butanoate (**10a**) was prepared from 3-(3-indolyl)butanoic acid (**10**) (100 mg, 0.049 mmol), employing the procedure described for ethyl 3-(3-indolyl)propanoate (**9a**). Ethyl 3-(3-indolyl)butanoate (**10a**) (107 mg, 95%) was obtained as a white solid; m.p.: 38–40 °C (39–40 °C, Bullock and Hand, 1956). (3-Indolyl)butanal oxime (**14**) was prepared from ethyl 3-(3-indolyl)butanote (**10a**) (50 mg, 2.99 mmol) employing the same procedure as described for compound **13**. 3-(3-Indolyl)butanal oxime (**14**) (17.5 mg, 40% yield) was obtained as a solid; m.p: 78–80 °C.

*3-(3-Indolyl)butanal oxime (**14**).* ^{1}H NMR ($CDCl_3$): *E*/*Z* (5:4) δ 8.81 (*br s*, 0.45H), 8.41 (*br s*, 0.55H), 7.94 (*s*, 1H), 7.63 (*dd*, *J* = 7.5, 2 Hz, 1H), 7.49 (*t*, *J* = 6 Hz, 0.55H, –*CH*═NHOH), 7.36 (*d*, *J* = 7.5 Hz, 1H), 7.22 (*t*, *J* = 7.5 Hz, 1H), 7.15 (*dd*, *J* = 7.5, 2 Hz, 1H), 6.98 (*d*, *J* = 7.5, 1H), 6.79 (*t*, *J* = 5.5 Hz, 0.45H, –*CH*═NHOH), 2.84 (*m*, 2H), 2.50 (*m*, 0.9H), 2.30 (*m*, 1.1H), 1.94 (apparent quintet, 2H). ^{13}C NMR ($CDCl_3$): δ 153.01, 152.43, 136.53, 127.57, 122.13, 121.63, 119.38, 119.04, 119.02, 115.88, 111.31, 111.30, 29.40, 27.03, 26.65, 25.04, 24.66. HR-EIMS: calc. for $C_{12}H_{14}N_2O$ $[M^+]$ *m*/*z* 202.1106, found 202.1104. FTIR ν_{max}: 3413, 3251, 3065, 2924, 1652, 1610, 1455, 1420, 1336, 906, 741 cm^{-1}.

*4.2.4. 3-(3-Indolyl)propanenitrile (**15**)*

Pyridine (13 μl, 0.16 mmol) was added to a solution of 3-(3-indolyl)propanal oxime (**13**) (15 mg, 0.079 mmol) in CH_2Cl_2 (4 ml) under Ar. After 12 h at 45 °C, the CH_2Cl_2 was removed under reduced pressure to yield crude product, which was subjected to FCC (EtOAc–hexane, 10:90, v/v) to afford 3-(3-indolyl)propanenitrile (**15**) (9.3 mg, 69% yield) as a solid, m.p.: 67–69 °C (66–68 °C, Black et al., 2006).

^{1}H NMR ($CDCl_3$): δ 8.11 (*br s*, 1H), 7.57 (*d*, *J* = 8 Hz, 1H), 7.40 (*d*, *J* = 8 Hz, 1H), 7.25 (*t*, *J* = 8 Hz, 1H), 7.17 (*t*, *J* = 8 Hz, 1H), 7.14 (*br d*, *J* = 1.5 Hz, 1H), 3.15 (*t*, *J* = 7 Hz, 2H), 2.72 (*t*, *J* = 7 Hz, 2H). ^{13}C NMR ($CDCl_3$): δ 136.48, 126.76, 122.65, 122.31, 119.94, 118.37, 112.90, 21.88, 18.9. HR-EIMS: calc. for $C_{11}H_{10}N_2$ $[M^+]$ *m*/*z* 170.0843, found 170.0840. FTIR ν_{max}: 3405, 3049, 2923, 2245, 1614, 1554, 1457, 1420, 1337, 1096, 743 cm^{-1}.

*4.2.5. 3-(3-Indolyl)butanenitrile (**16**)*

3-(3-Indolyl)butanenitrile (**16**) was prepared from 3-(3-indolyl)butanal oxime (**14**) (15 mg, 0.074 mmol) employing the procedure described for 3-(3-indolyl)propanenitrile (**15**). 3-(3-Indolyl)butanenitrile (**16**) (9.5 mg, 71% yield) was obtained as a clear liquid.

