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Induction and Characterization of an Indole-3-acetyl-L-alanine Hydrolase From *Arthrobacter Ilicis*

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Abstract

Indole-3-acetic acid (IAA)-amino acid amide conjugates have been found to be present in many plants, and they are proposed to function in the regulation of plant IAA metabolism in a variety of ways. IAA-amino acid conjugate hydrolase activities, and the genes that encode them, are therefore potentially important tools for modification of IAA metabolism, both for agronomic reasons as well as for determination of the mechanisms of IAA regulation. We have developed a simple and economical method to induce IAA-amino acid conjugate hydrolases in bacteria with *N*-acetyl-L-amino acids. Using this method, we identified four bacterial strains that can be induced to produce IAA-Ala hydrolases: *Arthrobacter ureafaciens* C-10, *Arthrobacter ureafaciens* C-50, *Arthrobacter ilicis* D-50, and *Cellulomonas fimi* D-100. The enzyme kinetics and the biochemical characteristics of IAA-Ala hydrolase from one specific bacterium, *Arthrobacter ilicis* D-50, have been determined. The enzyme has a unique substrate specificity for IAA-amino acid conjugates compared to a bacterial IAA-Asp hydrolase previously characterized.

Key words: *Arthrobacter;* Auxin metabolism; Enzyme activity; Enzyme induction; Hydrolase; IAA; IAA conjugate; Plant growth regulator

Introduction

Indole-3-acetic acid (IAA), the most studied auxin in plants, is an essential plant growth regulator controlling processes from embryogenesis to senescence. The biochemical processes that regulate IAA levels in plants are extremely complex (Normanly 1997). Factors affecting IAA levels should include: *de novo* IAA biosynthesis; conversion between IAA and indole-3-butyric acid (IBA), indole-3-lactic acid, and indole-3-ethanol; hydrolysis of IAA conjugates; amide-linked and ester-linked conjugation; oxidative catabolism; and physiological consumption. These factors combined form a complex network to regulate IAA levels so that plants maintain regular physiological processes for normal growth and development.

In recent studies, the novel phenomena of IAA conjugation and IAA conjugate hydrolysis have been found to play important roles in many aspects of a plant's physiology. The function of IAA conjugation, both amide-linked and ester-linked, includes the homeostatic control of free IAA levels (Bandurski 1980), storage and subsequent reuse of IAA (Cohen and Bandurski 1982), protection of IAA from other oxidase attack (Cohen and Bandurski

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1978), transport of IAA (Nowacki and Bandurski 1980), IAA non-decarboxylative oxidation (Tuominen and others 1994), and adaptation to high-temperature environments (Oetiker and Aeschbacher 1997). Because of the range of processes involving conjugation and hydrolysis, the study of IAA conjugate metabolism and its impact on IAA homeostatic control continue to be important to understanding the biochemistry and physiology of IAA in plants.

The Kosuge group (Glass and Kosuge 1986; Hutzinger and Kosuge 1968a,b) first discovered and investigated the enzyme, *N*-indole-3-L-ϵ-lysine (IAA-Lys) synthetase and cloned the *iaaL* gene from *Pseudomonas savastanoi*. The level of secreted IAA was reduced by a third in a non-*iaaL Pseudomonas savastanoi* strain that had been transformed with *iaaL* (Glass and Kosuge 1988), demonstrating that IAA conjugation-related enzymes can affect the IAA levels in IAA-containing organisms. Kowalczyk and Bandurski (1991) characterized the maize enzyme for IAA ester conjugation, 1-*O*-indole-3-acetyl-β-D-glucose (IAA-Glc) synthetase, and the gene, *iaglu*, was cloned and sequenced (Szerszen and others 1994). Transformation of tomato plants with *iaglu* in the sense orientation resulted in a rootless callus phenotype, whereas anti-sense *iaglu* resulted in a rooty callus phenotype (Iyer and others 1998). These results indicate that transformation with *iaglu* can alter IAA homeostasis to affect organogenesis in plant tissue culture. In a recent report, Staswick and others (2002) found that the products of *JAR1*-catalyzed reactions in the *Arabidopsis thaliana* adenylate plant hormones jasmonic acid, salicylic acid, and IAA. The adenylation product of IAA is the intermediate in the conjugation reaction from IAA to IAA-Asp (Staswick and others 2005).

Kuleck and Cohen (1992) found a large protein complex of 200 kDa from carrot that can hydrolyze several IAA amide conjugates, but this enzyme proved to be highly liable and difficult to purify. Bartel and Fink (1995) described the conjugate resistant *A. thaliana* mutant ILR1 and cloned the gene responsible for the phenotype. Wild-type plants treated with IAA-Lys exhibited IAA overdose symptoms. ILR1 hydrolyzes IAA-Lys and the *ILR1* mutant was, therefore, insensitive to IAA-Lys. In *A. thaliana*, *ILR1* belongs to an amidohydrolase gene family in which several genes encode IAA amino acid hydrolase activity (Davies and others 1999).

