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Partial Purification and Characterization of an Inducible Indole-3-Acetyl-L-Aspartic Acid Hydrolase from *Enterobacter agglomerans*[1]

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Indole-3-acetyl-amino acid conjugate hydrolases are believed to be important in the regulation of indole-3-acetic acid (IAA) metabolism in plants and therefore have potential uses for the alteration of plant IAA metabolism. To isolate bacterial strains exhibiting significant indole-3-acetyl-aspartate (IAA-Asp) hydrolase activity, a sewage sludge inoculation was cultured under conditions in which IAA-Asp served as the sole source of carbon and nitrogen. One isolate, *Enterobacter agglomerans*, showed hydrolase activity inducible by IAA-L-Asp or *N*-acetyl-L-Asp but not by IAA, $(NH_4)_2SO_4$, urea, or indoleacetamide. Among a total of 17 IAA conjugates tested as potential substrates, the enzyme had an exclusively high substrate specificity for IAA-L-Asp. Substrate concentration curves and Lineweaver-Burk plots of the kinetic data showed a Michaelis constant value for IAA-L-Asp of 13.5 mM. The optimal pH for this enzyme was between 8.0 and 8.5. In extraction buffer containing 0.8 mM Mg^{2+} the hydrolase activity was inhibited to 80% by 1 mM dithiothreitol and to 60% by 1 mm $CuSO_4$; the activity was increased by 40% with 1 mM $MnSO_4$. However, in extraction buffer with no trace elements, the hydrolase activity was inhibited to 50% by either 1 mM dithiothreitol or 1% Triton X-100 (Sigma). These results suggest that disulfide bonding might be essential for enzyme activity. Purification of the hydrolase by hydroxyapatite and TSK-phenyl (HP-Genenchem, South San Francisco, CA) preparative high-performance liquid chromatography yielded a major 45-kD polypeptide as shown by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

The naturally occurring plant hormone IAA serves as an important signaling molecule, and its concentration and metabolism are thought to be tightly regulated (Normanly et al., 1995). IAA levels are controlled in vivo in part by the formation and hydrolysis of conjugates (Szerszen et al., 1994; Bartel and Fink, 1995). Such conjugates may serve as storage for excess precursors or hormones during times of excess production and may also serve as a hormone source when IAA is needed for growth and development (Cohen and Bandurski, 1982).

IAA conjugation could function (a) in the transport of IAA (Nowacki and Bandurski, 1980), (b) in the storage and subsequent reuse of IAA (Epstein et al., 1980), (c) to protect IAA from enzymatic destruction (Cohen and Bandurski, 1978; Park and Park, 1987), (d) in the homeostatic control of IAA concentration in plants (Bandurski, 1980), or (e) as a means of entry into the catabolic pathway through IAA-Asp (Tuominen et al., 1994). Because of the potential involvement of IAA conjugation in hormonal regulation, a number of laboratories have focused on the role of both IAA-ester and IAA-amide conjugate synthesis and hydrolysis activities during development (Bialek et al., 1983; Hall and Bandurski, 1986; Glass and Kosuge, 1988; Roberto et al., 1990; Sitbon et al., 1991; Sasaki et al., 1992; Szerszen et al., 1994; Bartel and Fink, 1995). Recent findings implicate IAA-Asp as an intermediate in the oxidative catabolism of IAA in several species (Tsurumi and Wada, 1986; Monteiro et al., 1988; Tuominen et al., 1994). These studies are of interest because control of catabolic processes could be useful in the genetic engineering of plants with altered levels of IAA in specific tissues (Fig. 1).

IAA-L-Asp was the first naturally occurring IAA-amide conjugate to be chemically characterized (Andersson and Sandberg, 1982; Cohen, 1982). IAA-Asp is known to be an endogenous component of soybean seeds (Cohen, 1982), and available evidence indicates that it is present in many other plant species (Row et al., 1961; Olney, 1968; Tillberg, 1974; Andersson and Sandberg, 1982; Cohen and Ernstsen, 1991; Dunlap et al., 1996). However, despite its widespread existence in plants and two hopeful preliminary reports (Lantican and Muir, 1967; Higgins and Barnett, 1976), an in vitro system for IAA-Asp synthesis or hydrolysis by an enzymatic mechanism has yet to be achieved (Bandurski et al., 1995). The difficulty with studies of IAA-Asp hydrolysis has in part been due to the minute amount of free IAA that is released over time. This is explained by the low rate of IAA-Asp hydrolysis found in most higher plants (Bialek et al., 1983) and also by the fact that IAA-amino acid hydrolases isolated from plants appear to denature easily during purification (Cohen et al., 1988; Kuleck and Cohen, 1992). These considerations led us to investigate the potential for obtaining similar activities from bacterial cultures and to use such bacterial activities to understand and modify IAA metabolism in higher plants.

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Abbreviations: BSM, basal salts medium; LB, Luria broth.