^{1}H NMR ($CDCl_3$): δ 8.02 (*br s*, 1H), 7.59 (*d*, *J* = 8 Hz, 1H), 7.39 (*d*, *J* = 8 Hz, 1H), 7.22 (*t*, *J* = 7.5 Hz, 1H), 7.14 (*t*, *J* = 7.5 Hz, 1H), 7.05 (*br d*, 1H), 2.96 (*t*, *J* = 7 Hz, 2H), 2.35 (*t*, *J* = 7 Hz, 2H), 2.08 (quintet, *J* = 7 Hz, 2H). ^{13}C NMR ($CDCl_3$): δ 136.63, 127.27, 122.64, 122.45, 122.15, 120.01, 119.70, 118.85, 114.05, 111.48, 25.92, 24.10, 16.68. HR-EIMS: calc. for $C_{12}H_{12}N_2O$ $[M^+]$ *m*/*z* 184.1000, found 184.0999. FTIR ν_{max}: 3407, 3054, 2931, 2850, 2244, 1608, 1483, 1456, 1229, 743 cm^{-1}.

*4.2.6. 2-(1-Naphthyl)acetaldoxime (**19**)*

A solution of DIBAL-H (300 μl, 0.45 mmol) in 1.5 M toluene was added dropwise to a solution of 1-naphthyl acetonitrile (**17**) (50 mg, 0.299 mmol) in anhydrous CH_2Cl_2 (4 ml) cooled to

−78 °C under Ar. The reaction mixture, was stirred at −78 °C for 20 min, was diluted with ice-cold HCl (5 ml, 2 M), immediately extracted in EtOAc, dried over Na_2SO_4, and concentrated under reduced pressure to yield crude 2-(1-naphthyl)acetaldehyde, which was used for the next step without purification. A solution of $HONH_2{\cdot}HCl$ (31 mg, 0.45 mmol) and NaOAc (37 mg, 0.45 mmol) in H_2O (500 μl) was added to a cooled solution (0 °C) of crude 2-(1-naphthyl)acetaldehyde in 95% EtOH (4.5 ml). The reaction mixture was stirred at 0 °C for 5 min, and then at room temperature for 3 h. The reaction mixture was concentrated and the residue was taken in H_2O (5 ml), and was extracted with EtOAc. The combined organic extract was dried over Na_2SO_4 and concentrated under reduced pressure. Separation by FCC (EtOAc–hexane, 20:80, v/v) yielded 2-(1-naphthyl)acetaldehyde oxime (**19**) (25.4 mg, 46% yield) as a solid, m.p.: 127–129 °C (123–124 °C, Jensen and Dynesen, 1950).

^{1}H NMR ($CDCl_3$): *E*/*Z* isomers (1:0.6); δ 9.53 (*br s*, 1H), 8.77 (*br s*, 0.6H), 8.07 (*d*, *J* = 8 Hz, 0.6H), 7.99 (*d*, *J* = 8 Hz, 1H), 7.91 (*m*, 1.6H), 7.82 (*d*, *J* = 8 Hz, 1.6H), 7.70 (*t*, *J* = 6 Hz, 0.6H, –*CH*═NHOH), 7.61–7.53 (*m*, 3.2H), 7.47–7.40 (*m*, 3.2H), 6.95 (*t*, *J* = 5 Hz, 1H, –*CH*═NHOH), 4.20 (*d*, *J* = 5 Hz, 2H), 4.03 (*d*, *J* = 6 Hz, 1.2H). ^{13}C NMR ($CDCl_3$): δ 151.1, 150.76, 134.06, 132.99, 132.42, 132.15, 132.08, 128.99, 128.06, 127.92, 127.29, 127.06, 126.60, 126.54, 126.08, 126.03, 125.76, 123.88, 123.77, 33.58, 29.80. FTIR ν_{max}: 3189, 3067, 2867, 1657, 1596, 1509, 933, 796, 775 cm^{-1}. HR-EIMS: calc. for $C_{12}H_{11}O_2N$ [M$^+$] *m*/*z* 185.0840, found 185.0837.

