Nitrilase in biosynthesis of the plant hormone indole-3-acetic acid from indole-3-acetonitrile: Cloning of the *Alcaligenes* gene and site-directed mutagenesis of cysteine residues

(nitrile/nucleotide sequence/auxin/gene expression)

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ABSTRACT Indole-3-acetic acid is the major auxin in most plants. In Cruciferae, including Brassicaceae, indole-3-acetic acid is synthesized from indole-3-acetonitrile by nitrilase, after indole-3-acetonitrile is formed from tryptophan via indole-3-acetaldoxime or indole glycosinolates as the intermediate. We cloned and sequenced the gene for nitrilase (EC 3.5.5.1), which catalyzes the hydrolysis of indole-3-acetonitrile to indole-3-acetic acid, from *Alcaligenes faecalis* JM3. The amino acid sequence deduced from the nucleotide sequence of the nitrilase gene shows 34.7% identity with that of *Klebsiella ozaenae* nitrilase. A DNA clone containing the nitrilase gene expressed the active enzyme in *Escherichia coli* with excellent yield. Among five cysteine residues (Cys-40, Cys-115, Cys-162, Cys-163, and Cys-218) in the *Alcaligenes* nitrilase, only Cys-163 was conserved at the corresponding position in the *Klebsiella* nitrilase. Two mutant enzymes, in which Cys-162 and Cys-163 were replaced with Asn and Ala, respectively, were constructed by site-directed mutagenesis. A 35% increase of the specific activity and a large reduction of the K_m for thiophene-2-acetonitrile (which was used as a standard substrate for the nitrilase) were observed in the Cys-162 → Asn mutant enzyme. The Cys-163 → Ala mutation resulted in complete loss of nitrilase activity, clearly indicating that Cys-163 is crucial for the activity and Cys-162 could not provide the catalytic function of Cys-163.

Indole-3-acetic acid (IAA) is now regarded as the most significant auxin and regulates many aspects of plant growth and development (1, 2). IAA can be synthesized from tryptophan in plants (3, 4). A predominant biosynthesis route of IAA has been found, in which tryptophan is converted into indole-3-pyruvic acid or tryptamine and then transformed into IAA via indole-3-acetaldehyde. In another pathway, IAA is formed from tryptophan via indole-3-acetaldoxime in Cruciferae: tryptophan → indole-3-acetaldoxime → indole-3-acetonitrile → IAA. Naturally occurring in plants, indole-3-acetaldoxime (5) can be metabolized to IAA by indole-3-acetaldoxime dehydratase (EC 4.2.1.29) (6), leading to the formation of indole-3-acetonitrile, which can be hydrolyzed to IAA by nitrilase (EC 3.5.5.1). The nitrilase is found in Cruciferae (cabbage group and radish), Gramineae (grasses), and Musaceae (banana family) (7, 8). Indole-3-acetaldoxime is a precursor of indole glucosinolates such as glucobrassicin, which is widely distributed in Brassicaceae (9, 10), and glucobrassicin can be transformed into indole-3-acetonitrile and thereafter be converted to IAA through an action of nitrilase.

Recently we purified and characterized the nitrilase from *Alcaligenes faecalis* JM3 (11). This enzyme did not act on

compounds with a cyano group directly bound to an aromatic or heterocyclic ring, such as benzonitrile and cyanopyridine, which can be hydrolyzed by all microbial nitrilases so far reported from *Pseudomonas* sp. (12, 13), *Nocardia* sp. strains NCIB 11215 (14) and NCIB 11216 (15), *Fusarium solani* (16), *Arthrobacter* (17), *Klebsiella ozaenae* (18), and *Rhodococcus rhodochrous* strains J1 (19) and K22 (20). The *A. faecalis* enzyme is highly specific for arylacetonitriles such as indole-3-acetonitrile, benzyl cyanide, and thiophene-acetonitrile. No genetic studies concerning indoleacetonitrile-specific nitrilases have been reported. There are no reports on the structure–function relationships of the nitrilase except for that of bromoxynil (3,5-dibromo-4-hydroxybenzonitrile)-specific nitrilase from *K. ozaenae* (18). Identification of the active site will certainly help to elucidate its unique substrate specificity and subsequently help us to understand its physiological functions.