We (Chou and others 1996) induced and purified an IAA-Asp hydrolase from the bacterium *Enterobacter agglomerans* and subsequently cloned its gene, *iaaspH* (Chou and others 1998). This enzyme has important potential as a molecular tool for plant studies because IAA-Asp has been found to play many critical physiological and biochemical roles in plants. IAA-Asp levels can be dramatically increased in plants when high doses of active auxins are applied (Andreae and Good 1955). It is also the last intermediate retaining the indole ring in the IAA non-decarboxylative oxidation pathway in most plants (Tuominen and others 1994; Normanly 1997). In henbane cell cultures, IAA-Asp is the main IAA conjugate accumulated in a high-temperature–resistant line, whereas IAA-Glc is the main IAA conjugate in a non-resistant variety (Oetiker and Aeschbacher 1997). Therefore, it might be reasonable to expect a dramatic phenotype to occur when *iaaspH* is introduced into a plant. However, when Tam and Normanly (2002) transformed *A. thaliana* with *iaaspH*, they found no significant difference in morphological phenotype. The transgenic plants showed a significant decrease in IAA-Asp levels, but free IAA was maintained at wild-type levels. The results indicate that another regulatory mechanism was likely triggered to maintain IAA homeostasis, but it should also be pointed out that, unlike many other dicot species, *Arabidopsis* does not have IAA-Asp as a major conjugate (Ljung and others 2002). Indeed, IAA-Asp makes up less than 2% of total IAA in *A. thaliana* (Tarn and others 2000). Therefore, IAA-Asp hydrolase alone might not be sufficient to disturb free IAA levels in that species. Biochemical and physiological approaches are now pointing toward IAA-Asp being more involved in the regulation of IAA levels rather than being simply a storage form of IAA.

In earlier reports, IAA-Ala was considered a storage form of IAA in tomato, tobacco, and pea, and it was proposed to function as a slow-release agent for IAA through regulated enzymatic hydrolysis (Hangarter and others 1980; Hangarter and Good 1981). Additional studies with *in vivo* evidence of IAA-Ala hydrolysis in a variety of plants had been reported, including bean (Bialek and others 1983), *A. thaliana* (Campanella and others 1996), and *Lemna gibba* (Slovin 1997). A novel amidohydrolase from wheat was also found to cleave IBA-Ala instead of IAA-Ala (Campanella and others 2004). The enzyme catalyzing IAA-Ala hydrolysis in plants was not identified until the recent discovery of the *A. thaliana ILR1* gene family (LeClere and others 2002). A gene homolog, *ILL2*, which has strong IAA-Ala hydrolase activity was described. However, similar to other plant IAA hydrolases, this enzyme is not highly specific for IAA-Ala. *ILL2* can also efficiently hydrolyze many other IAA conjugates such as IAA-Lys, IAA-Met, IAA-Pro, IAA-Phe, IAA-Ser, IAA-Thr, IAA-Tyr, and IAA-Val, with hydrolase activities

higher than 100 nmol IAA released per minute per milligram of crude extract from *E. coli* GST-*ILL2* overexpression. Therefore, it remained both interesting and important to search for a more substrate-specific enzyme to use as a molecular tool to study the role of IAA-Ala in plant auxin metabolism, and to allow a new approach to molecular engineering of phytohormone metabolism. We (Chou and others 1996) found that the bacterial amidohydrolase exhibited a more restricted substrate requirement, and this specificity indicated a potentially fundamental difference between the best studied plant enzymes and microbial activities (Chou and others 2004). Based on these results, bacteria are a promising source to continue to search for highly substrate-specific amidohydrolases. This article presents the first report of an IAA-Ala hydrolase activity from bacteria. Through a systematic search based on a bacterial enrichment approach, we were able to find several bacteria with IAA-Ala hydrolase activities. One specific bacterial strain was chosen for further study, and we now describe the biochemical characteristics of this IAA-Ala hydrolase.

Material and Methods

Bacterial Strain Isolation and Characterization

Bacterial strains were isolated using standard enrichment techniques (Krieg 1981) from a variety of 20-cm-deep soil samples including soils from soybean, pineapple, and maize farms, as well as from a sewage sludge inoculation. The *N*-acetyl-L-alanine (Calbiochem-Novabiochem Corp., San Diego, CA) was added to the basal salt medium (BSM) (Tomasek and Karns 1989), as the sole carbon and nitrogen source to induce the IAA-Ala hydrolase activity. Four bacterial isolates were found to have an enzymatic activity inducible by *N*-acetyl-L-alanine, and these were subjected to fatty acid methyl ester GC analysis according to standard protocols (Microbial ID, MIDI, Newark, DE) for bacterial identification. The bacterial strains identified were further confirmed by tests for Gram's staining (Gram's staining solution kit, Kanto Chemicals, Tokyo, Japan), catalase activity, and oxidase activity (RapID Systems, Remel, Lenexa, KS).