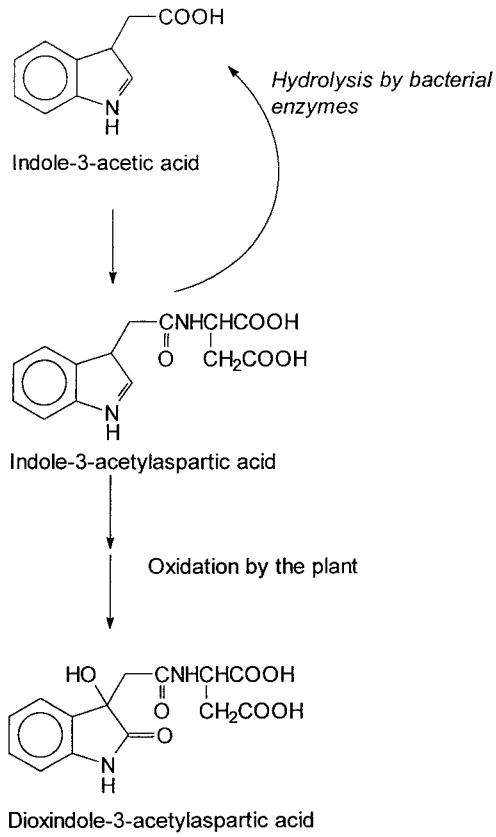

Figure 1. Possible pathway regulation for the catabolism of IAA-Asp in plants and the potential use of the bacterial enzyme.

We present here the first part of our ongoing study of an IAA-amino acid conjugate hydrolase with apparent specificity toward *N*-acyl-Asp hydrolysis, isolated from a bacterial strain of *Enterobacter agglomerans*. This enzyme can be induced in the bacteria by compounds with similar amide structures. The characterization of its cofactor requirements and optimal pH conditions suggests that the enzyme has a strong hydrophobic character and is rich in disulfide bonds. Its substrate specificity and kinetic constants reveal some interesting biochemical phenomena that might ultimately help in using this enzyme to understand the physiological processes regulating the metabolism of free IAA and IAA conjugates.

MATERIALS AND METHODS

Bacterial Strain Isolation and Characterization

Bacterial strains were isolated from a sewage sludge inoculation using standard enrichment techniques (Krieg, 1981) and IAA-DL-Asp or IAA-L-Ala (Aldrich) as the sole source of carbon and nitrogen. One bacterial isolate found to have an enzymatic activity highly inducible by IAA-Asp was subjected to Gram stain and oxidase tests. Two kinds of multimedia tubes (the Oxi/ferm tube and Enterotube II, Roche Diagnostic Systems, Nutley, NJ), were used for further bacterial characterization. The bacterial strain was also identified by fatty acid methyl ester analysis according to standard protocols (Microbial ID, MIDI, Newark, DE).

Induction and Extraction of IAA-Asp Hydrolase

Bacteria were grown in an LB-rich medium (GIBCO-BRL) for 24 h at 30°C and then transferred to BSM (Tomasek and Karns, 1989) containing different inducer treatments (Table I) for another 16 h to induce the IAA-Asp hydrolase activity. The bacterial cells were harvested and pelleted after enzyme induction by centrifugation at 12,000*g* for 5 min at 4°C. The pellet was resuspended in 4 mL of 50 mM potassium phosphate buffer (pH 7.0) containing 0.8 mM $MgSO_4$ (buffer A) and sonicated (Biosonic 4 unit, VWR Scientific) with a 20-mm probe using two pulses of 400 W for 15 s each at 4°C. Unbroken cells and cell debris were removed by centrifugation at 12,000*g* for 10 min. The supernatant was collected and labeled as crude extract and the IAA-Asp hydrolase activity was assayed.

Enzyme Activity Assays

Qualitative enzyme assays were measured by incubating 100 μL of a 10-fold buffer A-diluted crude extract with 1 mM IAA-L-Asp (no. I9387, Sigma) at 30°C for 10 min. The reaction was stopped by adding 20 μL of 85% H_3PO_4, and the IAA product was extracted into 200 μL of water-saturated ethyl acetate. The ethyl acetate extract was analyzed by silica gel 60-F_{254} TLC (no. 5719–2, E. Merck, Darmstadt, Germany) using a solvent of 2-butanone:ethyl acetate:ethanol:H_2O (3:5:1:1, v/v) as described by Labarca et al. (1965). After drying at room temperature, the TLC plate was developed by immersion in Ehmann's reagent (Ehmann, 1977) for about 5 s and incubated at 100°C for 1 to 5 min. Under these conditions, IAA had an R_F value of 0.8, and IAA-Asp had an R_F value of 0.2. Both could be visualized by their bright blue color after development.

Quantitative enzyme assays were measured using the same reaction and ethyl acetate extraction procedures. The ethyl acetate extract was then dried in vacuo and redissolved in 50% methanol:1% acetic acid (1:1, v/v). The so-

Table I. *IAA-Asp hydrolase induction by different inducers with BSM*

The bacteria were cultured in an LB-rich medium for 24 h and then transferred to BSM with inducers for another 16 h.

