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Purification and Characterization of a Novel Methyltransferase Responsible for Biosynthesis of Halomethanes and Methanethiol in *Brassica oleracea**

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A novel *S*-adenosyl-L-methionine:halide/bisulfide methyltransferase (EC 2.1.1.–) was purified approximately 1000-fold to apparent homogeneity from leaves of *Brassica oleracea*. The enzyme catalyzed the *S*-adenosyl-L-methionine-dependent methylation of the halides iodide, bromide, and chloride to monohalomethanes and of bisulfide to methanethiol. The dual function of the enzyme was demonstrated through co-purification of the halide- and bisulfide-methylating activities in the same ratio and by studies of competition between the alternative substrates iodide and bisulfide. The purification procedure included gel filtration, anion exchange chromatography, and affinity chromatography on adenosine-agarose. Elution of the protein from a chromatofocusing column indicated a pI value of 4.8. The pH optimum of halide methylation (5.5–7.0) was different from that of bisulfide methylation (7.0–8.0). The molecular mass values for the native and denatured protein were 29.5 and 28 kDa, respectively, suggesting that the active enzyme is a monomer. The enzyme had the highest specificity constant for iodide and the next highest for bisulfide. Substrate interaction kinetics and product inhibition patterns were consistent with an Ordered Bi Bi mechanism.

Interest in trace gas emissions has increased over the last two decades following the realization of their impact on atmospheric chemistry (1). Halogen- and sulfur-containing organic gases have attracted particular attention because of their respective effects on the integrity of stratospheric ozone (2) and the formation of acid rain (3).

Biological activity in the oceans is commonly viewed as a major source of organohalogen (4, 5) and organosulfur emissions (6, 7). Terrestrial microorganisms, fungi, and a few higher plants also emit these gases (8, 9). The biochemical bases for these emissions, however, remain poorly understood. Possible mechanisms involve spontaneous (10) or enzymatic (11, 12) reactions. A haloperoxidase-mediated incorporation of halides into a carbon skeleton was suggested as the main route for halomethane biosynthesis (11) but has never been actually shown to produce monohalomethanes. Subsequently, an enzymic mechanism involving a single-step conversion of halides into a monohalocarbon was proposed (13); this was supported by evidence for incorporation of the methionine methyl group into a monohalomethane (14). Recently, it was reported (15) that a methyl chloride transferase from the marine red alga *Endocladia muricata* could carry out the transfer of a methyl group from *S*-adenosyl-L-methionine (AdoMet)[1] to a halide (X^-) as follows.

$$X^- + \text{AdoMet} \longrightarrow CH_3X + \text{AdoHcy}$$

REACTION 1

Preliminary evidence was also presented for a similar activity in a wood-rotting fungus, *Phellinus pomaceus*, and a halophytic higher plant, *Mesembryanthemum crystallinum*.

Methanethiol was originally thought to come from methionine via a methioninase (L-methionine methanethiol-lyase) reaction (12). However, the observation that several bacterial isolates from soil and agricultural crops as well as cultures of marine algae evolved CH_3SH in the presence of bisulfide (HS^-) suggested that this gas may also be produced via a different mechanism (16). Thiol methyltransferases, capable of catalyzing the following reaction, were later found in a variety of organisms including bacteria (16), algae (17), and mammals (18, 19).

$$HS^- + \text{AdoMet} \longrightarrow CH_3SH + \text{AdoHcy}$$

REACTION 2

A survey of higher plants done in our laboratory (20) showed that many diverse species produce CH_3I when supplied with I^-, and CH_3SH when supplied with HS^-, in a manner similar to Reactions 1 and 2. The levels of the two activities were highly correlated among species. Plants from the family Brassicaceae exhibited some of the highest rates of CH_3I and CH_3SH production. To elucidate the biochemical basis for these environmentally important gas emissions, we developed an easy and reliable assay for their quantitation and proceeded to purify and characterize the halide- and bisulfide-methylating activities.

EXPERIMENTAL PROCEDURES

Plant Material

Plants of *Brassica oleracea* cv. April Red were grown in a greenhouse under a photoperiod of at least 12 h and were watered and fertilized as necessary. The two most expanded basal leaves of 3-month-old plants were used for enzyme extraction.

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[1] The abbreviations used are: AdoMet, *S*-adenosyl-L-methionine; X^-, halide ion; AdoHcy, *S*-adenosyl-L-homocysteine; TEMED, *N,N,N′,N′*-tetramethylethylenediamine; HPLC, high performance liquid chromatography; PAGE, polyacrylamide gel electrophoresis; BisTris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)-propane-1,3-diol.

Chemicals

S-Adenosyl-L-methionine was from Boehringer Mannheim. The inorganic phosphorus test kit, *S*-adenosyl-L-homocysteine, 5'-ADP-agarose, KI, KBr, and KCl were purchased from Sigma. Ammonium sulfide and authentic CH_3SH, CH_3Br, and CH_3I were obtained from Aldrich. Authentic CH_3Cl was purchased from Liquid Carbonic (Scarborough, Ontario, Canada). Sephadex G-100, Sephadex G-25, gel filtration calibration kit, and Polybuffer 74 were from Pharmacia Biotech Inc. Calf intestinal alkaline phosphatase was purchased from New England Biolabs (Beverly, MA). The protein dye reagent, TEMED, ammonium persulfate, and bisacrylamide were all from Bio-Rad (Mississauga, Ontario, Canada). All other chemicals were of reagent grade.