4.2.7. 2-(2-Naphthyl)acetaldoxime (**20**)

2-(2-Naphthyl)acetaldoxime (**20**) was prepared from 2-(2-naphthyl)acetonitrile (**18**) (50 mg, 0.299 mmol) employing the procedure described for 2-(1-naphthyl)acetaldoxime (**19**). 2-(2-Naphthyl)acetaldoxime (**20**) (26.8 mg, 51% yield) was obtained as a solid; m.p.: 126–129 °C (120 °C, Mayer et al., 1922).

^{1}H NMR ($CDCl_3$): *E*/*Z* isomers (1:0.7) δ 9.53 (*br s*, 1H), 8.77 (*br s*, 0.7H), 7.84 (*m*, 5.1H), 7.70 (*d*, *J* = 8 Hz, 1.7H), 7.66 (*t*, *J* = 6.5 Hz, 0.7H, –*CH*═NHOH), 7.49 (*m*, 3.4H), 7.38 (*m*, 1.7H), 7.03 (*t*, *J* = 5 Hz, 1H, –*CH*═NHOH), 3.94 (*d*, *J* = 5 Hz, 2H), 3.74 (*d*, *J* = 6.5 Hz, 1.4H). ^{13}C NMR ($CDCl_3$): δ 150.95, 150.82, 134.22, 133.73, 133.75, 132.56, 132.45, 128.68, 128.64, 127.87, 127.75, 127.70, 127.49, 127.41, 127.31, 127.24, 126.43, 125.95, 125.87, 36.26, 32.04. HR-EIMS: calc. for $C_{12}H_{11}ON$ [M$^+$] *m*/*z* 185.0840, found 185.0841. FTIR ν_{max}: 3221, 3053, 2956, 1659, 1598, 1507, 1458, 943, 821, 741 cm^{-1}.

4.2.8. Butanal oxime (**22**)

A solution of $HONH_2{\cdot}HCl$ (289 mg, 4.16 mmol) and NaOAc (341 mg, 4.16 mmol) in water (0.5 ml) was added to a solution of butanal (**21**) (150 mg, 2.08 mmol) in 95% EtOH (4 ml). The reaction mixture was stirred at room temperature for 3 h and concentrated (water bath temperature bellow 10 °C). The residue was taken in H_2O (5 ml) and extracted with CH_2Cl_2. The combined organic extract was dried over Na_2SO_4 and concentrated under reduced pressure (water bath temperature below 10 °C). Separation by FCC (EtOAc–hexane, 20:80, v/v) yielded butanal oxime (**22**) (172 mg, 95% yield) as a clear liquid.

^{1}H NMR ($CDCl_3$): *E*/*Z* isomers (1:1) 9.92 (*br s*, 1H), 9.56 (*br s*, 1H), 7.40 (*t*, *J* = 6 Hz, 1H, –*CH*═NOH), 6.71 (*t*, *J* = 5.5 Hz, 1H, –*CH*═NOH), 2.34 (*m*, 2H), 2.16 (*m*, 2H), 1.49 (*m*, 4H), 0.94 (*m*, 6H). ^{13}C NMR ($CDCl_3$): δ 152.75, 152.24, 31.47, 27.06, 20.07, 19.51, 13.96, 13.66. HR-EIMS: calc. for C_4H_9ON [M$^+$] *m*/*z* 87.0684, found 87.0685. FTIR ν_{max}: 3303, 2959, 2877, 1706, 933, 886 cm^{-1}.

4.2.9. 3-Methylbutanal oxime (**24**)

3-Methylbutanal oxime (**24**) was prepared from 3-methylbutanal (**23**) (100 mg, 1.161 mmol) employing the procedure described for butanal oxime (**22**). 3-Methylbutanal oxime (**24**) (103 mg, 88% yield) was obtained as pale yellow liquid (Ramon et al., 2010).

^{1}H NMR ($CDCl_3$): *E*/*Z* isomers (1:0.8) δ 7.44 (*t*, *J* = 6.5 Hz, 1H, –*CH*═NOH), 6.76 (*t*, *J* = 5.5 Hz, 0.8H, *CH*═NOH), 2.29 (*m*, 1.6H), 2.09 (*t*, *J* = 6.5 Hz, 2H), 1.84 (*m*, 1.6H), 0.97 (*m*, 10.8H). ^{13}C NMR ($CDCl_3$): δ 152.06, 151.7, 38.35, 33.96, 26.86, 26.37, 22.66, 22.47. FTIR ν_{max}: 3252, 3098, 2957, 1708, 1465, 923 cm^{-1}.