Chemicals and IAA-amino Acid Conjugate Preparation

Six commercially available IAA-amino acid conjugates (IAA-Asp, IAA-Ala, IAA-Leu, IAA-Ile, IAA-Phe, and IAA-Val) were purchased from Sigma (Sigma-Aldrich, St. Louis, MO). Other IAA-amino acid conjugates were synthesized by dicyclohexylcarbodiimide (DCCI)-activated condensation of IAA and L-amino acid esters, followed by base saponification to remove ester-protecting groups and yield IAA-amino acid conjugates, as described by LeClere and others (2002). IAA-α-L-Lys was a gift from Dr. Bonnie Bartel (Department of Biochemistry and Cell Biology, Rice University, Houston, TX). Other chemicals and reagents were analytical grade or higher.

Enzyme Induction and Extraction

Bacteria were grown in an LB medium (Alpha Biosciences Inc., Baltimore, MD) for 24 h at 30°C and then transferred to BSM. The bacterial concentration in BSM was adjusted to OD_{600} = 0.6, and then different inducers were added for another 16 h to allow for induction of the IAA-Ala hydrolase activities. The bacterial cells were then harvested and pelleted by centrifugation at 12,000 × *g* for 5 min at 4°C. The pellet was resuspended in 2 ml of 50 mM potassium phosphate buffer (pH 8.5) and sonicated (Microson, Misonix Inc., Farmingdale, NY) with a 3-mm probe using three pulses of 85 W for 15 s each at 4°C. Unbroken cells and cell debris were removed by centrifugation at 12,000 × *g* for 60 min. The supernatant was collected and labeled as crude extract, and the IAA-Ala hydrolase activity was assayed.

Enzyme Activity Assays

Qualitative enzyme assays were measured by incubating 100 µl of a crude extract with 1 mM IAA-Ala (Sigma, St. Louis, MO) at 30°C for 30 min. The reaction was stopped by the addition of 20 µl 85% H_3PO_4, and the IAA product was extracted into 200 µl water-saturated ethyl acetate. The ethyl acetate extract was analyzed by silica gel 60-F_{254} thin-layer cromatography (TLC) (Merck, Darmstadt, Germany) using a solvent of chloroform: methanol:H_2O (85:14:1, v/v/v), as described by Labarca and others (1965). After air drying at room temperature, the TLC plate was developed by immersion in Ehmann's reagent (Ehmann 1977) for about 5 s and was incubated at 100°C for 1 min. Both IAA and IAA-Ala could be visualized by their bright-blue color after development. Under these conditions, IAA had an R_f value of 0.5 and IAA- Ala had an R_f value of 0.1. Quantitative enzyme assays were performed using the same reaction and ethyl acetate extract procedures as described above. The ethyl acetate extract was dried *in vacuo* and redissolved in 60% methanol:1% acetic acid (1:1, v/v). The

Table 1. Systematic Tests of Four Bacteria Inducible for IAA-Ala Hydrolases

Bacteria identified	Gram staining	Oxidase activity	Catalase activity	Fatty acid composition identity scores by GC
Arthrobacter ureafaciens C-10	Positive	Positive	Positive	0.781
Arthrobacter ureafaciens C-50	Positive	Positive	Positive	0.726
Arthrobacter ilicis D-50	Positive	Negative	Positive	0.613
Cellulomonas fimi D-100	Positive	Negative	Positive	0.594

GC = gas chromatography.

solution was then analyzed by C_{18} reverse-phase high performance liquid chromatography (HPLC) (Whatman 5 μ, Partisil ODS-3 column, 4.6 × 150 mm) with a mobile phase of 60% methanol:1% acetic acid (1:1. v/v) and UV detection at 282 nm.

Protein Determination and SDS-PAGE

Protein concentrations were determined using a Coomassie dye-based protein assay kit (Pierce Biotechnology Inc., Rockford, IL) according to the method of Bradford (1976). Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) of proteins was performed by the method of Laemmli (1970).

Results

Bacterial Identification

Four bacterial isolates were found to have an IAA-Ala hydrolase inducible by *N*-acetyl-L-alanine. Bacterial identification was initially based on gas chromatography (GC) analysis of fatty acid ester composition and further confirmed by Gram's staining, oxidase activity, and catalase activity (Table 1). The four bacterial strains were Coryneform bacteria. Among them, bacterial strain D-50 correlates best with *Paenibacillus polymyxa* (0.708) and *Arthrobacter ilicis* (0.613). The numbers indicate the identity coefficient of fatty acid composition, with best identity at 1.000. The strain D-50, however, produces no spores and is positive for catalase activity, which should be negative for *P. polymyxa*. Therefore, the strain D-50 was identified as *A. ilicis D-50*. The same identification procedure was applied to bacterial strain D-100, which has a higher correlation to *P. polymyxa* (0.639) than *Cellulomonas fimi* (0.594), but also has no spore formation and was positive for catalase activity. We found that *A. ilicis* D-50 had the highest enzyme activity for IAA-Ala hydrolysis following induction (data not shown); therefore this bacterial strain was chosen for further biochemical analyses.

