THE JOURNAL OF BIOLOGICAL CHEMISTRY
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Vol. 263, No. 13, Issue of May 5, pp. 6310-6314, 1988
Printed in U.S.A.

Purification and Properties of a Nitrilase Specific for the Herbicide Bromoxynil and Corresponding Nucleotide Sequence Analysis of the *bxn* Gene*

(Received for publication, September 8, 1987)

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A *Klebsiella ozaenae* nitrilase which converts the herbicide bromoxynil (3,5-dibromo-4-hydroxybenzonitrile) to 3,5-dibromo-4-hydroxybenzoic acid has been expressed at 5–10% of the total protein in *Escherichia coli* from a cloned *K. ozaenae* DNA segment and purified 10.3-fold to homogeneity. The purified polypeptide is molecular weight 37,000 in size, but the active form of the enzyme is composed of two identical subunits. The purified enzyme exhibits a pH optimum of 9.2 and a temperature optimum of 35 °C. The purified enzyme is also quite sensitive to thiol-specific reagents. The nitrilase is highly specific for bromoxynil as substrate with a K_m of 0.31 mM and V_{max} of 15 μmol of NH_3 released/min/mg protein. Analysis of bromoxynil-related substrates indicates the enzyme exhibits preference for compounds containing two meta-positioned halogen atoms. Nucleotide sequence analysis of a 1,212-base pair *Pst*I-*Hin*cII DNA segment containing the locus (*bxn*) encoding the bromoxynil-specific nitrilase reveals a single open reading frame encoding a polypeptide 349 amino acids in length. The predicted sequence of the purified enzyme was derived from the nucleotide sequence of the *bxn* gene.

Recently, much attention has focused on the molecular mechanisms involved in the microbial degradation of herbicides and pesticides. For many xenobiotic compounds, metabolic pathways have been well characterized and, in some cases, genes encoding degrading activities have been cloned. However, little is known at the enzyme level about the polypeptides catalyzing these metabolic reactions.

We have focused our interest on the biodegradative process for the halogenated aromatic nitrile bromoxynil (3,5-dibromo-4-hydroxybenzonitrile), a widely used herbicide selective against broad-leaf weeds. Both microbial populations and tolerant plants can transform the cyano moiety of the herbicides bromoxynil (1, 2) and 2,6-dichlorobenzonitrile (3, 4). These compounds can be hydrolyzed to the corresponding amide and carboxylic acid products. These hydrolysis reactions are catalyzed by a class of enzymes termed nitrilases. A nitrilase activity has been partially purified from barley and shown to convert a variety of aromatic nitriles to their corresponding acid derivatives in the absence of any detectable amide intermediate (5). Nitrilases utilizing benzonitrile as a preferred substrate have also been purified from two *Nocardia* (rhodochrus group) species (6, 7), *Fusarium solani* (8) and an *Arthrobacter* sp. (9). These enzymes exhibit a broad substrate specificity for many aromatic nitriles, converting them to their corresponding acids. However, the benzonitrile-specific nitrilases obtained from both *Nocardia* sp. and *F. solani* were shown to be inefficient utilizing bromoxynil as substrate. Recently, a naturally occurring soil bacterium, *Klebsiella ozaenae*, has been shown to rapidly metabolize bromoxynil and express a bromoxynil-specific nitrilase activity (10). The gene locus (*bxn*) encoding the enzyme has been shown to be plasmid-encoded in *K. ozaenae* and was cloned in *Escherichia coli* on a 2.6-kilobase *K. ozaenae* DNA fragment (11).

In this communication, we present purification to homogeneity of the bromoxynil-specific nitrilase and properties of the purified enzyme. The amino acid composition of the purified protein is determined and compared with the nucleotide sequence analysis of the corresponding *bxn* gene locus.

EXPERIMENTAL PROCEDURES

Materials—All chemicals used were of reagent grade quality. DEAE-Sephadex A-50, Sephadex G-200, and chromatofocusing resins and reagents were purchased from Pharmacia (Uppsala, Sweden). Isopropylthio-β-D-galactoside and 5-bromo-4-chloro-3-indolylgalactoside were obtained from Bachem (Torrance, CA). Boehringer Mannheim was the source for dideoxy and deoxyribonucleotide triphosphates, while [α-^{32}P]dCTP (3000 Ci/mmol) and [γ-^{32}P]ATP (3000 Ci/mmol) were obtained from the Radiochemical Centre (Amersham, UK). The purified sodium salt of bromoxynil (3,5-dibromo-4-hydroxybenzonitrile), 5-bromo-4-hydroxybenzonitrile, and 3,5-dibromo-4-hydroxybenzamide were gifts from Rhône-Poulenc Agrochimie (Lyon, France). Benzonitrile, 4-hydroxybenzonitrile, and chloroxynil (3,5-dichloro-4-hydroxybenzonitrile) were from Aldrich. Ioxynil (3,5-diiodo-4-hydroxybenzonitrile) was purchased from Chem Services (West Chester, PA). Restriction endonucleases were purchased from Bethesda Research Laboratories and used according to the manufacturer's specifications. New England Biolabs was the source of *Bal*31 nuclease and *Bam*HI linkers, while Promega Biotec (Madison, WI) provided T_4 DNA ligase. Avian myeloblastosis virus reverse transcriptase was obtained from Life Sciences (Gainesville, FL).