When this work was started, the gene encoding the IAA-forming enzyme had not been cloned from plants (see Discussion). The existence of nitrilase, which catalyzes the conversion of indole-3-acetonitrile into IAA, in some species of the fungus *Taphrina*, causing hyperplastic diseases in plants, has been recently reported (21). As an initial step toward elucidating the evolution of nitrilase in IAA biosynthesis, we cloned, sequenced,[‡] and expressed in *Escherichia coli* the structural gene encoding the nitrilase specific for indole-3-acetonitrile from *A. faecalis* JM3. The role of cysteine residues in the *A. faecalis* nitrilase has been also investigated by means of site-directed mutagenesis.

MATERIALS AND METHODS

Bacterial Strains and Plasmids. *A. faecalis* JM3 was previously isolated from soil (22). *E. coli* JM105 (ref. 23, p. A.10) was used for pUC plasmid (24, 25) transformation.

PCR Amplification and DNA Sequence Analysis. The internal peptides of *A. faecalis* JM3 nitrilase, which was purified as described (11), were prepared by trypsin digestion and separated by HPLC. Oligonucleotide primers were synthesized on the basis of the amino acid sequences of the NH₂ terminus (11) and the peptides. The amino acid sequence Met-Gln-Thr-Arg-Lys-Ile-Val-Arg was used to model the oligodeoxynucleotide pool 5'-ATGCARACSMGVAAR-ATHGTVMG-3' (sense strand), and Gln-His-Glu-Ala-Ile-His-Ile-Ala to model 5'-GGGCATGCSGCDATRTGDATS-GCYTCRTGYTG-3' (antisense strand) (R = A or G; S = C or G; M = A or C; Y = C or T; V = A, C, or G; H = A, C, or T; and D = A, G, or T). Total DNA of *A. faecalis* JM3,

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Abbreviations: IAA, indole-3-acetic acid; IPTG, isopropyl β -D-thiogalactoside.

[‡]The sequence reported in this paper has been deposited in the GenBank data base (accession no. D13419).

which was cultured as described (11), was prepared after cell lysis (wet mass, 15 g) with lysozyme, *Achromobacter* peptidase (Wako Pure Chemical, Tokyo), and proteinase K (Merck), following the method of Saito and Miura (26). The single-stranded DNA was amplified by using the PCR (Perkin-Elmer/Cetus) as described by the manufacturer except that *Tth* (*Thermus thermophilus*) DNA polymerase (Toyobo, Osaka) was used instead of *Taq* DNA polymerase. The gel-purified PCR-synthesized product [560 base pairs (bp)] was cloned in the *HincII*-*Sph* I site of M13mp18 replicative-form DNA (25) and the construct was designated pNJM10. Nucleotides were sequenced by dideoxynucleotide chain termination (27).

Site-Directed Mutagenesis. Two oligonucleotides were synthesized for mutagenesis (28): 5'-CGGTGCCCTGA~~ACTGC~~TGGGAG-3' for replacement of Cys-162 by Asn and 5'-GGTGCCCTGTGC~~GCCT~~GGGAGC-3' for replacement of Cys-163 by Ala, where underlines indicate mismatched bases. To prepare single-stranded DNA, the *Pst* I and *Bam*HI sites of replicative-form M13mp18 DNA were ligated with the 1.16-kilobase (kb) *Pst* I-*Bam*HI fragment isolated from plasmid pNJM30. The desired mutants were selected by DNA sequencing. A 377-bp *Bgl* II-*Stu* I fragment (positions 742-1118) was excised from each of the mutated phage DNAs and inserted between the *Bgl* II and *Stu* I sites of plasmid pNJM30 instead of the parental fragment. The two mutant plasmids thus obtained were designated pNJM30-C162N and pNJM30-C163A, using the one-letter code and position number for each substituted amino acid.