Inducers	Concentration	Induction[a]
	mM	%
IAA-Asp	1	100
$(NH_4)_2SO_4$	5	0
Urea	5	0
IAA	1	0
Indoleacetamide	1	0
N-acetyl-L-Asp	0.2	10
N-acetyl-L-Asp	1	30
N-acetyl-L-Asp	5	100
N-acetyl-L-Asp	25[b]	200

[a] Inductions were measured visually based on IAA spot colorization of TLC with Ehmann's solution. [b] The 25 mM *N*-acetyl-L-Asp was the highest concentration tested.

lution was then analyzed by C_{18} reverse-phase HPLC (Whatman 5 μ Partisil ODS-3 column, 4.6 × 150 mm) with a mobile phase of 50% methanol:1% acetic acid (1:1, v/v) and UV detection at 282 nm.

Enzyme Purification

A 9-L bacterial culture was grown for 16 h with aeration in BSM supplemented with 25 mM *N*-acetyl-L-aspartate (no. A8901, Sigma) to induce the synthesis of the IAA-Asp hydrolase. Cells from the 16-h culture were pelleted by centrifugation (6,000*g* for 20 min at 4°C). The 40-g pellet (consisting of cells and a considerable amount of polysaccharide) was resuspended in 90 mL of buffer A. This cell suspension was passed through a chilled French pressure cell (1050 kg cm^{-2}) twice, and whole cells and debris were removed by centrifugation (12,000*g* for 10 min at 4°C). The supernatant was subjected to ultracentrifugation (200,000*g* for 1 h at 4°C), and the supernatant from this treatment (hereafter referred to as the "crude soluble fraction") was removed and used as a source of IAA-Asp hydrolase for further purification and characterization.

The crude soluble fraction was pumped (3 mL min^{-1}) onto a 2.0- × 19-cm ceramic hydroxyapatite preparative HPLC column (Macro-Prep, Bio-Rad) pre-equilibrated with 10 mM potassium phosphate buffer, pH 7.0. With this method separation is based primarily on the ionic strength of proteins. The column was washed with a 10 to 200 mM linear gradient of potassium phosphate buffer, pH 7.0, at a flow rate of 5 mL min^{-1}. Fractions containing IAA-Asp hydrolase activity were pooled and used for further purification.

The most active fractions from two column runs of the hydroxyapatite column containing IAA-Asp hydrolysis activity were pooled and brought to 0.5 M $(NH_4)_2SO_4$ by the addition of solid $(NH_4)_2SO_4$ and pumped at 3 mL min^{-1} onto a TSK-phenyl column (2.15 × 15 cm; HP-Genenchem, South San Francisco, CA) that had been equilibrated with buffer A containing 0.5 M $(NH_4)_2SO_4$. The column was washed with buffer A containing 0.5 M $(NH_4)_2SO_4$ until all unbound material was eluted and a stable baseline was obtained. Bound material was eluted using a linear gradient of 0.5 to 0 M $(NH_4)_2SO_4$ in buffer A at a flow rate of 5 mL min^{-1}. Active fractions were pooled and used for SDS-PAGE analysis and further purification.

Protein Determinations and SDS-PAGE

Protein concentrations in the crude extracts were determined by the method of Bradford (1976) and in the partially purified enzyme preparations were determined by the spectrophotometric method of Kalb and Bernlohr (1977). Size-exclusion chromatography of fractions from the TSK-phenyl column was carried out (Protein-Pak 300SW column, 7.8 × 300 mm; Waters), equilibrated, and run with buffer A at 1 mL min^{-1}. SDS-PAGE of proteins was performed by the method of Laemmli (1970).

RESULTS

Bacterial Identification

A total of 18 bacterial strains was isolated from the sewage sludge and used for further assays of IAA-Asp hydrolase induction. The IAA-Asp hydrolase activity in one bacterial strain, coded GK#12A, was found to be highly inducible by 1 mM IAA-Asp in BSM after 24 h of cell culture in LB-rich medium. This bacterial strain showed negative results on both Gram stain and oxidase tests, but the results were positive on anaerobic dextrose fermentations with gas production, aerobic dextrose oxidation, Xyl oxidation, urease activity, citrate consumption, and Lys decarboxylase activity. The multimedia test results strongly suggested that this bacterial strain belonged to the Enterobacteriaceae family (Martin and Washington, 1980; Richard, 1984). Further analysis consisted of testing the bacteria for fatty acid composition using GC, which showed that the GK#12A isolate was highly similar to *E. agglomerans* with a match correlation of 0.886 (the higher the correlation, with the maximal value at 1.000, the higher the similarity). Another strong match by these criteria was shown with *Actinobacillus lignieresii* (with a match correlation of 0.837) after 48 h of cell culture, but this possibility was ruled out because the reported oxidase test characteristic of *A. lignieresii* conflicted with the results obtained with GK#12A. Therefore, we concluded that GK#12A was a strain of *E. agglomerans*.