Table 2. IAA-Ala Hydrolase Induction by Different Inducers with Basal Salt Medium (BSM)

Inducers	Concentration (mM)	Induction (%)
N-Acetyl-L-Ala	10	100
N-Acetyl-L-Ala	5	60
N-Acetyl-D,L-Ala	10	60
N-Acetyl-D,L-Ala	5	40
IAA-Ala	5	≤ 5
Ala	5	10
$(NH_4)_2SO_4$	5	10
Acetamide	5	≤ 5
Urea	5	10

Inductions were measured visually based on IAA spot colorization of thin layer chromatography (TLC) with Ehmann's solution.
The bacteria were cultured in an LB (Luria broth)-rich medium for 24 h and then transferred to BSM with inducers for another 16 h.

IAA-Ala Hydrolase Inductions

Several compounds, as well as *N*-acetyl-L-alanine, were tested as inducers for IAA-Ala hydrolase in *A. ilicis* D-50 (Table 2). As expected from our previous studies of a bacterial IAA-Asp hydrolase, *N*-acetyl-L-alanine was the best inducer. Surprisingly, IAA-Ala was not an inducer, and alanine, $(NH_4)_2SO_4$, and urea have weak inducing activities. These results indicate a quite different induction pathway than was suggested by IAA-Asp hydrolase induction (Chou and others 1996). Because of their predicted metabolic roles, induction by alanine, $(NH_4)_2SO_4$, and urea may result from different genetic and response signaling systems.

Enzyme Optimal pH and Cofactor Effects

The bacterial IAA-Ala hydrolase activity was found to be highest under quite basic conditions, between pH 8 and pH 9 (Figure 1). We therefore prepared all

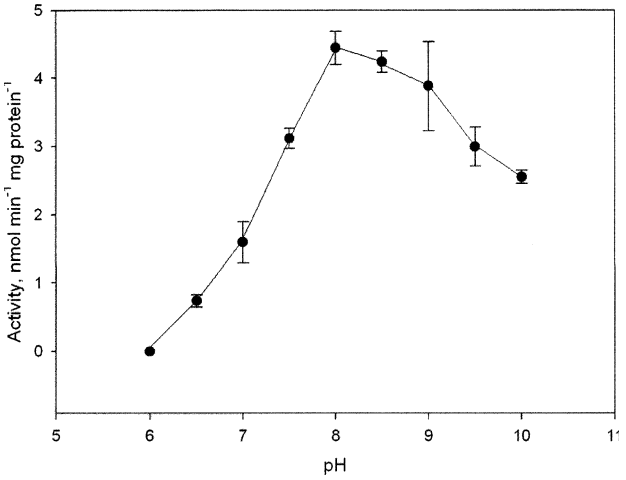

Figure 1. Optimal pH for activity of IAA-Ala hydrolase. The IAA released by hydrolysis was measured by quantitative high performance liquid chromatography (HPLC). Data are the averages ± SD of three experiments.

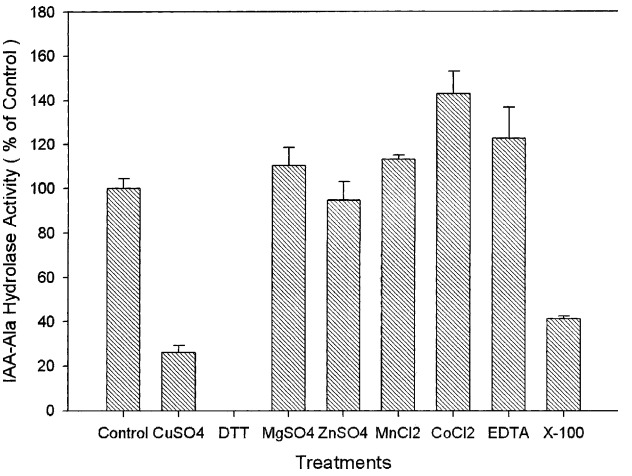

Figure 2. Effects of cofactors on IAA-Ala hydrolase activity. All treatments are at 1 mM, except Triton X-100, which was 1% (v/v). All values are relative to control (100%), which was 6.28 ± 0.28 nmol min^{-1} mg crude $protein^{-1}$. The IAA released by hydrolysis was measured by quantitative HPLC. Data are the means ± SD of three experiments.

of the routine extraction buffer and enzyme assays at pH 8.5. Possible cofactor effects on the bacterial IAA-Ala hydrolase activity are shown in Figure 2. It was found that 1 mM DTT completely inhibited the enzyme activity and 1 mM $CuSO_4$ and 1% (v/v) Triton X-100 decreased the enzyme activities by 75% and 60%, respectively. On the other hand, 1 mM $CoCl_2$ and 1 mM EDTA increased the enzyme activities by 40% and 20%, respectively.