Preparation of Crude Extracts from *E. coli* Transformants. Recombinant *E. coli* JM105 was cultured in 100 ml of 2× YT medium containing ampicillin (ref. 23, p. A.3) in a 500-ml shaking flask with isopropyl β-D-thiogalactoside (IPTG) added to a final concentration of 1 mM to induce the *lac* promoter. After various culture periods, the cells were harvested by centrifugation, suspended in 3 ml of 0.1 M potassium phosphate buffer (pH 7.0) containing 30% (mass/vol) 1,2-propanediol, which helps enzyme stability, disrupted by sonication for 5 min (Insonator model 201M; Kubota, Japan), and centrifuged at 12,000 × *g* for 30 min. SDS/PAGE was performed by the method of Laemmli (29).

Purification of Nonmutant and Mutant Nitrilases from *E. coli* Transformants. A nitrilase-overproducing strain was constructed by transforming *E. coli* JM105 with plasmid pNJM30, and it was cultured as described in *Results*. Cells of the transformants harvested were subjected to the following simple procedures at 0–4°C. Throughout purification steps, potassium phosphate buffer (pH 7.0) containing 10 mM dithiothreitol and 20% (mass/vol) glycerol was used. The supernatant of the ultrasonicated extracts derived from cells (wet mass, 7 g) was fractionated with ammonium sulfate (30–60%, mass/vol) and dialyzed against 0.01 M buffer. The dialyzed solution was applied to a phenyl-Sepharose CL-4B (Pharmacia) column equilibrated with 0.01 M buffer and eluted with 0.01 M buffer containing 20% (vol/vol) ethylene glycol. Active fractions were combined, dialyzed against 0.01 M buffer, and then concentrated by ultrafiltration with a Centrifo CF25 (Amicon).

Each mutant nitrilase was purified to homogeneity by SDS/PAGE from each mutant nitrilase-overproducing transformant according to the procedure used to purify the active nonmutant nitrilase described above.

Enzyme Assays. Nitrilase activity for *A. faecalis* JM3 was assayed by the method described previously (11). Thiophene-2-acetonitrile was used as a standard substrate instead of indole-3-acetonitrile, because the former is a more effective substrate and the sensitivity of the former's response in the assay is better (11).

RESULTS

Cloning and Nucleotide Sequence of the Nitrilase Gene from *A. faecalis* JM3. The nucleotide sequence corresponding to both primers was detected in cloned pNJM10 containing a 560-bp fragment generated by PCR amplification. To obtain the entire gene, after digestion of the total DNA with several restriction endonucleases, Southern hybridization (30) was carried out at 42°C using the radiolabeled (31) PCR-synthesized probe and a buffer containing 40% (vol/vol) formamide, 5× SSC (ref. 23, p. B.13), and 0.1% (mass/vol) SDS. A single 4-kb band was detected when total DNA was digested with *Sph* I. This fragment was ligated into *Sph* I-digested pUC19. Plasmid DNA containing the 4-kb *Sph* I fragment, designated as pNJM20, was prepared from a positive colony. Restriction endonuclease analysis together with Southern hybridization with the probe indicated the location of the nitrilase gene.

Fig. 1 shows the 1727-bp nucleotide sequence. The *Msp* I-*Mlu* I fragment of pNJM20 contained an open reading frame encoding 356 amino acids ($M_r = 38,908$), and the deduced amino acid sequences exactly corresponded to those determined with the purified nitrilase. The molecular mass of the *Alcaligenes* nitrilase as determined by SDS/PAGE was 44 kDa (11). This value is 13% higher than that predicted from the nucleotide sequence, probably due to poor SDS binding of the nitrilase.

The predicted amino acid sequence of *A. faecalis* JM3 nitrilase was compared with that of *K. ozaenae* nitrilase (18), which is highly specific for benzonitrile derivatives with two meta-positioned halogen atoms, such as bromoxynil and chloroxynil (3,5-dichloro-4-hydroxybenzonitrile) (Fig. 2). There was a 34.7% match of amino acids in 340 overlap residues between the two nitrilases. The amino acid sequence around Cys-163 was conserved. The neighboring amino acid residue is also cysteine (Cys-162). All nitrilases previously reported, including the *Alcaligenes* enzyme, were susceptible to thiol reagents and are therefore classified as sulfhydryl enzymes. On the other hand, three other cysteines (at positions 40, 115, and 218) in the *A. faecalis* JM3 nitrilase are not conserved at the corresponding position in the *K. ozaenae* enzyme. Considering the above results, Cys-162 or Cys-163 is expected to be a candidate as an active-site amino acid residue for the nitrilase.