Bacterial Strains, Plasmids, and DNA Manipulations—*E. coli* strains utilized were MM294 (*thi, endI, gyr*96, *hsd*R17, SupE44) and 71-18 (a *lac/pro, thi,* Su2, F′*lac* ΔM15 *lac* Iq). Plasmid pBrx9 has been described previously (11). Construction of plasmid pBrx16 is described in the text. DNAs were purified from sodium dodecyl sulfate/alkaline (12) lysates by two centrifugations in CsCl/ethidium bromide gradients. Plasmid DNAs were resuspended in TE buffer (0.02 M Tris-HCl, pH 8.0, 0.1 mM EDTA) prior to restriction analysis. Ligation reactions and transformation of *E. coli* strains with plasmid DNA were as before (11).

Purification of Nitrilase and Assay for Enzyme Activity—Nitrilase was prepared from 21 g of stationary phase *E. coli* cells containing the pBrx16 plasmid. Cells were pelleted at 8,000 × *g* for 15 min at 4 °C, washed in 0.1 M potassium phosphate buffer, pH 7.4, repelleted,

* This work was funded by Rhône-Poulenc Agrochimie (Lyon, France). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) J03196.

and frozen at −20 °C. The pellet was thawed at 4 °C and resuspended in 40 ml of KDE buffer (0.05 M potassium phosphate (pH 7.4), 1 mM dithiothreitol, 0.1 mM EDTA). The cell suspension was passed through a French pressure cell and centrifuged at 60,000 × *g* for 50 min at 4 °C. The resulting clear supernatant (crude extract) was diluted with KDE buffer to a protein concentration of 12 mg/ml (Fraction I). The crude extract was brought to 25% saturation with solid ammonium sulfate and the precipitate collected by centrifugation at 12,000 × *g* for 15 min at 4 °C. The supernatant was collected and brought to 35% saturation with solid ammonium sulfate. The precipitate was collected as above, resuspended in 10 ml of KDE buffer, and dialyzed extensively against the same buffer (Fraction II). All chromatographic operations were carried out at 4 °C. Fraction II was applied to a DEAE-Sephadex A-50 column (4.9 cm^2 × 40 cm) equilibrated in KDE buffer. The column was washed with 2 volumes KDE buffer and developed with a linear gradient of 0.1–0.4 M NaCl in KDE buffer. A single peak of nitrilase activity eluted at 0.27 M NaCl. The active fractions were pooled (Fraction III), precipitated with ammonium sulfate, resuspended in 10 ml, and dialyzed into 25 mM histidine, pH 6.2. A Pharmacia PBE94 chromatofocusing column (1.75 cm^2 × 20 cm) was prepared and equilibrated with 25 mM histidine, pH 6.2. The sample was applied and washed with 10 volumes of polybuffer 74, pH 4.0, to create a 6 to 4 pH gradient. The enzyme then was eluted with 1 M NaCl. The salt peak containing the active fractions was ammonium sulfate-precipitated, resuspended in KDE buffer, and dialyzed against KDE (Fraction IV).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of purified fractions was determined as described previously (13). Protein was determined by the method of Bradford (14) using bovine serum albumin as a standard. Nitrilase activity was assayed by measuring the production of ammonia (8) during the hydrolysis of bromoxynil. The standard assay was performed in duplicate at 35 °C in tubes containing, unless otherwise indicated, 1.0 ml of 3 mM bromoxynil in 0.1 M sodium carbonate buffer, pH 9.2. Aliquots of enzyme fraction were added and the mixtures incubated for periods of 5–20 min depending on the experiment. The reaction was terminated by the addition of 0.33 M sodium phenoxide (1 ml) followed by 0.01% sodium ferricyanide (1 ml) and 0.02 M sodium hypochlorite (1 ml). The assay mixture was vortexed, heated for 5 min in a boiling H_2O bath to allow color development, diluted with 6 ml of H_2O and the absorbance measured at 640 nm. Ammonium chloride was used to construct a standard curve. The limit of ammonia detection by this method was 5 nmol/ml. Specific activity measurements were expressed as micromoles of NH_3 released per min per mg protein.