Substrate Specificity of IAA-Ala Hydrolase

We tested 19 additional IAA-amino acid conjugates as possible alternative substrates for the bacterial IAA-Ala hydrolase (Table 3). Compared to the other conjugates tested, IAA-Ala was found to be the best substrate for the enzyme hydrolysis activity. Both IAA-Phe and IAA-Asp could be hydrolyzed, but at much lower rates. The assays were performed under three pH conditions because we found this enzyme to be very sensitive to acidic conditions (Figure 1). Under acidic conditions (pH 6), the bacterial enzyme lost activity for all 20 IAA-amino acid conjugates. Both IAA-Ala and IAA-Phe hydrolysis activities were stronger under alkali conditions (pH 8.5) than under neutral conditions (pH 7). IAA-Asp hydrolysis is stronger under neutral conditions, indicating the ionic characteristics of aspartate residue may affect the enzyme hydrolysis activity.

Kinetic Characteristics and SDS-PAGE Protein Profiles

The substrate concentration curve and Lineweaver-Burk plot of IAA-Ala hydrolase (Figure 3) shows

Table 3. Substrate Specificity of IAA-Ala Hydrolase to Twenty IAA-amino Acid Conjugates

	Hydrolysis rate (nmol min^{-1} mg $protein^{-1}$)		
Substrates	pH 6	pH 7	pH 8.5
IAA-Ala	nd	1.20 ± 0.23	3.94 ± 0.42
IAA-Asp	nd	0.218 ± 0.017	0.172 ± 0.020
IAA-Phe	nd	0.450 ± 0.048	0.840 ± 0.183

nd = non-detectable IAA peak in HPLC analysis.
Seventeen other IAA-amino acids, including IAA-Arg, IAA-Asn, IAA-Cys, IAA-Gln, IAA-Glu, IAA-Gly, IAA-His, IAA-Ile, IAA-Leu, IAA-Lys, IAA-Met, IAA-Pro, IAA-Ser, IAA-Thr, IAA-Trp, IAA-Tyr, and IAA-Val, were found non-detectable in this HPLC analysis at all pH values and therefore were not listed to simplify the table. Data are the means ± SD of three experiments.

that the enzyme has a K_m value at 5.65 mM when IAA-Ala is used as substrate. The K_m values of two other possible substrates, IAA-Phe and IAA-Asp, were found to be 27.5 mM and 35.7 mM, respectively (data not shown). The results indicate that the enzyme has a much stronger affinity for IAA-Ala than for IAA-Phe and IAA-Asp. The SDS-PAGE protein profiles of 10 mM *N*-acetyl-D,L-alanine induced and non-induced *A. ilicis* D-50 are shown in Figure 4. A major band around 40 kDa was consistently found in induced bacterial crude extract, and its presence correlated with the enzyme activity by TLC analysis (data not shown). The 40 kDa protein is therefore assigned as a possible candidate for the IAA-Ala hydrolase.

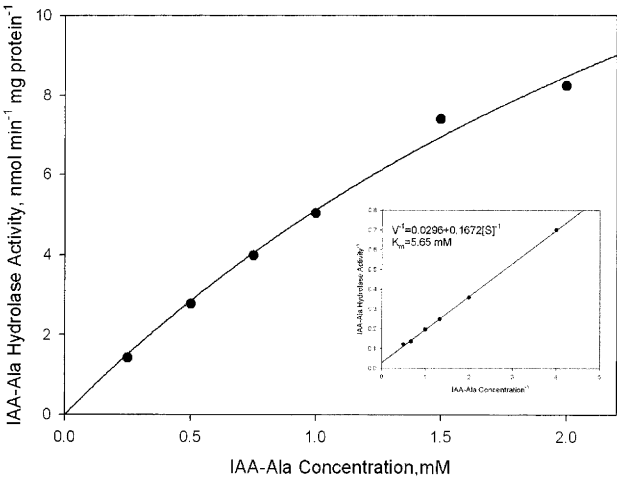

Figure 3. Substrate concentration curve and Lineweaver-Burk plot of IAA-Ala hydrolase. The IAA released by hydrolysis was measured by quantitative HPLC.