Production of the Nitrilase Protein in *E. coli*. Since there were no suitable restriction sites upstream of the presumed ATG initiation codon, it was necessary for expression that the sequence upstream of the ATG codon be modified. Modification was attained by using PCR with the recombinant plasmid as a template and the following two primers (Fig. 3). Primer 1 contained a *Pst* I recognition site, a ribosome-binding site, and a TGA stop codon in frame with the *lacZ* gene in pUC19 and 18 nucleotides of the nitrilase structural gene starting with the ATG start codon. Primer 2 contained 19 nucleotides of the gene (complementary to nucleotides 1425–1443 in Fig. 1) and a *Bam*HI recognition site. The plasmid designated as pNJM30 was constructed by ligation of the PCR product with pUC19/*Pst* I-*Bam*HI and was used to transform *E. coli* JM105.

When *E. coli* harboring pNJM30 was cultured for 12 hr at 37°C in 2× YT medium in the presence of IPTG, the nitrilase activity in the supernatant of the sonicated cell-free extract of the transformant was 63.4 units/mg, which was 11.6 times that in *A. faecalis* JM3 (with thiophene-2-acetonitrile as the substrate). At this time, as judged by quantitative evaluation of the gel track with a dual-wavelength TLC scanner (Shimadzu, Kyoto), the nitrilase formed corresponded to about 45% of the total soluble protein (Fig. 4). The nitrilase was easily purified to homogeneity on SDS/PAGE (data not shown). The properties such as specific activity and molec-