4.2.10. 1-Cyclopentanecarboxaldehyde oxime (**26**)

1-Cyclopentanecarboxaldehyde oxime (**26**) was prepared from 1-cyclopentanecarboxaldehyde (**25**) (50 mg, 0.510 mmol) employing the procedure described for butanal (**22**). 1-Cyclopentanecarboxaldehyde oxime (**26**) (50 mg, 88% yield) was obtained as a light yellow liquid.

^{1}H NMR ($CDCl_3$): *E*/*Z* isomers (1:0.4) δ 9.25 (*br s*, 1.4H), 7.36 (*d*, *J* = 7 Hz, 1H, –*CH*═NOH), 6.65 (*d*, *J* = 7 Hz, 0.4H, –*CH*═NOH), 3.26 (*m*, 0.4H), 2.62 (*m*, 1H), 1.92 (*m*, 0.8H), 1.84 (*m*, 2H), 1.69–1.57 (*m*, 5.6H), 1.49–1.36 (*m*, 2.8H). ^{13}C NMR ($CDCl_3$): 157.20, 155.93, 40.09, 35.28, 30.87, 30.17, 26.00, 25.61, 25.37. HR-EIMS: calc. for C_4H_9ON [M$^+$] *m*/*z* 113.0841, found 113.0840. FTIR ν_{max}: 3271, 2958, 1701, 1451, 935 cm^{-1}.

4.3. Cultures of Sclerotinia sclerotiorum for enzyme isolation

Sclerotia of *S. sclerotiorum* clone #33 initially obtained from C. Lefol from Agriculture and Agri-Food Canada (AAFC) collection (Saskatoon, SK) were used for inoculating PDA plates (one sclerotium per plate). The inoculated plates were incubated in the dark at 20 ± 2 °C for 15 days and sclerotia were collected up to 21 days. The optimum conditions for maximum enzymatic activity were studied by changing temperature (20, 23 and 28 °C), time of incubation (3, 4, 5 and 6 days), and light. The optimized cultures conditions used five sclerotia in 100 ml of PDB, incubated at 23 ± 2 °C under constant light for 5 days. The mycelia were filtered off and used for the preparation of crude protein extracts.

4.4. Protein extraction

The extraction buffer consisted of 10 mM Tris–HCl buffer pH 7.5 containing 1 mM DTT, and 3% glycerol (v/v). Five-day-old cultures prepared as described in Section 4.3 were filtered through cheesecloth, the mycelia were washed with purified ice-cold H_2O and the H_2O was wrung off. Mycelia were homogenized in a chilled mortar with equal weight of Ottawa sand and extracted with two volumes (w/v) of extraction buffer. The mycelial homogenate was centrifuged at 15,000*g* for 10 min to remove sand and uncrushed mycelia and then the supernatant was further subjected to centrifugation at 35,000*g* at 4 °C for 10 min. The resulting supernatant was taken as the crude protein extract for chromatographic purification of enzyme.

Protein estimation was performed by Bradford (1976) method using bovine serum albumin as the standard and Bradford reagent from Sigma Aldrich. One unit of activity is defined as the amount of enzyme that produces 1 μmol of indolyl-3-acetonitrile per min.

4.5. IADSs assay

The standard assay to determine IADSs activity contained 25 mM Tris–HCl buffer pH 7.5, 1.5 mM aldoxime substrate and 100 μl enzyme aliquot in a total volume of 500 μl. The reaction mixture was incubated for 60 min in darkness at 23 °C under constant shaking. For the aromatic substrates the reaction was stopped by adding EtOAc (2 ml), then the reaction products were extracted two times with EtOAc (2 ml). A control reaction was stopped similarly at 0 min. The organic layers were combined and concentrated to dryness. The extracts were dissolved in CH_3CN

(200 μl) and analyzed by HPLC. The amount of product formed was determined from calibration curves built using a commercial sample of indolyl-3-acetonitrile or other nitriles, as applicable. In experiments to determine the aliphatic nitriles, the reaction was stopped with an equal volume of dichloromethane, extracted and the extract analyzed by GC–MS.