DNA Sequence Determinations—DNA segments from plasmid pBrx9 were cloned into the modified M13 phages M13mp18 and M13mp19 (15). After selection on 5-bromo-4-chloro-3-indolylgalactoside, putative clones were size-screened by electrophoresis of the single-strand templates on 0.7% agarose gels. Dideoxy sequence reactions utilizing reverse transcriptase were carried out as described previously (16). For chemical sequencing,, chemical degradations were carried out accordingly (17), except methylene blue was used as reagent for guanine modifications (18). Products of the polymerization and degradation reactions were fractionated on polyacrylamide/urea sequencing gels. Gels were dried and subjected to autoradiography.

Composition Analysis of Purified Nitrilase—A 10-μg sample of Fraction IV enzyme was reduced, carboxymethylated, dialyzed against 6% acetic acid, and lyophilized. The sample was resuspended in either 100 μl of 5.7 N HCl or 10 μl of 4 N methanesulfonic acid (for determination of tryptophan residues) and hydrolyzed *in vacu* for 24 h at 110 °C. The lyophilized hydrolysate was resuspended in pH 2.2 citrate buffer containing internal standards, and amino acid composition was determined through sample loading to a Durrum D-500 amino acid analyzer.

Gel Filtration Analysis—A 4.9 cm^2 × 60-cm column of Sephadex G-200 was prepared and equilibrated in KDES buffer (0.05 M KPO_4, 1 mM dithiothreitol, 0.1 mM EDTA, and 0.17 M NaCl). The column was standardized with the molecular weight standards alcohol dehydrogenase (114,000), bovine serum albumin (68,000), ovalbumin (43,000), β-lactoglobin (34,000), and myoglobin (17,000). All chromatographic runs were carried out in KDES buffer, and 4-ml fractions were collected. 75-μl samples from each fraction were assayed for nitrilase activity. A protein elution profile was determined by monitoring the absorbance of each fraction at 280 nm.

RESULTS

Purification of the Bromoxynil-specific Nitrilase—The enzyme was purified, as described under "Experimental Procedures," from *E. coli* strain MM294 containing plasmid pBrx16. Plasmid pBrx16 is identical to the previously described plasmid pBrx9 (11), except that 39 base pairs of the untranslated region upstream from the putative AUG start codon have been deleted by *Bal*31 nuclease digestion. Plasmid pBrx9 consists of a pUC18 vector containing a cloned 2.6-kilobase segment of *K. ozaenae* DNA (11). High level expression of the *bxn* gene is obtained via transcription from the *lac* promoter located in the pUC18 vector plasmid (11). A sodium dodecyl sulfate-polyacrylamide gel containing protein samples from each purification step is shown in Fig. 1. The purified polypeptide has a molecular weight of approximately 37,000. Scanning of the final lane of the gel revealed that the Fraction IV enzyme preparation is greater than 99% homogeneous (data not shown). The faint band at approximately molecular weight 72,000 is the undenatured dimer form of the enzyme. Fig. 1 also shows that in crude extract preparations, expression of the bromoxynil-specific nitrilase accounts for between 5 and 10% of the total soluble protein in *E. coli*. Fraction IV nitrilase preparation was 10.3-fold purified from crude extracts and yielded a specific activity of 25 μmol of NH_3 released per min/mg protein (Table I). Approximately 80 mg of homogeneous enzyme can be obtained from 21 g of cells (1,520 mg of protein) accounting for an overall yield of 53% based on the starting material. Although the Fraction IV enzyme preparation exhibits a slightly lower specific activity

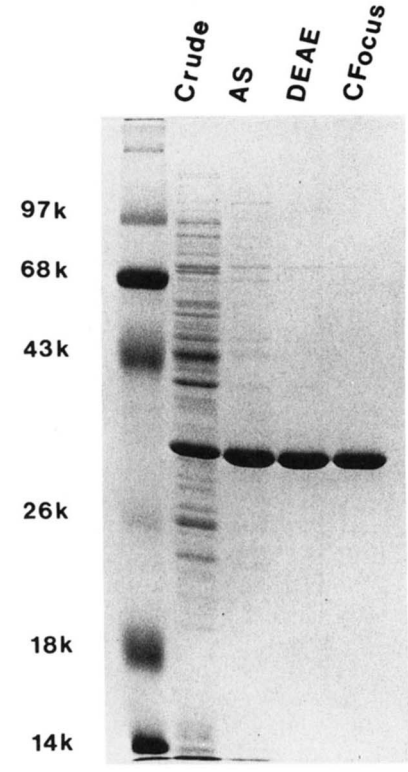

FIG. 1. **Purification of the bromoxynil-specific nitrilase from *E. coli*.** Protein fractions were added to an equal volume of 2 × sample buffer (13) and electrophoresed on a 11.25% denaturing polyacrylamide gel (13). Approximately 20 μg of crude extract (Fraction I), 16 μg of ammonium sulfate (*AS*) fraction (Fraction II), and 8–10 μg of the DEAE (Fraction III) and chromatofocusing (*CFocus*) fractions (Fraction IV) were applied to the gel. The molecular weight standards include phosphorylase B (*97k*), bovine serum albumin (*68k*), ovalbumin (*43k*), α-chymotrypsinogen (*26k*), β-lactoglobin (*18k*), and lysozyme (*14k*), respectively.