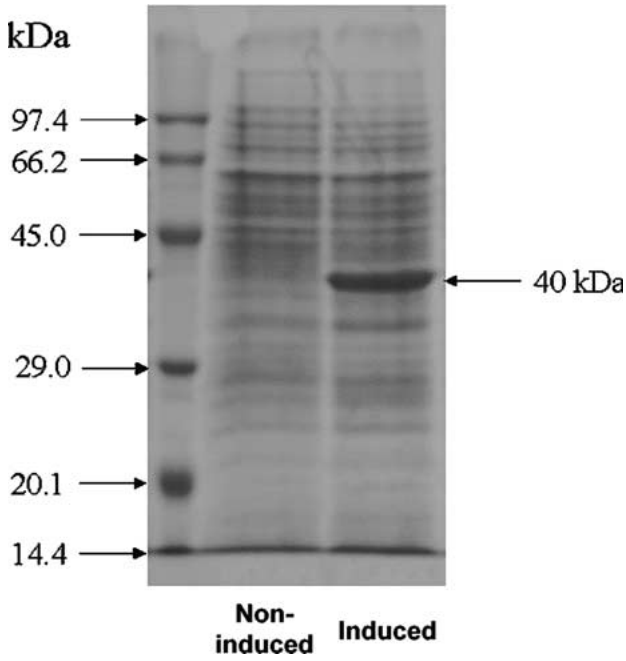

Figure 4. Protein profiles of 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of the crude soluble fraction of induced (**right**) and non-induced (**middle**) *A. ilicis* D-50, with left lane as the protein molecular weight markers. Each well was loaded with 15 μg crude extract protein. Arrow on the right indicates possible IAA-Ala hydrolase protein and its molecular weight.

Discussion

We have isolated and identified several bacterial strains (Table 1) capable of hydrolyzing IAA-Ala with a higher substrate specificity than other bacterial or plant IAA-amino acid hydrolases previously reported (Chou and others 1996, 2004; LeClere and others 2002). The enzyme from *A. ilicis* D-50 is inducible by *N*-acetyl-L-alanine, similar to the induction of IAA-Asp hydrolase of *Enterobacter agglomerans* by *N*-acetyl-L-aspartate, suggesting that *N*-acetyl-L-amino acids may be universal inducers for IAA-amino acid hydrolases from bacteria. If confirmed by further screens, it may prove possible to identify additional interesting IAA amino acid hydrolases for the study of auxin metabolism in plants through a similar induction approach.

In addition to *N*-acetyl-L-alanine, alanine, $(NH_4)_2SO_4$, and urea were found to slightly induce IAA-Ala hydrolysis activity (Table 2). This may result from a general induction of enzymes involved in aspects of nitrogen metabolism, including some that are capable of weakly hydrolyzing IAA-Ala. A surprising result was that IAA-Ala, the enzyme substrate, is not an inducer for the IAA-Ala hydrolase of *A. ilicis* D-50. This result differs from the bacterial IAA-Asp hydrolase that is inducible by both IAA-Asp and *N*-acetyl-L-aspartic acid (Chou and others 1996), and it indicates that the IAA-Ala hydrolase may have a more complex inducing mechanism and characteristics of enzyme–substrate associations. It is also possible that IAA-Ala is not the native substrate. In addition, it is possible that IAA-Ala may also function as a repressor for IAA-Ala hydrolase gene expression. Further studies of this enzyme's induction and signal transduction, as well as structural characteristics of the protein will be needed to elucidate these issues.

The IAA-Ala hydrolase of *A. ilicis* D-50 hydrolyzed IAA-Ala strongly, but IAA-Phe and IAA-Asp only weakly (Table 3), showing a considerable substrate preference toward IAA-Ala. Further analysis under acidic, neutral, and alkali conditions showed that both IAA-Ala and IAA-Phe hydrolysis activities were stronger at basic pH and IAA-Asp hydrolysis activity was stronger under more neutral conditions. The enzyme completely lost activity at acidic pH (Figure 1). This result may provide some insight into how this class of enzymes interacts with their substrates, especially those emzymes found in specific cellular compartments within plants. Plants may be able to regulate enzyme activities and levels of IAA and IAA-amino acid conjugates as needed based on general metabolism, for example, through responding to various pH changes in different organelles. The optimal pH of the IAA-Ala hydrolase activity was around pH 8 to pH 9 (Figure 1), similar to the bacterial IAA-Asp hydrolase (Chou and others 1996). This is significantly higher than IAA-amino acid hydrolysis enzymes reported in plants

(LeClere and others 2002), and the bacterial enzyme was less tolerant to acidic environments. This indicates that some fundamental catalytic characteristics of IAA-amino acid hydrolases may be variable on account of protein structural differences.