IAA-Asp Hydrolase Inductions

From different combinations of cell culture and induction experiments (Table I), we found that 24 h of culture on LB medium followed by culture for an additional 16 h on BSM containing 25 mM *N*-acetyl-L-Asp yielded the highest induction of IAA-Asp hydrolase. This protocol was thus adopted and used throughout the later experiments. Comparisons of the total protein amount in each induction experiment showed no distinguishable variation, ruling out the possibility that the negative induction results seen under some conditions might have come from an incomplete protein extraction.

Purification of IAA-Asp Hydrolase

IAA-Asp hydrolase was purified by chromatography of the crude soluble fraction (approximately 900 mg of total protein at a concentration of 1.2 mg mL^{-1}) on a ceramic hydroxyapatite preparative HPLC column, followed by chromatography of the hydroxyapatite HPLC active fractions on a TSK-phenyl preparative HPLC column (Table II). The final preparation had a specific activity of approximately 180 times that of the starting material and yielded a major band of approximately 45 kD when subjected to SDS-PAGE (Fig. 2).

Characterization of IAA-Asp Hydrolase

The native molecular size of the IAA-Asp hydrolase was estimated by size-exclusion chromatography to be between

Table II. *Purification of IAA-Asp hydrolase from E. agglomerans*

Purification Step	Total Protein	Total Activity[a]	Specific Activity	Recovery	Purification
	mg	*μmol min^{-1}*	*μmol min^{-1} mg^{-1} protein*	*%*	*fold*
Crude extract	886.08	52.81	0.0596	100	1
Hydroxyapatite	207.4	48.32	0.233	91.5	4
TSK-phenyl[b]	0.746	7.87	10.551	14.9[c]	177

[a] The hydrolysis assays were measured by quantitative HPLC analysis. [b] The TSK-phenyl column was loaded with the combined active fractions from two hydroxyapatite columns. [c] The activity tested here was from the peak-active fraction of 5 mL of eluate, and thus does not include the total activity recovered.

40 and 55 kD. Taken together with the size estimated by SDS-PAGE, these results suggest that the enzyme is a monomer of 45 kD.

Assays were performed to test the effect of different metal salts or divalent metal chelators, the reductant DTT, and the detergent Triton X-100 on activity. $MgSO_4$, $ZnSO_4$, EDTA, and $CoSO_4$ showed no effect on enzyme activity at 1 mM. Assays with 1 mM DTT or 1% Triton X-100 showed approximately 50% inhibition of IAA-Asp hydrolase activity (Fig. 3A). In contrast, if the extraction buffer contained 0.8 mM Mg^{2+}, similar assays showed 60 and 80% inhibition by 0.1 mM $CuSO_4$ and DTT, respectively, and an approximately 40% increase by 1 mM $MnCl_2$ on the IAA-Asp hydrolase activity (Fig. 3B).

A total of 17 different IAA conjugates were assayed in a study of alternative substrates for IAA-Asp hydrolase. The results showed that the enzyme had an exclusively high specificity for IAA-L-Asp as the substrate (Table III). In addition, the optimal pH condition for IAA-Asp hydrolase activity was found to be between pH 8.0 and 8.5 (Fig. 4).

To define the substrate requirements of the IAA-Asp hydrolase, K_m values were estimated from substrate concentration curves and Lineweaver-Burk plots using IAA-L-Asp as the substrate. The kinetic experiments showed a K_m value of approximately 13.5 mM (Fig. 5).

DISCUSSION

IAA-Asp makes up more than 50% of the total IAA conjugates in some plant tissues, such as soybean seeds (Cohen, 1982), and yet direct biochemical studies of its hydrolysis are still rare. In this report we focus on IAA-Asp hydrolase activity because it has now been shown that in certain plant species the regulation of IAA-Asp synthesis and hydrolysis play a key role in IAA metabolism and, thus, plant growth and development (Cohen and Baldi, 1983; Nordstrom and Eliasson, 1991; Bialek and Cohen, 1992; Magnus et al., 1992; Bandurski et al., 1995). In par-

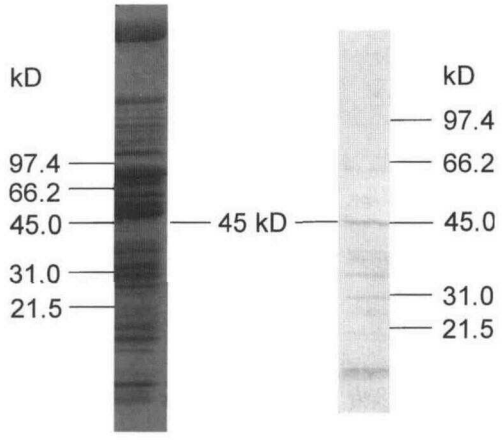

Figure 2. SDS-PAGE of the crude soluble fraction (left) and the fraction after hydroxyapatite and TSK-phenyl column purification (right).