Buffers

The buffers used were as follows: 100 mM Tris acetate, pH 7.5, containing 10% glycerol (v/v) and 14 mM 2-mercaptoethanol (buffer A); 25 mM Tris acetate, pH 7.4, containing 10% glycerol and 14 mM 2-mercaptoethanol (buffer B); 25 mM Tris acetate, pH 7.4, containing 14 mM 2-mercaptoethanol (buffer C); buffer C with 100 mM NaCl (buffer D); 25 mM BisTris/iminodiacetic acid, pH 7.0 (buffer E); and Polybuffer 74/iminodiacetic acid (1:10, v/v), pH 4.0 (buffer F). All buffers were filtered before use through a 0.22-μm membrane.

Measurement of Halide / Bisulfide Methyltransferase Activity

Enzyme Assay—Since I^- was the preferred substrate among halides, it was used as the halide substrate throughout the purification procedure. The enzyme activity was assayed in a 1-ml mixture containing 0.5 mM AdoMet, 50 mM KI (halide methylation), or 20 mM $(NH_4)_2S$ (bisulfide methylation) all prepared in buffer A. Enzyme preparations containing up to 200 μg of protein were used to start the reaction. The mixture was incubated in a 5-ml glass vial sealed with a screw cap fitted with a Teflon-lined septum (Supelco, Oakville, Ontario, Canada) and maintained on an orbital shaker (150 rpm) at room temperature. The reaction rate was linear for at least 45 min at all enzyme and substrate concentrations. A standard incubation time of 30 min was adopted.

Gas Chromatography—The products formed were analyzed by gas chromatography using a flame ionization detector. One-ml headspace samples were injected in a 210 × 0.3-cm stainless steel column packed with 80/100-mesh Porapak Q (Supelco) in a Hewlett-Packard 5890 series II gas chromatograph. Column temperatures were 160 °C for CH_3I and CH_3Br, 145 °C for CH_3SH, and 130 °C for CH_3Cl. The carrier gas (helium, ultrapure) flow rate was 40 ml/min. The column was purged by heating to 200 °C between injections. Products were quantified by peak area and identified by comparison of their retention times with those of authentic methyl halides or CH_3SH, which were used to calibrate the instrument. Recoveries of 75 nl of CH_3I and 28 nl of CH_3SH injected to vials containing the assay mixture were 69 and 87%, respectively, after 30 min. The data presented here were not corrected for recoveries. The identity of CH_3I and CH_3SH in the headspace of enzyme assays was confirmed by mass spectrometry using a KRATOS MS 50 mass spectrometer operated in the electron impact mode.

Protein Estimation

Total soluble proteins were determined by the method of Bradford (21) using the Bio-Rad protein reagent, following the microassay procedure, and using bovine serum albumin as the standard.

Preparation of Adenosine-Agarose Affinity Gel

5'-ADP-agarose (4 ml) was washed under vacuum according to the manufacturer's instructions and incubated with 800 units of calf intestinal alkaline phosphatase and 5 ml of calf intestinal alkaline phosphatase buffer (1 ×), pH 7.5, in a total volume of 9 ml. The gel was dephosphorylated overnight at 37 °C in a continuously rotating reaction vial, transferred to a column, washed with 10 ml of buffer C containing 2 M NaCl, and washed again with 20 ml of buffer C alone. The washing was done in fractions of ~10 ml, which were subsequently assayed for phosphate content. The dephosphorylation reaction was repeated until no more phosphate was detected. The gel was washed with an excess of deionized water before each reaction. Free phosphate was determined colorimetrically with the method of Fiske and Subbarow (22).

Purification of Halide / Bisulfide Methyltransferase

Extraction—All manipulations were carried out at 4 °C unless stated otherwise. Halide- and HS^--methylating activities were monitored simultaneously throughout the purification procedure. Generally, 1 kg of freshly harvested leaves were cut into small squares, immediately frozen with liquid nitrogen, and then homogenized with buffer A (1:3, w/v) and polyvinylpolypyrrolidone (10%, w/w) for 5 min in a blender at full speed. The homogenate was filtered through eight layers of cheesecloth and centrifuged for 20 min at 15,000 × *g*. The supernatant (crude extract) was recovered, and enzyme activity and protein concentration were assayed in an aliquot desalted by passage through a PD-10 column (Pharmacia). The crude extract was fractionated with solid $(NH_4)_2SO_4$, and the proteins that precipitated between 60 and 85% saturation, containing halide and bisulfide methyltransferase activities, were recovered by centrifugation at 12,000 × *g* for 20 min.

Gel Filtration on Sephadex G-100—The pellet from the 60–85% $(NH_4)_2SO_4$ fraction was resuspended in 15 ml of buffer B and applied to a Sephadex G-100 column (2.6 × 90 cm) that had been previously equilibrated in the same buffer. The column was eluted with buffer B at 25 ml/h, and 2-ml fractions were collected. The active fractions from this step were pooled and concentrated by diafiltration in a stirred Amicon cell fitted with a YM-30 membrane (Amicon, Danvers, MA).