TABLE I

Purification of the bromoxynil-specific nitrilase

	Volume	Protein	Total units[b]	Yield	Specific activity	
	ml	*mg*		*%*		*-fold*
Fraction I						
Crude extract	130	1560	3900		2.50	
Fraction II						
Ammonium sulfate	15.5	302	3333	85	11.03	4.4
Fraction III						
DEAE-Sephadex	84	89	2306	59	25.90	10.4
Fraction IV						
Chromatofocusing	11.5	80.5	2069	53	25.70	10.3

[a] Starting material 21 g of cells.
[b] unit = μmol of NH_3 released per min.

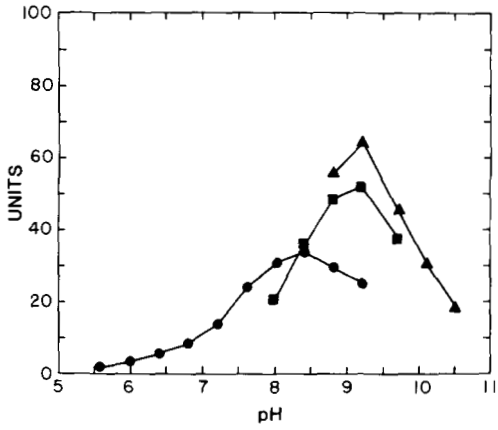

FIG. 2. **pH optimum for the bromoxynil-specific nitrilase.** Assays were performed in duplicate as described under "Experimental Procedures." Approximately 1.2 μg of Fraction IV protein was used in each assay, and the reaction time was 10 min. Substrate concentration was 3 mM bromoxynil. Buffer concentration in each assay was 0.1 M. Buffers used were KPO_4 (●), sodium borate (■), and sodium carbonate (▲).

than Fraction III, the final chromatofocusing step was utilized to remove slight contaminants observed in the Fraction III preparation. These values are consistent with the observation that the bromoxynil-specific nitrilase expressed from plasmid pBrx16 in *E. coli* constitutes between 5 and 10% of the total soluble protein in crude extracts.

pH and Temperature Optima for the Bromoxynil-specific Nitrilase—The purified enzyme exhibited activity in a fairly narrow pH range (Fig. 2). Optimal enzyme activity was obtained in sodium carbonate buffer at pH 9.2. Approximately 15% of optimal activity was demonstrated at pH 7.0 with a rapid rise to pH 9.2 and a steep decline approaching pH 10.6. The pH optimum remained relatively constant (optimum of pH 9.2) when different substrate concentrations were assayed. This narrow pH curve is in contrast to broad pH ranges exhibited by the benzonitrile-specific nitrilases purified from *Nocardia* sp. (6, 7) and *F. solani* (8). The bromoxynil-specific nitrilase was active over a broad range of temperature with optimal activity expressed at 35 °C (data not shown). Approximately 15% of optimal activity was obtained at 10 and 55 °C, respectively.

Effect of Metal Ions and Inhibitors on Nitrilase Activity—The action of possible inhibitors and metal ions on enzyme activity is shown in Table II. The enzyme is very susceptible to inhibition by thiol-specific reagents such as *p*-hydroxymercuribenzoate and phenylmercuriacetate, demonstrating that thiol groups are probably important for active site function, a conclusion supported by the sensitivity of the enzyme to

TABLE II

Effect of inhibitors and metal ions on the bromoxynil-specific nitrilase

Assays were performed in duplicate as described under "Experimental Procedures." 1.2 μg of Fraction IV enzyme was utilized per assay. Substrate concentration was 3 mM bromoxynil, and the reaction time was 10 min. Inhibitors were added to the reaction mixture prior to addition of the enzyme. The following metal ions at 100 μM showed no inhibition of enzyme activity: Ca^{2+}, Co^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2}.