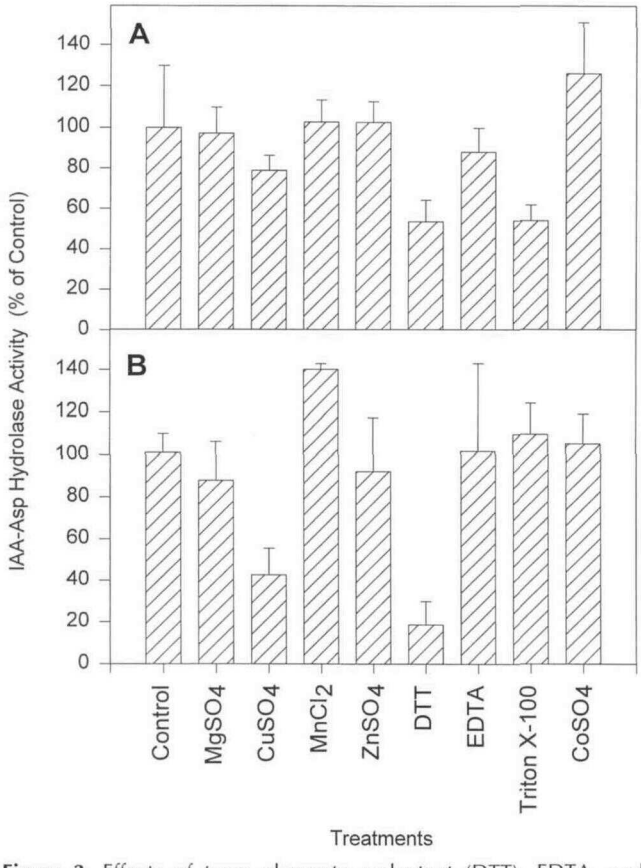

Figure 3. Effects of trace elements, reductant (DTT), EDTA, and detergent (Triton X-100) on IAA-Asp hydrolase. A, Crude extract without Mg^{2+} in the buffer; B, crude extract with the standard Mg^{2+}-containing buffer (see "Materials and Methods"). All treatments are at 1 mM except Triton X-100, which was 1% (v/v). All values are relative to control (100%), which was 23.9 ± 4.0 nmol min^{-1} mg^{-1} protein in the experiment reported in A and 35.8 ± 1.8 nmol min^{-1} mg^{-1} protein in B. The IAA released by hydrolysis was measured by quantitative HPLC. Data are the averages ± SD of three experiments.

Table III. *Assays for alternative substrates of IAA-Asp hydrolase*

All assays were performed using 0.5 mg of protein from the crude extract or 0.01 mg of purified protein from the TSK-phenyl column with 1 mM of substrates at 30°C for 24 h, except for IAA-L-Asp, which was done with 0.1 mg of protein at 30°C for 10 min. The hydrolysis was measured visually based on IAA spot colorization of TLC with Ehmann's solution, except for the purified enzyme on IAA-L-Asp, which was measured by quantitative HPLC.

Substrate	Hydrolysis Rates[a]	
	Crude enzyme	Purified enzyme
IAA-L-Asp	98,740	10.6×10^6
IAA-D-Asp	<0.8	
IAA-L-isoLeu	<0.8	
IAA-L-Phe	<0.8	
IAA-L-Val	<0.8	
IAA-L-Leu[b]	0.8	<0.8
IAA-L-Gly[b]	2.4	<0.8
IAA-L-Ala	<0.8	
IAA-L-Lys	<0.8	
IAA-L-Glu[b]	1.1	<0.8
IAA-L-nonVal[b]	0.8	<0.8
IAA-β-Ala	<0.8	
IAA-δ-amino-valeric acid	<0.8	
IAA-γ-amino-butyric acid	<0.8	
IAA-L-Orn	<0.8	
IAA-L-norLeu	<0.8	
N_α,N_ε-$(IAA)_2$-L-Lys	<0.8	

[a] The hydrolysis rates are pmol min^{-1} mg^{-1} protein. Hydrolysis was not detectable below the rate of 0.8 pmol min^{-1} mg^{-1} protein for 24-h incubation (less than 0.2 μg IAA on the TLC plate).
[b] Substrates (IAA-L-Leu, IAA-L-Gly, IAA-L-Glu, and IAA-L-norVal) with trace hydrolysis were further tested with more purified protein.

ticular, recent studies show that IAA conjugation to aspartate is the entry step into the IAA catabolic processes in some plants (Tsurumi and Wada, 1986; Pluss et al., 1989); therefore, in such plants IAA-Asp formation would be the last potentially reversible step prior to the irreversible oxidation of the indolic ring. Previous direct biochemical approaches and molecular analyses of IAA-conjugate-resistant mutants have focused on IAA-amino acid hydrolases, but these in vitro studies yielded proteins with no reported hydrolytic activity toward IAA-Asp (Cohen et al., 1988; Kuleck and Cohen, 1992; Bartel and Fink, 1995). In vivo studies, however, suggest that plants are able to carry out such hydrolysis reactions (Bialek et al., 1983; Cohen and Baldi, 1983). Because of the expected low level of IAA-Asp hydrolysis activity in plants and the failure of prior plant studies to show in vitro hydrolytic activity toward IAA-Asp, we decided to determine whether such an activity could be found in prokaryotes. Sewage sludge has been shown to be a rich source from which to isolate bacterial strains. We found 18 possible candidates from sewage sludge showing the ability to grow by consuming IAA conjugates such as IAA-Asp and IAA-Ala. Of these, GK#12A was identified as a strain of *E. agglomerans* containing the strongest inducible activity capable of hydrolyzing IAA-Asp. This strain was therefore chosen for further investigation.