Affinity Chromatography on Adenosine-Agarose—The concentrated sample was applied to an adenosine-agarose column (1.5 × 3 cm) that had been previously equilibrated with buffer B. The column was washed at a constant flow rate of 10 ml/h with 15 ml of buffer B, followed by 8 ml of the same buffer containing 300 mM $(NH_4)_2SO_4$. The salt was then removed by washing the column with buffer B. The halide- and bisulfide-methylating activities were recovered by elution with a 40-ml linear gradient of 0–4 mM AdoMet in buffer B. One-ml fractions were collected and assayed for activity and protein content. The halide and bisulfide methyltransferase activities eluted between 2 and 3 mM AdoMet. The remaining bound proteins were washed off the column with 2 M NaCl in buffer B; no methyltransferase activity was detected in this fraction.

Anion Exchange on Protein Pak Q—Active eluate from the affinity step was submitted to high performance liquid chromatography (HPLC) (Millipore, Milford, MA) on a 1 × 10-cm Protein Pak Q anion exchange column (Millipore) equilibrated with buffer C. The sample was loaded at a flow rate of 0.5 ml/min, and the column was washed with the same buffer until no further UV-absorbing material was eluted. Enzyme activity was then eluted with NaCl in buffer C in a two-step linear gradient of 0–200 mM in 80 min and 200–500 mM in 20 min, collecting 0.5-ml fractions.

Gel Filtration on Superdex 75—The fraction containing the first peak of halide and bisulfide methyltransferase activity from the previous step was loaded on an HPLC Superdex 75 gel filtration column (1 × 30 cm) (Pharmacia) previously equilibrated with buffer D. Proteins were eluted in the same buffer at a flow rate of 0.5 ml/min, collecting 0.5-ml fractions. At this level of purification the halide and bisulfide methyltransferase preparation was apparently free from contaminating proteins.

Chromatofocusing

An enzyme preparation from the Sephadex G-100 gel filtration step was subjected to HPLC chromatofocusing on a Mono P HR (5/20) column (Pharmacia) previously equilibrated with buffer E. Unbound proteins were removed by washing the column with the same buffer. Enzyme activity was then eluted with a pH gradient of 7.0–4.0 generated with 45 ml of buffer F at a flow rate of 1 ml/min. One-ml fractions were collected in 0.2 ml of 500 mM Tris acetate buffer, pH 7.7, containing 50% glycerol.

Molecular Weight Determination

Molecular weight of the native halide/bisulfide methyltransferase was determined by gel filtration on a Sephadex G-100 column (2.5 × 44 cm) calibrated with the following markers: ribonuclease A (M_r 13,700), chymotrypsinogen A (M_r 25,000), ovalbumin (M_r 43,000), and albumin (M_r 67,000). The column void volume was determined with blue dextran 2000. Buffer B was used for column equilibration and elution. One-ml samples were applied to the column, which was operated at a flow rate of 20 ml/h. In order to achieve better resolution, column calibration was done in two steps, the first involving ribonuclease A and ovalbumin and the second involving albumin and chymotrypsinogen A.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

Proteins from different purification steps were separated by SDS-PAGE under denaturing conditions (23) using 12% acrylamide gels. Gels were stained with silver nitrate or Coomassie Blue. The molecular mass of the denatured enzyme was determined from a plot of log molecular mass against the migration distance of standard proteins.

TABLE I
Purification of halide/bisulfide methyltransferase

Step	Total activity[a]		Total protein	Specific activity		CH_3I/CH_3SH Ratio	Purification		Recovery	
	CH_3I	CH_3SH		CH_3I	CH_3SH		CH_3I	CH_3SH	CH_3I	CH_3SH
	microkatals		*mg*	*microkatals/mg*			*-fold*		%	
Crude extract	39.2	28.6	4276.0	0.009	0.007	1.37	1	1	100	100
85% $(NH_4)_2SO_4$	31.2	26.4	860.5	0.036	0.031	1.18	4.0	4.43	79.7	92.3
G-100	16.6	11.8	300.7	0.055	0.039	1.41	6.11	5.57	42.5	41.1
Adeno-agarose	8.3	7.1	0.722	11.5	9.834	1.17	1278	1405	21.3	24.7
PP-Q peak 1	0.61	0.47	0.041	14.88	11.46	1.29	1653	1637	1.55	1.65
Superdex 75	0.12	0.105	0.0135	9.231	8.077	1.14	1025	1154	0.3	0.36

[a] One katal of halide/bisulfide methyltransferase is defined as the amount of enzyme that catalyzes the conversion of 1 mol of substrate/s under the assay conditions.

Determination of Kinetic Properties

The kinetic properties of the halide/bisulfide methyltransferase were determined using a preparation purified through a Sephadex G-100 gel filtration step.

Alternative substrate competition experiments were performed by varying the concentration of one substrate at each of a series of concentrations of the other. The concentration of AdoMet was kept constant at 0.5 mM. Data were presented as double-reciprocal plots of initial velocity (**v**) *versus* varying substrate (S) concentrations.

Substrate interaction studies were done by fixing the concentration of one substrate while changing that of the other. Linear regressions were fitted to the data in double-reciprocal plots. Replots of the data were used to determine the kinetic parameters.

RESULTS

B. oleracea leaf extracts catalyzed the AdoMet-dependent methyl transfer to X^- or HS^-. The enzyme responsible for both of the reactions was purified to homogeneity, and its dual function was demonstrated.