Analysis of cofactor effects of this enzyme (Figure 2) showed similar characteristics to all other reported IAA-amino acid hydrolases and reconfirmed that there are fundamental mechanisms involved in enzyme catalysis of the hydrolysis of IAA amino acid conjugates. Based on a computer simulation and point mutation study, a histidine residue of bacterial IAA-Asp hydrolase was shown to be essential for enzyme activity and to be absolutely conserved in the class of enzymes so far reported (Chou and others 2004). It will be interesting to examine the protein structure of the IAA-Ala hydrolase to see if this structural analysis can be universally applied.

The substrate concentration curves and Lineweaver-Burk plots of IAA-Ala hydrolase (Figure 3) revealed a lower K_m value of 5.65 mM compared to bacterial IAA-Asp hydrolase (Chou and others 1996). However, the K_m value is significantly higher than those found for plant IAA-amino acid hydrolases (LeClere and others 2002). This result supports our earlier work (Chou and others 2004) which noted that there are some fundamental and critical differences between bacterial IAA-amino acid hydrolases and plant IAA-amino acid hydrolases in terms of enzyme substrate association. The other two alternative substrates have a K_m at 24.4 mM for IAA-Phe and 35.7 mM for IAA-Asp (data not shown), indicating a very low enzyme affinity for these two substrates. Based on these findings, we conclude that IAA-Ala is the preferred substrate of this bacterial enzyme.

The protein profile by SDS-PAGE analysis (Figure 4) shows a major 40 kDa protein found only in bacterial protein extracts induced by *N*-acetyl-D,L-alanine. This protein is consistently and proportionally associated with the hydrolase activities when the SDS-PAGE protein profiles are correlated with the enzyme activity assays by TLC analysis. Based on these results, we are continuing our effort to clone the gene for this bacterial IAA-Ala hydrolase to further analyze the protein structure and molecular characteristics of this enzyme.

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REFERENCES

Andreae WA, Good NE. 1955. The formation of indoleacetylaspartic acid in pea seedlings. Plant Physiol 30:380–382.

Bandurski RS. 1980. Homeostatic control of concentrations of indole-3-acetic acid In: Skoog F Plant growth substances 1979 Berlin Heidelberg New York: Springer-Verlag. pp 37–49.

Bartel B, Fink GR. 1995. *ILR1*, an amidohydrolase that releases active indole-3-acetic acid from conjugates. Science 268:1745–1748.

Bialek K, Meudt WJ, Cohen JD. 1983. Indole-3-acetic acid (IAA) and IAA conjugates applied to bean stem sections: IAA content and the growth response. Plant Physiol 73:130–134.

Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254.

Campanella JJ, Ludwig-Mueller J, Town CD. 1996. Isolation and characterization of mutants of *Arabidopsis thaliana* with increased resistance to growth inhibition by indoleacetic acid-amino acid conjugates. Plant Physiol 112:735–745.

Campanella JJ, Olajide AF, Magnus V, Ludwig-Muller J. 2004. A novel auxin conjugate hydrolase from wheat with substrate specificity for longer side-chain auxin amide conjugates. Plant Physiol 135:2230–2240.

Chou JC, Kuleck GA, Cohen JD, Mulbry WW. 1996. Partial purification and characterization of an inducible indole-3-acetyl-L-aspartic acid hydrolase from *Enterobacter agglomerans*.. Plant Physiol 112:1281–1287.

Chou JC, Mulbry WW, Cohen JD. 1998. The gene for indole-3-acetyl-L-aspartic acid hydrolase from *Enterobacter agglomerans:* molecular cloning, nucleotide sequence, and expression in *Escherichia coli*.. Mol Gen Genet 259:172–178.

Chou JC, Welch WH, Cohen JD. 2004. His-404 and His-405 are essential for enzyme catalytic activities of a bacterial indole-3-acetyl-L-aspartic acid hydrolase. Plant Cell Physiol 45:1335–1341.

Cohen JD, Bandurski RS. 1978. The bound auxins: protection of indole-3-acetic acid from peroxidase-catalyzed oxidation. Planta 139:203–208.

Cohen JD, Bandurski RS. 1982. Chemistry and physiology of the bound auxins. Annu Rev Plant Physiol 33:403–430.

Davies RT, Goetz DH, Lasswell J, Anderson MN, Bartel B. 1999. IAR3 encodes an auxin conjugate hydrolase from Arabidopsis. Plant Cell 11:365–376.

Ehmann A. 1977. The Van Urk-Salkowski reagent—a sensitive and specific chomogenic reagent for silica gel thin-layer chromatographic detection and identification of indole derivatives. J Chromatogr 132:267–276.

Glass NL, Kosuge T. 1986. Cloning of the gene for indoleacetic acid-iysine synthetase from *Pseudomonas syringae* subsp. *savastanoi*.. J Bacteriol 166:598–602.

Glass NL, Kosuge T. 1988. Role of indoleacetic acid-lysine synthetase in regulation of indoleacetic acid pool size and virulence of *Pseudomonas syringae* subsp. *savastanoi*. J Bacteriol 170:2367–2373.