Hsp1
 CCGGCTTGCTGATGCGCCCCATCCCTGATTCTGTGGGTCGTGGGCATGGGGTGACGGTGAATACAGCTTCGCGCTTAAAGCCAGGAGCCCCAACCCCTCTGT 108
 TCACTGCACGACGCGCCAGGCCAGGATGGGGAGCTCCCGCTCCCTGCTGCGACTCCAGTCAAGCATCTCGCGCTGTCAGTCAATTCATCGCCACCTTCCCTCTCTA 219
 CACTGACGCAAAACCAAGACGACCGGGCTCGGTAAAGCAGCGGGTCTGGCGATCAGGATCGCGGATACCGCGCAAGGAGGATGTCAGACAGAAATAATCGTCCGGGCA 330
MetGlnThrArgLysIleValArgAla
 GCGCCGTACAGCCGCGCTCTCCCACTACGATCTGGCAACGGGTGTGATAAAACATTAGCTGGCTCGTCAGGCCCCGATGAGGGCTGTGACCTGATCGTGTGTTGGT 441
AlaAlaValGlnAlaAlaSerProAsnTyrAspLeuAlaThrGlyValAspLysThrIleGluLeuAlaArgGlnAlaArgAspGluGlyCysAspLeuIleValPheGly
 GAAACCTGGCTGCGCGGCTATCCCTTCCACGTCTGGCTGGGCGCACCGGCTGGTGGCTGAAATACAGTGGCCGCTACTATGCCAATCGCTCTCGCTGGACAGTGCGAG 552
GluThrTrpLeuProGlyTyrProPheHisValTrpLeuGlyAlaProAlaTrpSerLeuLysTyrSerAlaArgTyrTyrAlaAsnSerLeuSerLeuAspSerAlaGlu
 TTTCAACGATTGCCAGGCGCGACGGACTTGGGTATTTTCATCGCACTGGGTATAGCGAGCGCAGCGCGCGCCTTTACCTGGGCAATGCCTGATCGACGACAAG 663
PheGlnArgIleAlaGlnAlaAlaArgThrLeuGlyIlePheIleAlaLeuGlyTyrSerGluArgSerGlyGlySerLeuGlyGlnCysLeuIleAspAspLys
 GGCCAGATGCTGTGGTTCGCGTCGCAAACTCAAACTACATGTTGAGCGCACCGTGTGGTGAAGGTTATGCGCCGAGATCTGATTGTGTCGCGACACCGAGCTGGGCGCG 774
GlyGlnMetLeuTrpSerArgArgLysLeuLysProThrHisValGluArgThrValPheGlyGluGlyTyrAlaArgAspLeuIleValSerAspThrGluLeuGlyArg
 GTCGGTGCCTGTGCTGCTGGGAGCACCTGTCCCTTGGAGCAAGTACGGCTGTACTCCAGCAGCAGGCAATTCACATTGCCCGCTGGCGCTCTTTTCGCTGTACAGC 885
ValGlyAlaLeuCysCysTrpGluHisLeuSerProLeuSerLysTyrAlaLeuTyrSerGlnHisGluAlaIleHisIleAlaAlaTrpProSerPheSerLeuTyrSer
 GAACAGGCCATCGCGCTCAGCGCAAGGTGAACATGGCTGCTCGCAATCTATTTCGGTTGAAGGCCAGTCTTACCATCGCGCCAGCAGTGTCTGTCACCCAGGAGACA 996
GluGlnAlaHisAlaLeuSerAlaLysValAsnMetAlaAlaSerGlnIleTyrSerValGluGlyGlnCysPheThrIleAlaAlaSerSerValValThrGlnGluThr
 CTGGACATGCTGGAAGTAGGTGAACACACGCTCCCTGCTGAAAGTGGGCGCGGAGTTCATGATTTTGGCGCGGACGACGACATGGCTCCCTACCTGCCACAC 1107
LeuAspMetLeuGluValGlyGluHisAsnAlaSerLeuLeuLysValGlyGlyGlySerSerMetIlePheAlaProAspGlyArgThrLeuAlaProTyrLeuProHis
 GATGCCAGGCGCTGATCATTGCCGATCTGAACATGGAAGAAATGCTTCCGCAAGGCGATCAACGCCCTGTGGGCCACTACTCCAAACCCGAGGCCACCGCTGTGTA 1218
AspAlaGluGlyLeuIleIleAlaAspLeuAsnMetGluGluIleAlaPheAlaLysAlaIleAsnAspProValGlyHisTyrSerLysProGluAlaThrArgLeuVal
 CTGGACCTGGGCGACCGTGAGCCCATGACTCGGGTGATTCGAAAGCGTGATCCGGAAGAAGCTCCGAGCGCAGTGCAAAAGTACGGCTGCGCCGCTGCCGTCAGC 1329
LeuAspLeuGlyHisArgGluProMetThrArgValHisSerLysSerValIleGlnGluGluAlaProGluProHisValGlnSerThrAlaAlaProValAlaValSer
 CAGACTCAGGACTCGGATACGCTACTGTTGCAAGAACCGTCTGACCCCAAGATGACAGGCCGCGCAAACTGTCCGGGCTTGTATCTCTGCTGCCCGATCCCC 1440
GlnThrGlnAspSerAspThrLeuLeuValGlnGluProSer***
 CATCCGCTTCGCTCGTGGCGAAATATCTGCTGCCTGTCACTCTTCTGGTCTGGCTCAAACAGATATCTTGCTGATCTAGTCAGTCCAGTTGATCGCAGTCCCTGC 1551
 CGTCAGCCTTTGGAACCAAGACGAGAGGTCAATGAGCAATGGTCCACACGCGAGTTCGCGCTATCCGCGCGCGGCCCTATGGATGGAGGCCGTCAACAGGCCT 1662
 ATGTGCAACTGGAATGCGCGTTCCTCCCGCTCCAGCGCCCTTTCTCGGAGGCCATTACGCGT 1727
EcorV
HluI

FIG. 1. Nucleotide and amino acid sequences of the nitrilase gene. The underlined amino acid sequences were determined by Edman degradation. Potential ribosome-binding sequence is marked as SD, and a relevant stop codon is indicated by asterisks. An inverted repeat sequence downstream of the nitrilase gene is indicated by opposing arrows.

ular mass were almost the same as those of the parental nitrilase.