Purification of Halide/Bisulfide Methyltransferase—The enzyme was extracted from *B. oleracea* by subjecting leaf tissue to a freeze-thaw cycle prior to homogenization and was purified using $(NH_4)_2SO_4$ precipitation followed by gel filtration chromatography on Sephadex G-100, affinity chromatography on adenosine-agarose, and HPLC on Protein Pak Q anion exchange and Superdex 75 gel filtration columns. The specific activity of the enzyme was enriched more than 1000-fold over the crude preparation with a recovery of 0.35%. Table I summarizes the purification procedure, and Fig. 1 shows a typical pattern of proteins from various purification steps separated on SDS-PAGE and visualized by Coomassie Blue.

Affinity chromatography on adenosine-agarose was efficient in removing most of the contaminating proteins (Fig. 2). Further purification was achieved by anion exchange HPLC, which resolved four peaks of halide/bisulfide methyltransferase activity (Fig. 3). The first and largest peak of activity eluting from this column contained only two proteins as seen on the SDS gel (Fig. 1). Further purification of this peak by HPLC on a Superdex 75 gel filtration column resolved two protein peaks, only one of which contained halide/bisulfide-methylating activity. This peak appeared free of contaminants when visualized on SDS-PAGE (Fig. 1). The Superdex-purified halide/bisulfide methyltransferase migrated as a single band on SDS-PAGE with an apparent molecular mass of 28,000 daltons. Since this is close to the value of 29,500 determined for the native protein on a G-100 gel filtration column, the active enzyme probably exists as a monomer.

Demonstration of Dual Activity—The halide and bisulfide methyltransferase activities co-purified to homogeneity in the same ratio (1.26 ± 0.14) during a five-step procedure (Table I, Figs. 2 and 3). Moreover, double-reciprocal plots of the data from competition kinetics between the halide and bisulfide substrates gave regression lines that intersected at the $1/v$ axis (Fig. 4, *A* and *B*). These results established that the halide and bisulfide methylations are catalyzed by the same protein, halide/bisulfide methyltransferase.

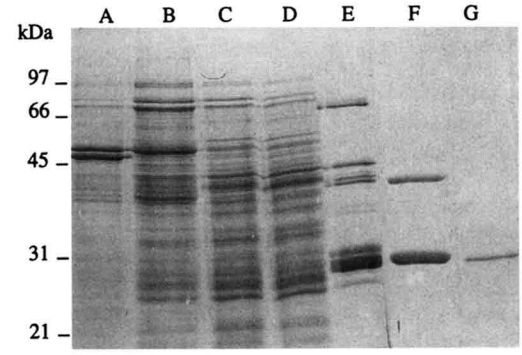


FIG. 1. **SDS-PAGE of fractions with halide/bisulfide methyltransferase activity from the successive steps of purification.** *Lane A*, crude extract (~10 μg); *lane B*, 60–85% $(NH_4)_2SO_4$ (~5 μg); *lane C*, Sephadex G-100 (~5 μg); *lane D*, Amicon YM-30 (~5 μg); *lane E*, adenosine-agarose (~5 μg); *lane F*, Protein Pak Q (~5 μg); *lane G*, Superdex 75 (~1 μg). The molecular mass markers are indicated to the *left* in kDa. The gel was stained with high sensitivity Coomassie Brilliant Blue G-250.

Effect of pH on Halide/Bisulfide Methyltransferase—The halide/bisulfide methyltransferase activity was assayed over a wide range of pH, using buffers with overlapping ranges. The pH optimum for I^- methylation was fairly broad, between 5.5 and 7.0 (Fig. 5*A*), whereas that for HS^- methylation was sharper and more alkaline, between 7.0 and 8.0 (Fig. 5*B*). In addition, halide/bisulfide methyltransferase activity eluted at pH 4.8 upon chromatofocusing on a Mono P column, suggesting that the enzyme has a pI value of 4.8 ± 0.2.

Enzyme Stability—After Sephadex G-100 gel filtration, the enzyme was stable for over 2 months at −80 °C in buffer B. After affinity chromatography, the halide/bisulfide methyltransferase became extremely labile, losing all activity after overnight storage at −80 °C. Addition of 20% glycerol to the preparation and storage at −20 °C conserved 12% of the activity after 48 h. In contrast, after the Protein Pak Q anion exchange step, the enzyme retained more than 70% of its activity after 24 h and 55% after 48 h at 4 °C in buffer C containing 175 mM NaCl.

Kinetic Analysis

Substrate Interaction Kinetics—Double-reciprocal plots with I^- as the variable substrate at several fixed concentrations of AdoMet gave converging lines (Fig. 6*A*). The same pattern was observed when HS^- was the variable substrate at different fixed concentrations of AdoMet (Fig. 6*B*).

Product Inhibition Kinetics—The order of substrate binding and product release was determined from product inhibition studies. *S*-Adenosyl-L-homocysteine was a competitive inhibitor with respect to AdoMet (Fig. 7*A*) and noncompetitive with respect to iodide (Fig. 7*B*) or bisulfide (Fig. 7*C*). The kinetics of product inhibition for the gaseous CH_3I or CH_3SH could not be