Inhibitor/metal ion	Final concentration	% Control activity
p-Hydroxymercuribenzoate	0.5 μM	2.5
Phenylmercuriacetate	0.5 μM	4.3
Iodoacetate	10 μM	5
$AgNO_3$	10 μM	93.8
$AgNO_3$	100 μM	2.5
$CuSO_4$	10 μM	75
$CuSO_4$	100 μM	31.3
KCl	50 mM	107
NaCl	500 mM	114

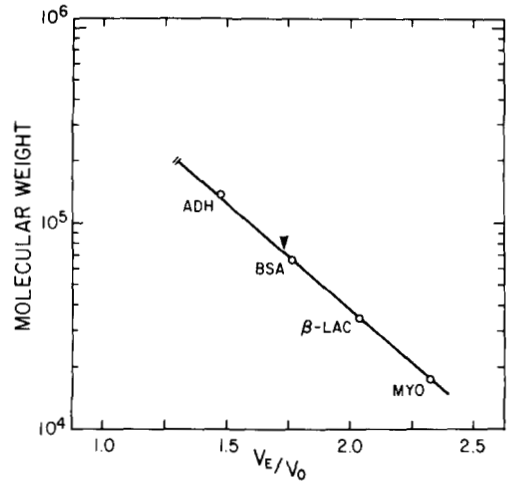

FIG. 3. **Size determination of the active form of the bromoxynil-specific nitrilase.** A Sephadex G-200 column was prepared, run, and assayed as described under "Experimental Procedures." Reaction time was 20 min. Approximately 3 mg of Fraction IV enzyme was applied to the column, and 85% of the initial activity was recovered. *ADH*, *BSA*, *β-LAC*, and *MYO* refer to the molecular weight protein standards alcohol dehydrogenase, bovine serum albumin, β-lactoglobin, and myoglobin, respectively. The *arrowhead* points to the V_E/V_O for the bromoxynil-specific nitrilase.

Ag^+ ions. The nitrilase was unaffected by a number of divalent cations that were inhibitory to the benzonitrile-specific nitrilase isolated from *Nocardia* sp. 11216 (6). Metal-chelating agents such as EDTA, azide, and cyanide did not influence activity, demonstrating that the enzyme did not possess a metal ion requirement. Monovalent cations had no effect on nitrilase activity even at 500 mM. The Cu^{2+} sensitivity of the enzyme is similar to that exhibited by the benzonitrile-specific nitrilases isolated from *Nocardia* sp. 11216 (6) and *Arthrobacter* (9).

Determination of Molecular Weight—The molecular size of the active enzyme was determined by gel filtration of Fraction IV enzyme on a Sephadex G-200 column. As shown in Fig. 3, the active enzyme eluted as a single peak with a relative elution volume of 1.74 corresponding to a molecular weight of 72,000. This lower molecular weight is closer to the benzonitrile-specific nitrilases isolated from *Arthrobacter* (9), in contrast to the high molecular weight aggregates formed by the enzymes found in *Fusarium* and *Nocardia* sp. (6–8). Protein analysis of fractions eluted from the G-200 column detected no protein at the monomer position (37,000) indicating that the active form of the enzyme is composed of two identical

subunits and that, in a native environment, all enzyme molecules exist in the dimer form.

Enzyme Kinetics and Substrate Specificity—Experiments on the effect of varying enzyme concentration on the initial velocity of the nitrilase reaction demonstrated that the rate of ammonia release was linear with respect to time and was directly proportional to enzyme concentration between 0.05 and 3 μg protein/ml. The initial velocity of nitrile hydrolysis was measured on various substrates and the results described in Table III. The enzyme exhibited simple single order saturation kinetics on all substrates tested and K_m and V_{max} values were derived from Lineweaver-Burke plots. Bromoxynil is the preferred substrate with a K_m of 0.31 and a V_{max} of 15 μmol of NH_3 released per min/mg. The bromoxynil-specific nitrilase exhibits a marked requirement for halogens at the meta-positions of the substrate molecule. The V_{max} for a singly substituted meta-positioned bromine substrate was 10-fold less than the value obtained for bromoxynil, and almost no detectable activity can be obtained utilizing *p*-hydroxybenzonitrile as substrate. The enzyme exhibits no detectable activity when benzonitrile is utilized as the substrate. These data also suggest that the enzyme exhibits a degree of halogen specificity. Although chlorine and iodine atoms can be substituted efficiently for bromine at the meta-positions, K_m and V_{max} measurements are lower when 3,5-dichloro-4-hydroxybenzonitrile and 3,5-diiodo-4-hydroxybenzonitrile are utilized as substrates. Interestingly, the enzyme exhibits a slightly increased V_{max}, but a 3-fold higher K_m compared with bromoxynil when 3,5-dichloro-4-hydroxybenzonitrile is utilized as substrate. The corresponding benzoic acid derivatives and ammonia are the sole detectable compounds produced on hydrolysis of these compounds by the bromoxynil-specific nitrilase. Formation of the corresponding bromoxynil amide derivative was not detected by high pressure liquid chromatographic analysis (10) nor did 3,5-dibromo-4-hydroxybenzamide act as a substrate for the enzyme.