Expression of Mutant Nitrilase Genes. To investigate the function of consecutive cysteines in the *Alcaligenes* nitrilase, we constructed two mutant enzymes; one is the C162N mutant, in which Cys-162 was replaced with Asn, and the other is the C163A mutant, in which Cys-163 was replaced with Ala. In the former mutation, Cys was altered to Asn, because Asn was situated next to the conserved Cys in the *Klebsiella* nitrilase. In the latter mutation, alanine was chosen to minimize possible conformational change caused by the substitution. The mutated enzymes were successfully overproduced in *E. coli* JM105. SDS/PAGE of cell-free extracts from the transformant cells harboring plasmids pNJM30-C162N or pNJM30-C163A, which were cultured under the conditions used to overproduce the active nitrilase in the transformant carrying pNJM30, gave a major protein band with a mobility identical to that of the nonmutated nitrilase. Both mutant proteins were purified to give a single band on SDS/PAGE (data not shown).

No nitrilase activity was detected in the C163A mutant, even when large amounts of enzyme were used in the reaction for 20 hr. The C162N mutant enzyme catalyzed the hydrolysis of thiophene-2-acetonitrile to thiophene-2-acetic acid at 195 units/mg under the standard reaction conditions. This

value was higher than that of native *A. faecalis* JM3 (144 units/mg) (11) by 35%, clearly demonstrating that the substitution of Asn for Cys-162 brings about an increase in enzyme activity. The K_m value for this mutant enzyme for thiophene-2-acetonitrile was below 10 μ M, and this value was lower than that of the native nitrilase ($K_m = 91 \mu$ M) (11).

The far-UV circular dichroism spectra and molecular masses of the mutant enzymes were virtually identical with those of the parental enzyme (data not shown). These results indicate that the disappearance of enzymatic activities of mutants was not due to marked changes in tertiary structure.

DISCUSSION

Many plants synthesize nitrile compounds. Cyanide is a coproduct of ethylene synthesis (32), after which it is detoxified to β -cyano-L-alanine (33), leading to the formation of asparagine (34). Cyanoglycoside (35) and cyanolipids (36) occur in plants. In the biosynthesis of the cyanoglucoside dhurrin, *p*-hydroxyphenylacetone nitrile is formed from (*Z*)-*p*-hydroxyphenylacetaldoxime (37) in sorghum. The conversion of an aldoxime into a nitrile is also found in banana leaf (7) and in cabbage (38).

The biosynthetic pathways of IAA from L-tryptophan fall roughly into two types in terms of plant intermediates.

JM3	MQTRKIVRAAAVQAASPNYDLATGVDKTIELARQARDEGCDLIVFGETWLPYGFPHVWLGAPAWS-LKYSARYYANSLSLDSAEFQRIAQ	89
Bxn	MDTTFKAAAVQAEPVWMDAAATADKTIVLAKAAAAGALVAFPELVIPGPGFM-LTHNQETELPFIKYRKQAIADGPEIEKIRC	87
JM3	AARTLGFIFIALGYSERSGGSLYLGGCLIDDKGQMLWSRRKLKPTHVERTVFEGYARDLIVSDTELGRVGLCCWEHLSPLSKYALYSQH	179
Bxn	AAQEHNIALSFGYSERAGRTLYMSQMLIDADGTTKIRRRKLKPTFRFERELFEGEDGSDQLQAQTSVGRVGLNCAENQLSKFALAAEG	177
JM3	EAIHIAAWPFSFSLYSEQAHALSAKNMAASQIYSVEGQCFTIAASSVVQTETLDMLEVGE-HNASLLKVGGSMSIFAPDGRTLAPYLP	268
Bxn	EQIHISAWP-FTLGGSPVLVGDSIGAINQV--YAAETGTPTVLMSTQVVGPTGIAAFEIEDRYNPNQY-LGGGYARIYGPDMQLKSKSLSP	262
JM3	DAEGLIADLNMEIEIAFAKAINDPVGHYSKPEATRLVLDLGHREPMTRVHKSQVIEEAPHPVQSTAAPVAVSQTQDSDTLLVQEPS	356
Bxn	TEEGIVYAEIDLMSLEAAKYSLDPTGHYSRPDVF-SVSINRQRPVSEVIDSNGDEDPRACEPDEGDRVVISTAIGVLPRYCCHS	349

FIG. 2. Comparison of the deduced amino acid sequences of the nitrilases from *A. faecalis* JM3 and *K. ozaenae*. The two sequences were aligned by introducing gaps (hyphens) to maximize identities. Identical residues are denoted by asterisks between the sequences.