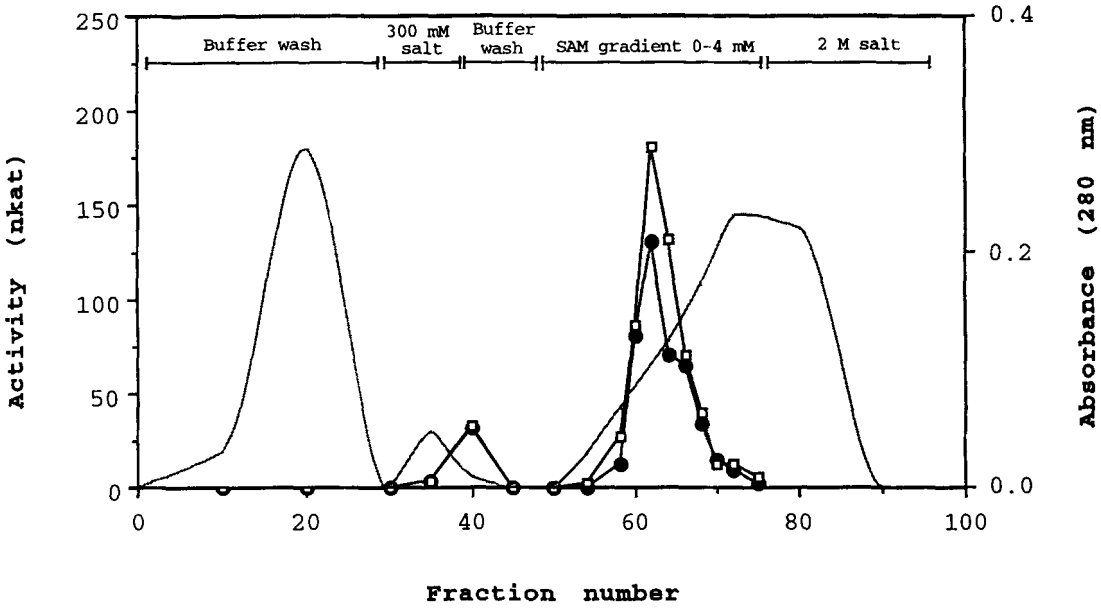


FIG. 2. **Elution of halide/bisulfide methyltransferase from an adenosine-agarose affinity column.** Specifically bound proteins were eluted in a 0–4 mM AdoMet (*SAM*) gradient in buffer B. Fractions of the eluate were assayed for halide (□) and bisulfide (●) methyltransferase activities. Both activities overlapped up to fraction 55. Proteins were continuously monitored by absorbance at 280 nm (*solid line*). Most of the increase in the absorbance with the gradient elution was due to the presence of AdoMet. *nkat*, nanokatals.

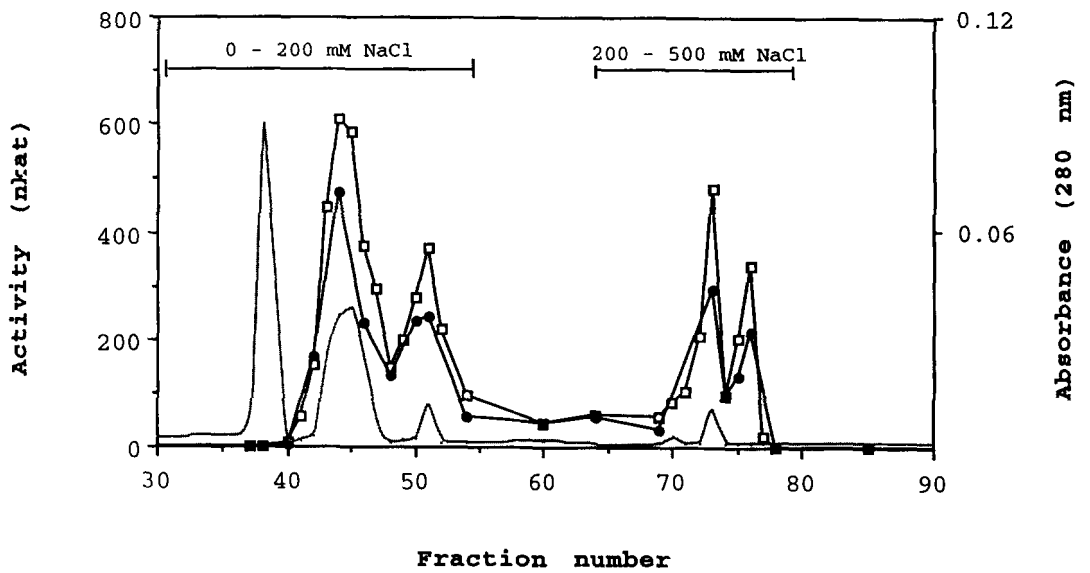


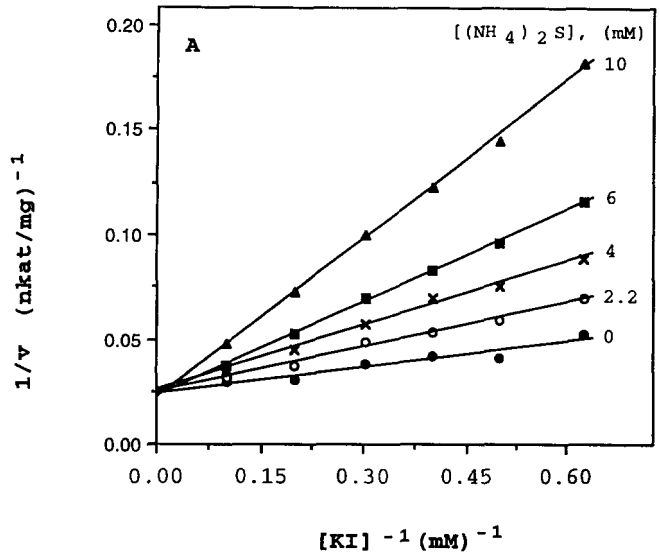
FIG. 3. **Elution of halide/bisulfide methyltransferase from a Protein Pak Q column upon anion exchange high performance liquid chromatography.** Bound proteins were eluted in a 0–200 mM and 200–500 mM two-step linear gradient of NaCl in buffer C. Halide- (□) and bisulfide-methylating (●) activities were assayed in each fraction. Proteins were monitored by absorbance at 280 nm (*solid line*). *nkat*, nanokatals.

determined under the present assay conditions.