N-acetyl-L-Asp and IAA-Asp both served as strong inducers of IAA-Asp hydrolase (Table I). *N*-acetyl-L-Asp was used rather than IAA-Asp for large-scale cultures because *N*-acetyl-L-Asp is less expensive and easier to obtain in large amounts. This alternative provided an additional benefit in that it prevented possible contamination by residual IAA-Asp from induced cultures in the later enzyme assay experiments. The induction by multiple substrates

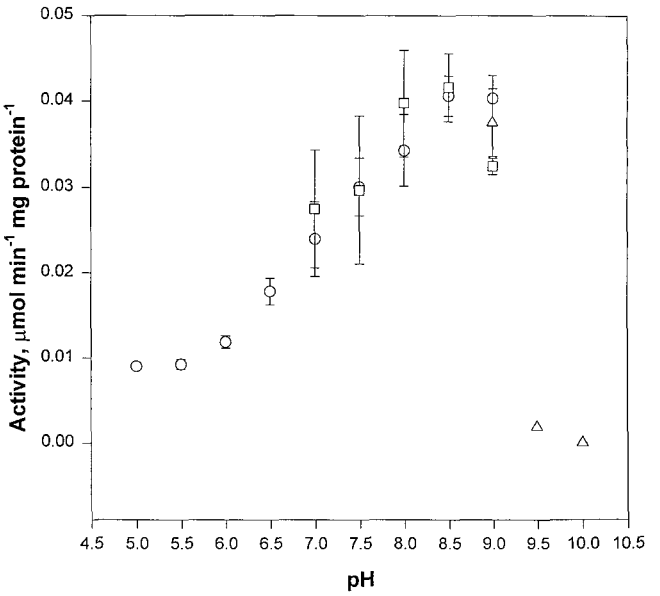

Figure 4. Optimal pH for activity of IAA-Asp hydrolase. The buffers used were 50 mM potassium phosphate (○), 50 mM Tris-HCl (□), and 50 mM boric acid (△). The IAA released by hydrolysis was measured by quantitative HPLC. Data are the averages ± SD of three experiments.

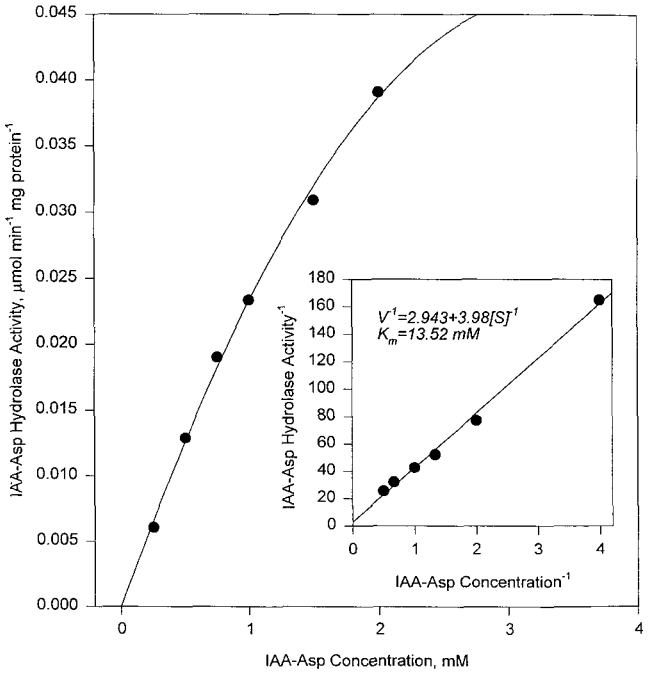

Figure 5. Substrate concentration curve and Lineweaver-Burk plot of IAA-Asp hydrolase. The IAA released by hydrolysis was measured by quantitative HPLC.

and the substrate specificity of the enzyme activity (Table III) both indicated that induction was probably triggered by a special amide structure (i.e. *N*-acylation on aspartate). Alternatively, induction might have been a multiple-step process in which IAA-Asp served as the original inducer and triggered a sequential induction involving the production of different activities. However, the induction of IAA-Asp hydrolase activity by *N*-acetyl-aspartate and the reverse process argues against such a relationship. A more detailed series of induction experiments will be necessary to elucidate fully the induction mechanism.

Enzyme purification utilizing the ceramic hydroxyapatite column for separation based on ionic strength and the TSK-phenyl column for separation by hydrophobic character (Table II) increased the purification by 10-fold in comparison with the combination of the DEAE ion-exchange column and the TSK-phenyl column (data not shown). The peak active fraction from the TSK-phenyl column showed a major band at 45 kD, which consistently corresponded with the enzyme activity assays and was thus identified as the protein having the IAA-Asp hydrolase activity. This size estimation was confirmed by size-exclusion chromatography, in which fractions containing strong enzyme activity eluted in the molecular mass range calculated from marker protein standards to be 40 and 55 kD (data not shown). After elution from the TSK-phenyl column the protein could be easily purified by SDS-PAGE and electroelution electrophoresis. This method has previously yielded proteins suitable for protein sequencing and, thus, sequence-based gene cloning.