FIG. 3. Sequences of the oligonucleotide primers for expression of the nitrilase gene. Primer 1 was used as a sense primer; primer 2 was used as an antisense primer. The sequences are shown in the 5'-to-3' direction. SD, ribosome-binding site.

Although several investigators have studied the route via indole-3-acetaldehyde as an intermediate, none have reported the structure of the gene encoding the enzyme synthesizing IAA. On the other hand, the route via indole-3-acetaldoxime and indole-3-acetonitrile is present in Brassicaceae (6). In cabbage, the *in vitro* conversion of L-tryptophan to indole-3-acetaldoxime by microsomal membranes (39) and the presence of indole-3-acetonitrile have been reported (40). Thimann and Mahadevan demonstrated in 1964 the existence of nitrilase activity in intact leaf tissue from several *Brassica oleracea* varieties (7). Rausch and Hilgenberg described the partial purification of nitrilase from Chinese cabbage (41).

Indole-3-acetonitrile and phenylacetoneitrile (benzylcyanide) are suitable substrates for the *Alcaligenes* nitrilase (11). The K_m values for indole-3-acetonitrile and phenylacetoneitrile are 0.009 mM and 0.010 mM, respectively, and they are less than those for other nitriles. This nitrilase acts upon acetonitrile derivatives at an aromatic ring with an indolyl, phenyl, or thiophenyl group but not upon benzonitrile derivatives and aliphatic nitriles except for acetonitrile and acrylonitrile. Phenylacetic acid is present in *Phaseolus mungo* (42) and it may be a commonly occurring natural auxin (43), as is IAA. These observations suggest that auxin precursors such as indole-3-acetonitrile and phenylacetoneitrile are physiological substrates for *Alcaligenes* nitrilase.

Furthermore, the specific activities of all nitrilases from *Nocardia* sp. strains NCIB 11215 (14) and NCIB 11216 (15),

FIG. 4. SDS/PAGE of the supernatant prepared from *E. coli* containing pNJM30. Lanes 5 and 10 were loaded with the following molecular mass standards: phosphorylase (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20 kDa), and α -lactalbumin (14 kDa). Lanes 1-4, 6-9, and 11, supernatants of sonicates (70 μ g of protein). Lane 1, *E. coli* containing pNJM30, sample taken after a 6-hr incubation at 28°C with IPTG; lane 2, as lane 1, but after a 12-hr incubation; lane 3, as lane 1, but after a 7-hr incubation, during which IPTG was added 4 hr from the start; lane 4, as lane 3, but after a 12-hr incubation; lane 6, as lane 1, but after a 6-hr incubation at 37°C; lane 7, as lane 6, but after a 12-hr incubation; lane 8, as lane 6, but after a 7-hr incubation, during which IPTG was added 4 hr from the start; lane 9, as lane 8, but after a 12-hr incubation; lane 11, *E. coli* JM105 containing the vector plasmid pUC19, as a control.

Fusarium (16), *Arthrobacter* (17), *R. rhodochrous* J1 (19), *Klebsiella* (18), *R. rhodochrous* K22 (20), and *A. faecalis* JM3 (11) were, in units/mg, 1.74, 15.7, 1.66, 1.31, 15.9 (benzonitrile as the substrate), 25.7 (bromoxynil as the substrate), 0.737 (crotononitrile as the substrate), and 144 (thiophene-2-acetonitrile as the substrate), respectively. The specific activity of *Alcaligenes* nitrilase for indole-3-acetonitrile as the substrate is 14.8 units/mg. Partially purified nitrilase from barley has a specific activity of 2.69×10^{-3} unit/mg for indole-3-acetonitrile (7). In contrast, the specific activity of nitrile hydratase, which catalyzes the hydration of a nitrile to an amide, is very high. The values of nitrile hydratases from *Pseudomonas chlororaphis* B23 (44), *Brevibacterium* R312 (45), and *Rhodococcus* sp. N-774 (46) are, in units/mg, 1840, 1890, and 1260 (propionitrile as a substrate), respectively. Although nitrilase and nitrile hydratase are both nitrile-degrading enzymes, there is a marked difference between them in specific activity.