As calculated from replots of the data, the V_{max} for the formation of CH_3I was 1140 nanokatals/mg of protein; that for CH_3SH was 804 nanokatals/mg of protein. The respective K_m values for I^- and HS^- were 1.3 and 4.7 mM. There were two different K_m values for AdoMet depending on the second substrate used, 0.03 mM with I^- and 0.226 mM with HS^-. Table II summarizes the kinetic parameters of the halide/bisulfide methyltransferase for its different substrates.

DISCUSSION

The halide and bisulfide methyltransferase activities from *B. oleracea* were purified to apparent homogeneity. The two activities co-purified at a constant ratio (1.26 ± 0.14) throughout the procedure (Table I, Figs. 2 and 3). The kinetics of mutual inhibition between the substrates I^- and HS^- were characteristic of competitive inhibition (Fig. 4, *A* and *B*). Taken together, these results establish that both halide- and bisulfide-methylating activities reside on the same active site of a single protein, the halide/bisulfide methyltransferase. The homogeneous protein was obtained through a five-step procedure that resulted in an overall purification of approximately 1000-fold with a recovery of 0.35% (Table I). Affinity chromatography on an adenosine-agarose column was the key step, in which the enzyme specifically bound to the matrix while most of the contaminating proteins were washed through during sample loading (Fig. 2). The enzyme was selectively eluted with the co-substrate AdoMet in a linear gradient resulting in a 1300-fold increase in the specific



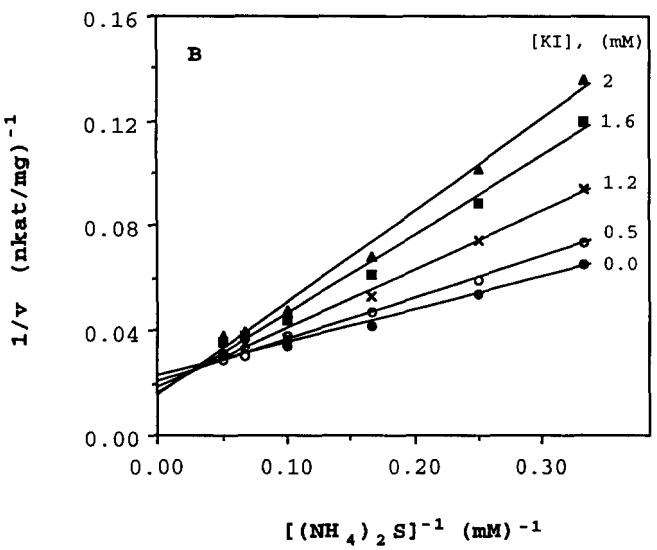
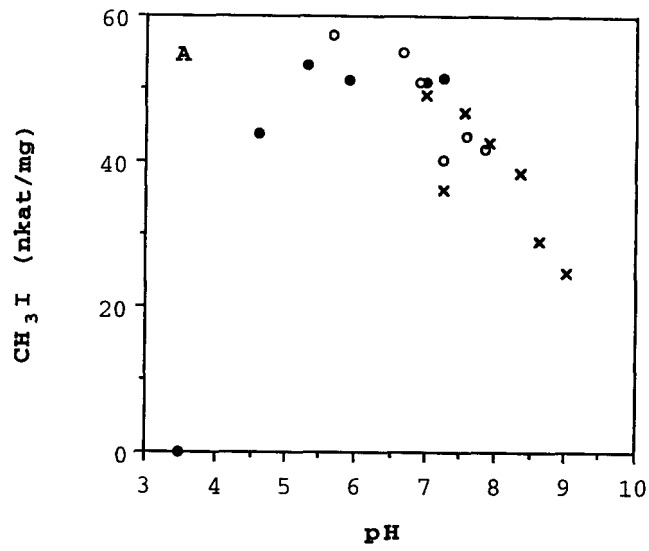


FIG. 4. **Competition between the alternative substrates of the halide/bisulfide methyltransferase.** *A*, inhibition of iodide methylation by different fixed concentrations of bisulfide. *B*, inhibition of bisulfide methylation by different fixed concentrations of iodide. The concentration of AdoMet was kept constant at 0.5 mM. *nkat*, nanokatals.