4.6. Cellular localization of IADSs

For cellular localization, the enzyme was fractionated by the method of Bridge et al. (2003) with modifications, as follows. Mycelia (10 g) were ground with equal weight of sand and 20 ml of buffer A (1 mM DTT and 5% glycerol, v/v). The homogenate was centrifuged for 20 min at 10,000*g* to yield supernatant and pellet (P1). The supernatant was further centrifuged at 100,000*g* for 60 min to yield supernatant and pellet (P2). The resulting supernatant containing the soluble protein fraction was assayed for IADSs activity. The pellet P2 was solubilized in ice-cold buffer B (10 mM Tris–HCl, pH 7.5 containing 1 mM DTT, 2% glycerol v/v, 0.015% Triton-X-100 v/v, and 50 mM NaCl) ground in a mortar and centrifuged for 15 min at 30,000*g*. The pellet, P1, was solubilized in buffer C (10 mM Tris–HCl pH 7.5, 1 mM DTT, 2% glycerol v/v, 0.015% Triton-X-100 v/v, and 150 mM NaCl) ground in a mortar, centrifuged at 30,000*g* and the supernatant was collected as the cell wall proteins. IADSs assay was carried out using the supernatant.

4.7. Purification of IADSs

An Äkta FPLC (GE Healthcare) was used for all protein separations. All separation steps were carried out at 5 °C.

4.7.1. DEAE Sephacel chromatography

The soluble protein extract (10 ml, ca. 20 mg) obtained from mycelia (ca. 2 g from 100 ml culture) was equilibrated by dialyzing against 10 mM Tris–HCl buffer pH 7.5, 0.015% Triton X-100 v/v, 1 mM DTT, 3% glycerol v/v, and loaded onto a DEAE Sephacel column (1.6 × 14 cm) pre-equilibrated with the same buffer. The enzyme was eluted with a NaCl gradient of 0–0.2 M in the same buffer at a flow rate of 0.5 ml/min and 5 ml fractions were collected. Fractions showing IADSs activity (43–45) were pooled and used in the second step of purification.

4.7.2. Hydroxyapatite chromatography

The pooled active fractions from the first step were dialyzed against 20 mM phosphate buffer pH 7.0, 1 mM DTT, 0.015% Triton X-100, 2% glycerol and loaded onto a hydroxyapatite column of 1.6 × 8.0 cm (Bio-Rad). The enzyme was eluted with a gradient of 0.025–0.30 M phosphate buffer pH 7.0 at a flow rate of 0.5 ml/min, fractions of 1.8 ml were collected and assayed for IADSs activity. Fractions 38–40 were pooled and used in the next step of purification.

4.7.3. Gel filtration on Superdex-75

The pooled fractions showing IAD activity were concentrated to 2 ml using an Amicon Ultra Centrifugal (10 K) and subjected to gel filtration on Superdex 75 (1.6 × 60 cm column dimension). The enzyme was eluted with 10 mM Tris–HCl buffer pH 7.5 containing 1 mM DTT, 0.015% Triton X-100, 2% glycerol (v/v), and 25 mM NaCl. The column was calibrated with protein markers consisting of blue dextran (M_r, 200 kDa), albumin (M_r, 64 kDa), trypsinogen (M_r, 43 kDa), ribonuclease (M_r, 25 kDa), and ovalbumin (M_r, 13.7 kDa). Fractions of 1.0 ml were collected at a flow rate of 0.6 ml/min and assayed for IADSs activity. Fractions 59–61 were pooled and concentrated to 100 μl (Nanosep 10 K), then used for biochemical analysis.

4.7.4. Partial chromatographic purification of the IADSs using HiTrap DEAE FF chromatography

The soluble protein extract from mycelia (10 ml, ca. 7 mg) was equilibrated by dialyzing against 20 mM Tris–HCl buffer pH 7.5, 0.015% Triton X-100, 1 mM sodium dithionate, 3% glycerol and then loaded onto a HiTrap DEAE FF (GE Healthcare) anion-exchange column (5 ml). Proteins were eluted with 20 ml of the same buffer, first alone and then with a 60 ml of 0.0–0.45 M NaCl gradient. Fractions of 1.8 ml were collected at a flow rate of 0.5 ml min^{-1}, and 100 μl was assayed for IADSs activity. Fractions (19–21) showing activity were pooled and used for testing the substrate specificity.