Sequence Analysis of the bxn Gene Locus—Previously described plasmid pBrx9 (11) was used as a source for the determination of the nucleotide sequence of the *bxn* gene locus. The boundaries of the functional *bxn* gene are defined by *Pst*I and *Hinc*II restriction sites at the 5′ and 3′ ends, respectively (11). Unique *Xmn*I, *Cla*I, *Sal*I, *Sca*I, and *Sph*I sites were located within the *bxn* gene as defined by insertional inactivation of gene function or subcloning specific DNA fragments and assaying whole cells for enzyme activity (11). These restriction sites, along with the *Pst*I and *Hinc*II sites defined cloned fragments from which sequencing reactions were derived. Alternatively, seven *Hae*III fragments spanning the *bxn* gene were cloned into M13 vectors and sequenced. This strategy facilitated determination of each nucleotide for both strands of the entire 1212-base pair sequence displayed in Fig. 4. The sequence reveals a putative coding region 349 amino acids in length beginning with an AUG codon at nucleotide position 66 and ending with a UAA termination codon at nucleotide position 1,115. This sequence encodes a putative polypeptide of molecular weight 38,100 which is in close agreement with the molecular weight 37,000 polypeptide observed in denaturing polyacrylamide gels of the purified

TABLE III

Velocity analysis of various substrates

Assays were performed in duplicate at pH 9.2 as described under "Experimental Procedures." Substrate concentrations utilized were between 0.05 and 3 mM. Reactions contained 1 μg of Fraction IV enzyme and were for 5 min.

Substrate	K_m	V_{max}[a]
	mM	
3,5-Dibromo-4-hydroxybenzonitrile (bromoxynil)	0.31	15.0
3,5-Dichloro-4-hydroxybenzonitrile (chloroxynil)	0.83	18.0
3,5-Diiodo-4-hydroxybenzonitrile (ioxynil)	0.55	12.2
5-Bromo-4-hydroxybenzonitrile	0.91	1.5
4-Hydroxybenzonitrile	ND[b]	0.23

[a] Expressed as μmol of NH_3 released per min/mg.

[b] Initial velocity estimates could not be determined (ND) due to insensitivity of the assay.

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                                                                        65
                CTGCAGGATAGTAGGGGCTTGAAGAGATACGCTGTTTGTCGAGCCATCAAAATAAGGGGATTTTC

                                        95                             125
ATG GAC ACC ACT TTC AAA GCA GCC GCT GTT CAG GCC GAA CCG GTA TGG ATG GAT GCC GCT
Met Asp Thr Thr Phe Lys Ala Ala Ala Val Gln Ala Glu Pro Val Trp Met Asp Ala Ala
                                                                        20

                                       155                             185
GCA ACA GCC GAT AAG ACC GTG ACG CTA GTA GCT AAA GCC GCA GCG GCT GGC GCG CAG CTC
Ala Thr Ala Asp Lys Thr Val Thr Leu Val Ala Lys Ala Ala Ala Ala Gly Ala Gln Leu
                                                                        40

                                       215                             245
GTC GCA TTT CCC GAA TTG TGG ATT CCG GGC TAC CCA GGA TTC ATG CTC ACG CAC AAC CAA
Val Ala Phe Pro Glu Leu Trp Ile Pro Gly Tyr Pro Gly Phe Met Leu Thr His Asn Gln
                                                                        60

                                       275                             305
ACC GAA ACC CTA CCA TTC ATC ATT AAA TAC CGC AAG CAG GCA ATC GCC GCC GAT GGA CCA
Thr Glu Thr Leu Pro Phe Ile Ile Lys Tyr Arg Lys Gln Ala Ile Ala Ala Asp Gly Pro
                                                                        80

                                       335                             365
GAA ATC GAA AAA ATT CGC TGC GCG GCT CAG GAG CAT AAC ATT GCG CTC TCC TTT GGG TAC
Glu Ile Glu Lys Ile Arg Cys Ala Ala Gln Glu His Asn Ile Ala Leu Ser Phe Gly Tyr
                                                                       100

                                       395                             425
AGC GAA CGG GCT GGC CGT ACG CTC TAC ATG TCA CAA ATG CTT ATC GAT GCC GAT GGC ATC
Ser Glu Arg Ala Gly Arg Thr Leu Tyr Met Ser Gln Met Leu Ile Asp Ala Asp Gly Ile
                                                                       120