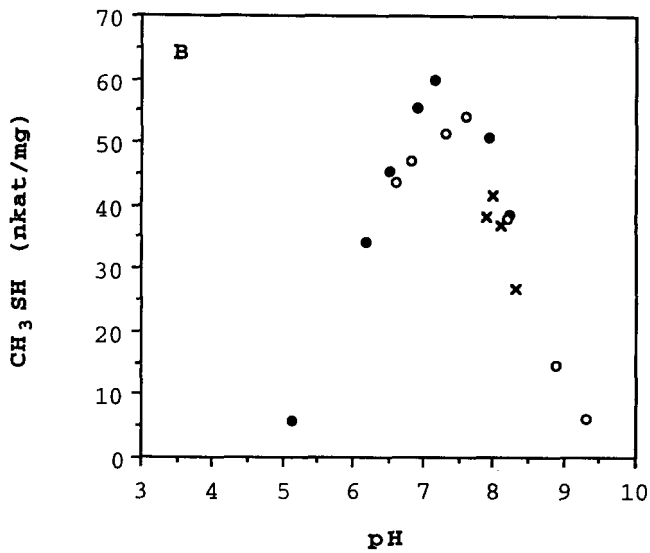


FIG. 5. **Effect of pH on halide/bisulfide methyltransferase activity.** The pH dependence of halide- (*A*) and bisulfide-methylating (*B*) activities was investigated using 100 mM of the following buffers: phosphate-citrate (●), phosphate (○), and Tris acetate (X). *nkat*, nanokatals.

activity over the crude preparation. Adenosine, linked via its adenine-C_8 to agarose (ag-adenosine, Pharmacia), has been successfully used to purify other plant methyltransferases (24, 25). Our adenosine-agarose matrix was highly stable and was repeatedly used without any loss of its binding or eluting efficiencies. Other affinity matrices prepared with either AdoMet or AdoHcy linked via their free amino or carboxyl groups to Affi-Prep 10 (Bio-Rad) or EAH-Sepharose (Pharmacia) failed to bind the enzyme.

High performance liquid chromatography on Protein Pak Q anion exchange column resolved multiple peaks of halide/bisulfide methyltransferase activity (Fig. 3). An enzyme preparation processed through a procedure in which salt precipitation was replaced with polyethylene glycol precipitation also gave multiple peaks in this anion exchange step (not shown). These observations suggest that the enzyme may exist in multiple charge isoforms, although this possibility remains to be confirmed through further experiments. Interestingly, rat liver thiol methyltransferase, with which the halide/bisulfide methyltransferase shares many properties, was reported in five different cellular compartments (26). The harsh freeze-thaw treatment needed to maximize the extraction of halide/bisulfide methyltransferase was also similar to the procedure used to extract the compartmented rat liver enzyme (18).

The protein from a fraction containing peak halide/bisulfide methyltransferase activity from the Superdex 75 step migrated as a single band of 28,000 Da on SDS-PAGE (Fig. 1). Moreover, when consecutive fractions across the first peak of activity after anion exchange HPLC were applied to SDS-PAGE, the Coomassie Blue staining intensity of the 28,000-Da protein band peaked in the fraction containing peak enzyme activity (not shown). These observations confirmed that the molecular mass of the denatured halide/bisulfide methyltransferase was 28,000 Da. Since the molecular weight of the enzyme, calculated from

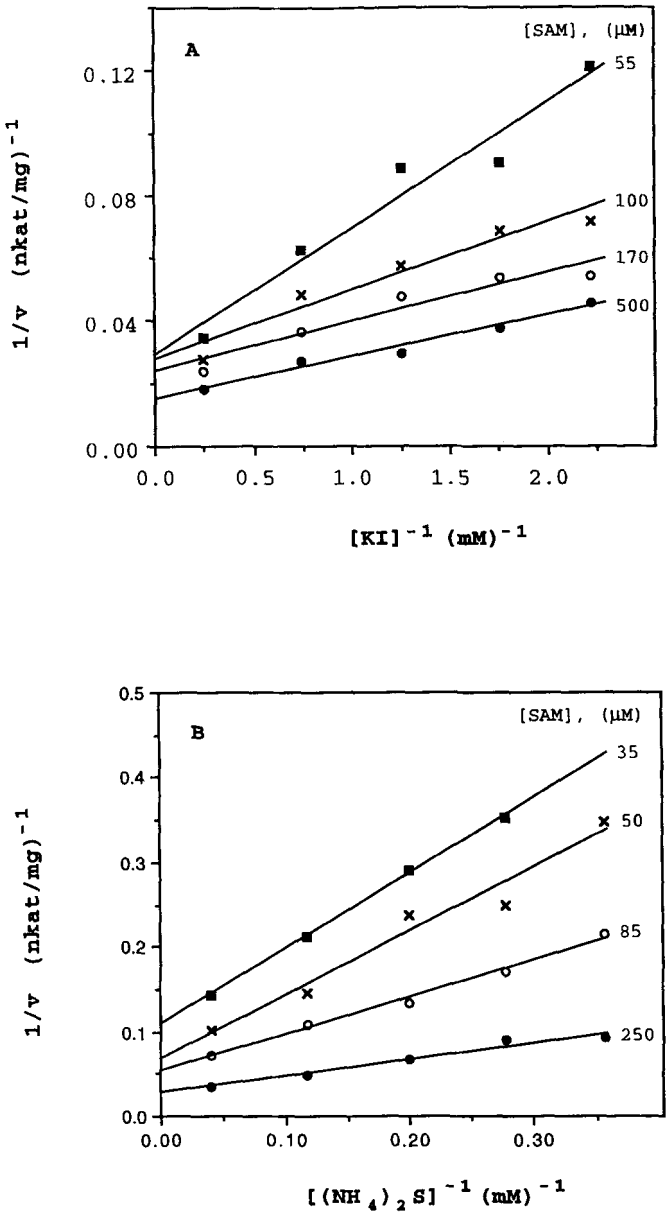


FIG. 6. **Double-reciprocal plots of initial velocity (*v*) *versus* substrate concentration for the methylation of iodide and bisulfide.** The effect of iodide (*A*) or bisulfide (*B*) concentrations on the initial velocity of the methylation reaction at different fixed concentrations of AdoMet (*SAM*) is shown. *nkat*, nanokatals.