4.8. SDS–PAGE analysis

Protein-denaturing sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was carried out using 10% polyacrylamide gels. Standard molecular mass marker proteins with a molecular mass range from 15 to 225 kDa obtained from Bio-Rad (V849A), Hercules, CA, USA, were used to determine the approximate molecular mass of proteins. The protein bands were stained with Coomassie brilliant blue R-250.

4.9. Identification of tryptic peptides of IADSs by LC–ESI-MS/MS

Analyses were carried out by the Plant Biotechnology Institute, National Research Council of Canada, Saskatoon, SK. Protein gel slices were manually excised from Coomassie stained gels and placed in a 96-well microtitre plate. The protein was then automatically de-stained, reduced with DTT, alkylated with iodoacetamide, digested with porcine trypsin (Rosenfeld et al., 1992), and Q-TOF LC–ESI-MS/MS analysis carried out as described previously (Pedras et al., 2008). LC–ESI-MS/MS data were processed using mascot distiller (version 2.1.1.0; Matrix Science, London, UK; available at http://www.matrixscience.co.uk). The main search parameters were methionine oxidation as differential modification. Finally, proteins were identified using mascot distiller. Protein homology analysis was carried out using identified protein by NCBI BLAST (http://ca.expasy.org/tools/blast/). Multiple sequence alignments were generated at http://ca.expasy.org/tools/sim-prot.html (Duret et al., 1996) and at http://www.genebee.msu.su/services/malign_reduced.html (Nikolaev et al., 1997). The analysis to predict cellular localization of the enzyme was performed using PSORT WWW Server (http://psort.ims.u-tokyo.ac.jp/). Molecular mass calculations were carried out with pI/Mw tool software (http://ca.expasy.org/tools/pi_tool.html).

4.10. Characterization and substrate specificity of IADSs

The kinetic parameters of IADSs were determined using as substrate *E*/*Z*-indolyl-3-acetaldoxime. The concentration of the substrate in the assay mixture was varied from 0 to 1.2 mM.

The substrate specificity of the purified IADSs was tested using the *E*/*Z*-aldoximes shown in Table 4. IADSs activity was assayed in presence of 1.5 mM of each oxime in 25 mM Tris–HCl buffer pH 7.5. Product formation was determined by HPLC using as standards the nitriles corresponding to dehydration of each oxime. Calibration curves were built using synthetic compounds.

4.11. Effect of pH and temperature on the activity of IADSs

The effect of pH on the enzyme activity was studied by assaying the purified enzyme in buffers with pHs ranging from 5.0 to 9.5 in 50 mM diethanolamine, 50 mM *N*-ethylmorpholine, and 100 mM morpholine-ethanesulfonic acid buffer.

To determine the optimum temperature for IAD activity, assay mixtures were incubated at different temperatures starting from 6 to 60 °C. The reactions were stopped after incubating for 60 min and indolyl-3-acetonitrile was estimated as described above.

4.12. Effect of additives and anaerobic conditions on IADSs activity

The activity of IADSs in 25 mM Tris–HCl buffer pH 7.5 was examined in presence of several compounds and cofactors at 1 mM: DTT, $Na_2S_2O_4$, $FeCl_2$, $FeCl_3$, NaN_3, PLP and FAD. The enzyme activity was measured and expressed as the percentage of activity calculated as the ratio of the specific activity of indole-3-acetaldoxime dehydratase in the presence of additives and a control reaction (aerobic conditions, assay without additives) (Table 4).

For anaerobic conditions, the enzymatic assays were performed under argon; the reaction mixture (2.0 ml), stored in a capped and sealed vial, was first purged with a stream of argon for 5 min and then kept under a steady stream of argon for the duration of the experiment; 1.0 ml of the reaction mixture was collected after 60 min using a needle and syringe and the reaction was stopped by adding to 2 ml of EtOAc. The products were extracted and quantified as described above for the IADSs standard assay.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.phytochem.2010.10.002.

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