IAA is thought to have auxin activity at concentrations below 10^{-7} M in plants. Even though the specific activity of nitrilase is generally low, it is enough to form IAA exhibiting hormonal action in plant cells. Moreover, nitrilases from *A. faecalis* JM3, *R. rhodochrous* K22, and *R. rhodochrous* J1 were easily decomposed by self-digestion in solution even at 10°C (unpublished results). Our recent studies indicated that partially purified nitrilase from *Brassica campestris* var. *campestris* cv. *tsukena* is very unstable (unpublished results). These features of nitrilases may be responsible for a controlling IAA levels to prevent the presence of remaining nitrilase activity in plants.

Mahadevan and Thimann proposed the postulated reaction mechanism in which a nitrile carbon atom is subjected to a nucleophilic attack by a sulfhydryl group in the active site of the enzyme (8). We have used site-directed mutagenesis to examine the potential roles of the single conserved Cys-163 and the neighboring Cys-162 in the *Alcaligenes* nitrilase in the catalytic mechanism. The resulting loss of activity when Cys-163 is replaced with Ala suggests that Cys-163 plays important roles in this nitrilase reaction or in maintenance of enzyme structure, and that Cys-162 cannot replace the function of Cys-163 participating in catalysis. The striking increase in activity and decrease in K_m value by the replacement of Cys-162 with Asn remain unexplained, but this finding also supports that Cys-162 is not the amino acid in the active site of the enzyme.

While this manuscript was being written, cDNA cloning of the nitrilase from *Arabidopsis thaliana*, which converts indole-3-acetonitrile into IAA, was reported (47). This plant nitrilase was significantly similar to the *Klebsiella* and *Alcaligenes* nitrilases in amino acid sequences. As expected for a catalytically or structurally important amino acid, Cys-163 in the *A. faecalis* nitrilase is conserved in the *Arabidopsis* nitrilase. Therefore, these sequences are clustered into a superfamily (48).

Microbial production of IAA seems to be essential for the virulence of bacteria in their host plants (49). In *Agrobacterium tumefaciens* (50) and *Pseudomonas savastanoi* (51, 52), L-tryptophan is converted into indole-3-acetamide by tryptophan-2-monooxygenase and this is converted to IAA by indole-3-acetamide hydrolase. In contrast, in virulent fungus *Taphrina* species causing hyperplastic diseases in plants such as cherry, peach, and plum, IAA is synthesized not only from L-tryptophan via indolepyruvate and indoleacetaldehyde as intermediates but also from indole-3-acetonitrile by the nitrilase (21). If the nitrilases in microorganisms were understood in detail at the gene and protein levels, then understanding the nature of the evolutionary relationship of microorganisms to plants, and IAA biosynthesis in plants, would be a step closer.

Recently, it has been proposed that the nontryptophan pathway is the primary route of IAA biosynthesis in maize, on the basis of experiments using a maize tryptophan auxotroph, orange pericarp (*orp*) (53). Although the presence of indole-3-acetonitrile in plants has not been fully investigated, the nitrilase route in IAA biosynthesis cannot be ruled out. The cloning of the nitrilase mRNA provides the potential to manipulate endogenous IAA production and consequently growth promotion, with correlation to other plant hormones such as cytokinins, gibberellins, etc. It may be possible to control IAA levels in plants by reducing expression of the plant nitrilase gene with antisense RNA technology (54) or by introducing the *A. faecalis* JM3 nitrilase gene into plants with a particle gun (55). These approaches may make a substantial contribution toward agricultural industry. It should also be possible to clone the nitrilase gene from plants in addition to *Arabidopsis*.

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