Hangarter RP, Good NE. 1981. Evidence that IAA conjugates are slow-release sources of free IAA in plant tissues. Plant Physiol 69:1424–1427.

Hangarter RP, Peterson MD, Good NE. 1980. Biological activities of indoleacetyl amino acids and their use as auxins in tissue culture. Plant Physiol 65:761–767.

Hutzinger O, Kosuge T.. 1968a. 3-indoleacetyl-L-lysine, a new conjugate of 3-indoleacetic acid produced by *Pseudomonas savastanoi*. In: Wightman F, Setterfield G Biochemistry and physiology of plant growth substances Ottawa, Ontario, Canada: The Runge Press, Ltd.. pp 183–194.

Hutzinger O, Kosuge T. 1968b. Microbial synthesis and degradation of indole-3-acetic acid. III. The isolation and characterization of indole-3-acetyl-ϵ-L-lysine. Biochemistry 7:601–605.

Iyer M, Epstein E, Cohen JD, Slovin JP. 1998. Molecular manipulation of IAA-glucose formation in tomato. Plant Physiol 117:S–132.

Kowalczyk S, Bandurski RS. 1991. Enzymatic synthesis of l-*O*-(indol-3-ylacetyl)-ß-D-glucose: Purification of the enzyme from *Zea mays* and preparation of antibodies to the enzyme. Biochem J 279:509–514.

Krieg NR. 1981. Enrichment and isolation In: Gerhardt P, Murray RGB, Costilow RN, Nester EW, Wood WA, Krieg NR, Phillips GB Manual of methods for general bacteriology Washington, DC: American Society for Microbiology. pp 112–142.

Kuleck GA, Cohen JD. 1992. The partial purification and characterization of IAA-alanine hydrolase from *Daucus carota* (abstract no. 103). Plant Physiol 99:S–18.

Labarca C, Nicholls PB, Bandurski RS. 1965. A partial characterization of indoleacetylinositols from *Zea mays*. Biochem Biophys Res Commun 20:641–646.

Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685.

LeClere S, Tellez R, Rampey RA, Matsuda SPT. 2002. Characterization of a family of IAA-amino acid conjugate hydrolases from Arabidopsis. J Biol Chem 277:20446–20452.

Ljung K, Hull AK, Kowalczyk M, Marchant A, Celenza J. 2002. Biosynthesis, conjugation, catabolism and homeostasis of indole-3-acetic acid in *Arabidopsis thaliana*. Plant Mol Biol 50:309–332.

Normanly J. 1997. Auxin metabolism. Physiol Plant 100:431–442.

Nowacki J, Bandurski RS. 1980. *Myo*-inositol esters of indole-3-acetic acid as seed auxin precursors of *Zea mays* L. Plant Physiol 65:422–427.

Oetiker JH, Aeschbacher G. 1997. Temperature-sensitive plant cells with shunted indole-3-acetic acid conjugation. Plant Physiol 114:1385–1395.

Slovin JP. 1997. Phytotoxic conjugates of indole-3-acetic acid: potential agents for biochemical selection of mutants in conjugate hydrolysis. J Plant Growth Regul 21:215–221.

Staswick PE, Serban B, Rowe M, Tiryaki I, Maldonado MT. 2005. Characterization of an Arabidopsis enzyme family that conjugates amino acids to indole-3-acetic acid. Plant Cell 17:616–627.

Staswick PE, Tiryaki I, Rowe ML. 2002. Jasmonate response locus JAR1 and several related Arabidopsis genes encode enzymes of the firefly luciferase superfamily that show activity on jasmonic, salicylic, and indole-3-acetic acids in an assay for adenylation. Plant Cell 14:1405–1415.

Szerszen JB, Szczyglowski K, Bandurski RS. 1994. *iaglu*, a gene from *Zea mays* involved in conjugation of growth hormone indole-3-acetic acid. Science 265:1699–1701.

Tam YY, Epstein E, Normanly J. 2000. Characterization of auxin conjugates in Arabidopsis: low steady-state levels of indole-3-acetyl-aspartate, indole-3-acetyl-glutamate, and indole-3-acetyl-glucose. Plant Physiol 123:589–595.

Tam YY, Normanly J. 2002. Overexpression of a bacterial indole-3-acetyl-L-aspartic acid hydrolase in *Arabidopsis thaliana*.. Physiol Plant 115:513–522.

Tomasek PH, Karns JS. 1989. Cloning of a carbofuran hydrolase gene from *Achromobacter* sp. strain WM111 and its expression in Gram-negative bacteria. J Bacteriol 171:4038–4044.

Tuominen H, östin A, Sundberg B, Sandberg G. 1994. A novel metabolic pathway for indole-3-acetic acid in apical shoots of *Populus tremula* (L.) × *Populus tremuloides* (Michx.). Plant Physiol 106:1511–1520.