We tested 17 IAA conjugates as possible substrates, including IAA-amino acid conjugates and both L- and D-forms of IAA-Asp. Surprisingly, we did not find any comparable level of activity toward any other IAA conjugates (Table III). We detected weak activity with IAA-Gly using less purified protein and extending the incubation period to 24 h at 30°C (IAA-L-Asp was completely hydrolyzed within 30 min under the same conditions). No activity toward IAA-Gly was found, however, when the more highly purified active fraction from the TSK-phenyl column was used as the enzyme source. These results indicate that the weak hydrolytic activity toward IAA-Gly might be caused by some nonenzymatic degradation due to contaminating compounds or the presence of contaminating enzyme(s) that catalyze IAA-Gly hydrolysis. IAA-L-Leu, IAA-L-Glu, and IAA-L-norVal were also weakly hydrolyzed by less pure preparations and, as with IAA-Gly hydrolysis, enzyme preparations from the TSK-phenyl column did not have this hydrolytic activity. The high degree of IAA-amino acid specificity of this IAA-Asp hydrolase is especially interesting since this bacterial enzyme is more specific than enzymes isolated so far from higher plants. For example, the activity of ILR1 from Arabidopsis could hydrolyze five IAA-amino acid conjugates tested, IAA-Phe, IAA-Leu, IAA-Ala, IAA-Gly, and IAA-Val (Bartel and Fink, 1995), and the IAA-Ala hydrolase from carrot cells showed cross-activity to IAA-Phe (G.A. Kuleck and J.D. Cohen, unpublished data).

Analyzing the effect of trace elements and reducing agents on IAA-Asp hydrolase revealed that it might contain a very strong hydrophobic and disulfide-bond-rich structure, since 1 mM DTT inhibited as much as 80% of the enzyme activity and the presence of Mg^{2+} prevented the inhibitory effect of 1% Triton X-100 (Fig. 3). It is, however, unusual that 1 mM $CuSO_4$ inhibited the activity by 60% in the presence of Mg^{2+} but had no effect on enzyme activity when Mg^{2+} was omitted from the extraction buffer. The optimal activity under mildly basic conditions, pH 8.0 to 8.5 (Fig. 4), could indicate that these conditions stabilize the enzyme structure by preventing proton attack on disulfide bonds.

The substrate concentration curves of IAA-Asp hydrolase and Lineweaver-Burk plots revealed a relatively high K_m value of 13.52 mM, showing that the enzyme has a lower affinity for the substrate than might be optimal for phytohormone conjugate hydrolysis. However, the enzyme effectively hydrolyzed IAA-Asp at substrate levels well below its K_m. In addition, substrate concentrations might be higher in specific cellular organelles within the plant (Sitbon et al., 1993).

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LITERATURE CITED

Andersson B, Sandberg G (1982) Identification of endogenous N-(3-indoleacetyl) aspartic acid in Scots pine (*Pinus sylvestris* L.) by combined gas chromatography-mass spectrometry, using high-performance liquid chromatography for quantification. J Chromatogr **238:** 151–156

Bandurski RS (1980) Homeostatic control of concentrations of indole-3-acetic acid. *In* F Skoog ed, Plant Growth Substances 1979. Springer-Verlag, Berlin, pp 37–49

Bandurski RS, Cohen JD, Slovin JP, Reinecke DM (1995) B1: auxin biosynthesis and metabolism. *In* PJ Davies ed, Plant Hormones: Physiology, Biochemistry and Molecular Biology, Ed 2. Kluwer Academic, Dordrecht, The Netherlands, pp 39–65

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

Bialek K, Cohen JD (1992) Amide-linked indoleacetic acid conjugates may control levels of indoleacetic acid in germinating seedlings of *Phaseolus vulgaris*. Plant Physiol **100:** 2002–2007

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

Cohen JD (1982) Identification and quantitative analysis of indole-3-acetyl-L-aspartate from seeds of *Glycine max* L. Plant Physiol **70:** 749–753

Cohen JD, Baldi BG (1983) Studies of endogenous indole-3-acetyl-L-aspartate during germination of soybeans. Proc Plant Growth Regul Soc Am **10:** 117–122

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

Cohen JD, Ernstsen A (1991) Indole-3-acetic acid and indole-3-acetylaspartic acid isolated from seeds of *Heracleum laciniatum* Horn. Plant Growth Regul **10:** 95–101

Cohen JD, Slovin JP, Bialek K, Chen KH, Derbyshire MK (1988) Mass spectrometry, genetics and biochemistry: understanding the metabolism of indole-3-acetic acid. *In* GL Steffens, TS Rumsey, eds, Biomechanisms Regulating Growth and Development. Kluwer Academic, Dordrecht, The Netherlands, pp 229–241