                                       455                             485
ACC AAA ATT CGT CGT CGA AAG CTC AAA CCA ACC CGC TTT GAA CGA GAA CTC TTT GGC GAA
Thr Lys Ile Arg Arg Arg Lys Leu Lys Pro Thr Arg Phe Glu Arg Glu Leu Phe Gly Glu
                                                                       140

                                       515                             545
GGT GAC GGA TCG GAC TTA CAG GTC GCC CAA ACT AGC GTT GGT CGG GTG GGT GCC CTC AAC
Gly Asp Gly Ser Asp Leu Gln Val Ala Gln Thr Ser Val Gly Arg Val Gly Ala Leu Asn
                                                                       160

                                       575                             605
TGC GCG GAG AAT TTG CAG TCG CTA AAC AAG TTT GCG CTT GCT GCC GAG GGT GAA CAG ATA
Cys Ala Glu Asn Leu Gln Ser Leu Asn Lys Phe Ala Leu Ala Ala Glu Gly Glu Gln Ile
                                                                       180

                                       635                             665
CAT ATC TCC GCC TGG CCA TTC ACG CTT GGA AGC CCT GTG CTC GTC GGA GAC TCC ATC GGC
His Ile Ser Ala Trp Pro Phe Thr Leu Gly Ser Pro Val Leu Val Gly Asp Ser Ile Gly
                                                                       200

                                       695                             725
GCC ATC AAC CAG GTC TAC GCG GCC GAG ACG GGG ACC TTC GTT CTC ATG TCG ACG CAG GTG
Ala Ile Asn Gln Val Tyr Ala Ala Glu Thr Gly Thr Phe Val Leu Met Ser Thr Gln Val
                                                                       220

                                       755                             785
GTT GGA CCG ACC GGC ATC GCC GCC TTC GAG ATC GAA GAC AGG TAC AAC CCG AAT CAG TAT
Val Gly Pro Thr Gly Ile Ala Ala Phe Glu Ile Glu Asp Arg Tyr Asn Pro Asn Gln Tyr
                                                                       240

                                       815                             845
CTT GGT GGT GGG TAC GCG CGG ATC TAC GGG CCT GAC ATG CAG TTG AAG AGC AAG TCG TTG
Leu Gly Gly Gly Tyr Ala Arg Ile Tyr Gly Pro Asp Met Gln Leu Lys Ser Lys Ser Leu
                                                                       260

                                       875                             905
TCA CCG ACC GAA GAG GGC ATC GTC TAC GCC GAG ATC GAC CTG TCG ATG CTT GAG GCA GCA
Ser Pro Thr Glu Glu Gly Ile Val Tyr Ala Glu Ile Asp Leu Ser Met Leu Glu Ala Ala
                                                                       280

                                       935                             965
AAG TAC TCG CTC GAT CCC ACG GGC CAC TAT TCG CGC CCT GAT GTG TTC AGC GTG TCG ATT
Lys Tyr Ser Leu Asp Pro Thr Gly His Tyr Ser Arg Pro Asp Val Phe Ser Val Ser Ile
                                                                       300

                                       995                            1025
AAC CGG CAA CGG CAG CCT GCG GTG TCA GAA GTT ATC GAC TCA AAC GGT GAC GAG GAC CCG
Asn Arg Gln Arg Gln Pro Ala Val Ser Glu Val Ile Asp Ser Asn Gly Asp Glu Asp Pro
                                                                       320

                                      1055                            1085
AGA GCA GCA TGC GAG CCC GAC GAG GGG GAT CGT GAG GTC GTA ATC TCT ACG GCA ATA GGG
Arg Ala Ala Cys Glu Pro Asp Glu Gly Asp Arg Glu Val Val Ile Ser Thr Ala Ile Gly
                                                                       340

                                      1115                            1155
GTT CTA CCC CGT TAT TGC GGA CAT TCC TAATAAAAAGAGACACGTTGTACCAAAGGGGTGTTCATGTCCA
Val Leu Pro Arg Tyr Cys Gly His Ser

                                             1200
GACGCAGAAAATATAGCCCAGAGTTAAAACGCGAAGCCATCGCTTTAACCCGTCAAC
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FIG. 4. **Antisense strand of the 1212-base pair nucleotide sequence of the *bxn* gene.** Codon specified amino acids are displayed *below* the sequence, and base pairs and amino acids are numbered accordingly.