Dunlap JR, Slovin JP, Cohen JD (1996) Indole-3-acid, ethylene, and abscisic acid metabolism in developing muskmelon (*Cucumis melo* L.) fruit. Plant Growth Regul **19:** 45–54

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

Epstein E, Cohen JD, Bandurski RS (1980) Concentration and metabolic turnover of indoles in germinating kernels of *Zea mays* L. Plant Physiol **65:** 415–421

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

Hall PJ, Bandurski RS (1986) [^{3}H]Indole-3-acetyl-*myo*-inositol hydrolysis by extracts of *Zea mays* L. vegetative tissue. Plant Physiol **80:** 374–377

Higgins SL, Barnett NM (1976) Cell-free synthesis of indoleacetylaspartic acid (abstract no. 95). Plant Physiol **57:** S-18

Kalb VJ, Bernlohr RW (1977) A new spectrophotometric assay for proteins in cell extracts. Anal Biochem **82:** 362–371

Krieg NR (1981) Enrichment and isolation. *In* P Gerhardt, RGE Murray, RN Costilow, EW Nester, WA Wood, NR Krieg, GB Phillips, eds, Manual of Methods for General Bacteriology, Chap 8. American Society for Microbiology, Washington, DC, 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

Lantican BP, Muir RM (1967) Isolation and properties of the enzyme system forming indoleacetic acid. Plant Physiol **42:** 1158–1160

Magnus V, Hangarter RP, Good NE (1992) Interaction of free indole-3-acetic acid and its amino acid conjugates in tomato hypocotyl cultures. J Plant Growth Regul **11:** 67–75

Martin WJ, Washington JA II (1980) Enterobacteriaceae. *In* A Balows, WJ Hausler, JP Truant, eds, Manual of Clinical Microbiology, Ed 3, Chap 16. American Society for Microbiology, Washington, DC, pp 195–219

Monteiro AM, Crozier A, Sandberg G (1988) The biosynthesis and conjugation of indole-3-acetic acid in germinating seeds of *Dalbergia dolchipetala*. Planta **174:** 561–568

Nordstrom AC, Eliasson L (1991) Levels of endogenous indole-3-acetic acid and indole-3-acetylaspartic acid during adventitious formation in pea cuttings. Physiol Plant **82:** 599–605

Normanly J, Slovin JP, Cohen JD (1995) Rethinking auxin biosynthesis and metabolism. Plant Physiol **107:** 323–329

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

Olney HO (1968) Growth substances from *Veratrum tenuipetalum*. Plant Physiol **43:** 293–302

Park RD, Park CK (1987) Oxidation of indole-3-acetic acid conjugates by horseradish peroxidase. Plant Physiol **84:** 826–829

Pluss R, Titus J, Meier H (1989) IAA-induced adventitious root formation in greenwood cuttings of *Populus tremula* and formation of 2-indolone-3-acetylaspartic acid, a new metabolite of exogeneously applied indole-3-acetic acid. Physiol Plant **75:** 89–96

Richard C (1984) Genus. VI. *Enterobacter*. *In* NR Krieg, ed, Bergey's Manual of Systematic Bacteriology, Vol 1. Williams & Wilkins, Baltimore, MD, pp 465–476

Roberto FF, Klee H, White F, Nordeen R, Kosuge T (1990) Expression and fine structure of the gene encoding N^{ϵ}-(indole-3-acetyl)-L-lysine synthetase form *Pseudomonas savastanoi*. Proc Natl Acad Sci USA **87:** 5797–5801

Row VV, Sanford WW, Hitchcock AE (1961) Indol-3-acetyl-D,L-aspartic acid as a naturally-occurring indole compound in tomato seedlings. Contrib Boyce Thompson Inst **21:** 1–10

Sasaki K, Sakai S, Kamada H, Harada H (1992) Regulation of IAA-amino acid conjugate formation in carrot tissues transformed by *Agrobacterium tumefaciens*. J Exp Bot **43:** 1491–1495

Sitbon F, Edlund A, Gardestrom P, Olsson O, Sandberg G (1993) Compartmentation of indole-3-acetic acid metabolism in protoplasts isolated from leaves of wild-type and IAA-overproducing transgenic tobacco plants. Planta **191:** 274–279

Sitbon F, Sundberg B, Olsson O, Sandberg G (1991) Free and conjugated indoleacetic acid (IAA) contents in transgenic tobacco plants expressing the *iaa*M and *iaa*H IAA biosynthesis genes from *Agrobacterium tumefaciens*. Plant Physiol **95:** 480–485

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

Tillberg E (1974) Occurrence of endogenous indol-3yl-aspartic acid in light and dark-grown bean seedlings (*Phaselous vulgaris*). Physiol Plant **31:** 271–274

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

Tsurumi S, Wada S (1986) Dioxindole-3-acetic acid conjugates formation from indole-3-acetylaspartic acid in *Vicia* seedlings. Plant Cell Physiol **27:** 1513–1522

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