TABLE IV
Amino acid composition of the K. ozaenae bromoxynil-specific nitrilase

Amino acid	Residues in polypeptide	
	Observed	Predicted from nucleotide sequence
	mol/ml	
Ala	45.5 ± 1.0	43
Arg	17.3 ± 2.3	18
Asx	30.5 ± 1.0	30
Cys	2.5 ± 1.3	4
Gly	34.5 ± 0.8	29
Glx	43.5 ± 1.8	42
His	5.5 ± 1.3	5
Ile	20.2 ± 1.0	23
Leu	25.8 ± 1.3	25
Lys	13.5 ± 0.8	13
Met	7.0 ± 2.1	8
Phe	12.5 ± 0.8	12
Pro	18.0 ± 1.4	19
Ser	21.2 ± 1.4	22
Thr	19.7 ± 0.5	19
Trp	2.5 ± 1.5	3
Tyr	11.8 ± 0.4	13
Val	19.2 ± 1.3	21

nitrilase fraction. The calculated pI derived from the amino acid composition is 5.4. A putative ribosome binding site (19) is also observed at nucleotide positions 54–60. The amino acid composition of the polypeptide revealed by the nucleotide sequence is similar to the composition observed when the purified nitrilase fraction is subjected to amino acid analysis (Table IV). The nitrilase polypeptide contains 44 negatively charged residues (Asp and Glu) and 31 positively charged residues (Lys and Arg), indicating an overall net negative charge for the protein. This fact is supported by the observation that the enzyme binds tightly to DEAE-Sephadex and is still retained on a chromatofocusing column at pH 4.0. The enzyme also contains a high proportion of aliphatic amino acids (38%) and contains 11% aromatic residues.

DISCUSSION

An enzyme, nitrilase, that converts the herbicide bromoxynil (3,5-dibromo-4-hydroxybenzonitrile) to its corresponding acid form (3,5-dibromo-4-hydroxybenzoic acid) has been expressed at high level in *E. coli* from a cloned *K. ozaenae* DNA segment, purified to homogeneity, and characterized. The bromoxynil-specific nitrilase exhibits properties similar to benzonitrile-specific nitrilases purified from *Fusarium solani* (8), *Nocardia* sp. (6, 7), and *Arthrobacter* (9) in terms of inhibition by sulfhydryl blocking agents and metal ions, temperature, and pH optima. The active form of the bromoxynil-specific enzyme is a homo-dimer composed of two identical molecular weight 38,100 subunits. In this respect, the enzyme is more closely related to the size of the smaller *Arthrobacter* benzonitrile-specific nitrilases (9) as opposed to the active high molecular weight aggregates formed by the enzymes isolated from *Fusarium* (8) and *Nocardia* (6, 7). The benzonitrile-specific nitrilases identified from the above three organisms exhibit activity on a wide variety of substituted benzyl, toluidyl, and pyridinyl nitrile-containing compounds. These enzymes, like the bromoxynil-specific nitrilase, also cannot utilize their corresponding amide derivatives as substrates, even though an amide form is a probable reaction intermediate. The possibility exists that this class of nitrilases, to which the bromoxynil-specific nitrilase belongs, requires a cyano moiety (as opposed to an amide) for recognition and catalysis and does not release the substrate from a single catalytic site during conversion to the corresponding acid form. The above enzymes, however, are very inefficient when bromoxynil is utilized as a substrate. Although a detailed substrate analysis has not been undertaken, the *K. ozaenae* bromoxynil-specific nitrilase exhibits a preference for two meta-positioned halogens with bromine atoms resulting in the highest degree of activity. It will be interesting to determine whether the *K. ozaenae* bromoxynil-specific nitrilase exhibits any degree of homology at the gene or protein level to the benzonitrile-specific enzymes identified from *Fusarium*, *Nocardia*, and *Arthrobacter* when DNA and/or protein sequences for these enzymes become available. The polypeptide deduced from the nucleotide sequence exhibits a calculated pI of 5.4. This is in contrast to the final purification step using a chromatofocusing column. The active form of the enzyme binds to the column at pH 4.0 and must be eluted with high salt. This observation indicates that the actual pI for the bromoxynil-specific nitrilase must be lower than 4.0. This could be due to the existence of the active enzyme form as a homo-dimer and/or the availability of the majority of negatively charged amino acid residues on the active enzyme surface.

The primary amino acid sequence of the bromoxynil-specific nitrilase coupled with the ease in preparing large quantities of homogeneous enzyme open the way for a detailed analysis of the catalytic site as it relates to the three-dimensional enzyme structure. The DNA sequence of the *bxn* gene and purification and characterization of the bromoxynil-specific nitrilase have also been undertaken in preparation for the introduction and expression of the enzyme in bromoxynil sensitive plants to confer resistance. Recent experiments[1] indicate that expression of this bacterial nitrilase in transgenic plants results in high levels of resistance to the herbicide bromoxynil.

Acknowledgments—We wish to thank Jim Kenny for amino acid composition analysis of the purified enzyme and Bill Hiatt and Georges Freyssinet for critically evaluating the manuscript.

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