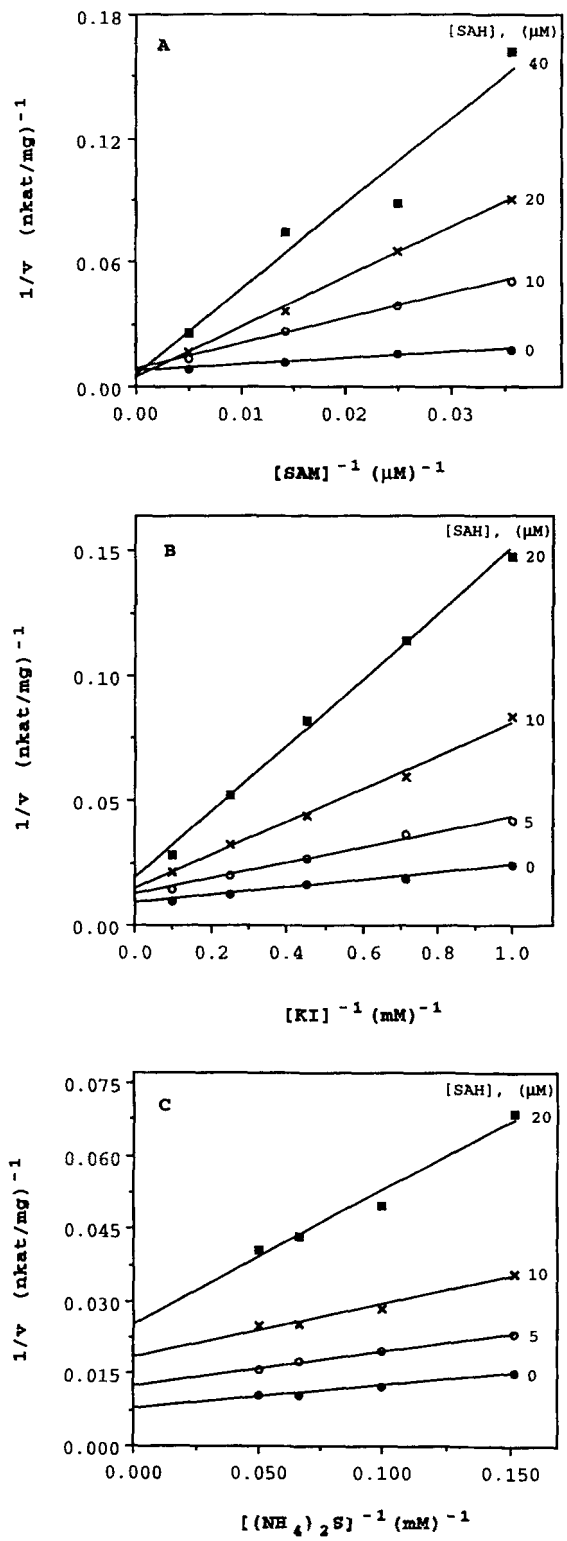


FIG. 7. **Product inhibition of halide/bisulfide methyltransferase reaction, presented as double-reciprocal plots.** *A*, inhibition of the methylation reaction by *S*-adenosyl-L-homocysteine (*SAH*) with respect to *S*-adenosyl-L-methionine; the concentration of iodide was fixed at 50 mM. *B*, inhibition of the methylation reaction by *S*-adenosyl-L-homocysteine with respect to iodide; the concentration of AdoMet was fixed at 400 μM. *C*, inhibition of the methylation reaction by *S*-adenosyl-L-homocysteine with respect to bisulfide; the concentration of AdoMet was fixed at 400 μM. *nkat*, nanokatals.

gel filtration chromatography, was 29,500, the native protein probably functions as a monomer. The molecular weight of this enzyme was similar to the algal methyl chloride transferase (15) and mammalian thiol methyltransferase (18, 26).

The halide/bisulfide methyltransferase exhibited different pH optimum values for its alternative halide (Fig. 5*A*) and bisulfide (Fig. 5*B*) substrates. A likely reason for this difference is the pH dependence of the HS^- ion concentration. Since H_2S has a pK_a1 of 7.02 (27), [HS^-] would be expected to fall sharply as the pH drops below 7. The halide- and bisulfide-methylating activities had the same pI value at pH 4.8, which was consistent with the observation that both activities existed on a single protein.

The pattern of substrate interaction kinetics involving AdoMet with either I^- or HS^- (Fig. 6, *A* and *B*) was symptomatic of a sequential substrate binding mechanism (28). Product inhibition kinetics were used to determine the reaction mechanism of the halide/bisulfide methyltransferase. The last product to be released would act as a competitive inhibitor to

TABLE II
Kinetic parameters of halide/bisulfide methyltransferase from B. oleracea

Substrate	Kinetic parameters[a]			
	K_m	K_i	V_{max}	K_{cat} (V_{max}/K_m)
	mM	*mM*	*nanokatals/mg*	
KI	1.3	0.95	1140	877
KBr	29		2.04	7×10^{-2}
KCl	85		0.39	4.6×10^{-3}
$(NH_4)_2S$	4.7	1.35	804	171.1
AdoMet[b]	0.03		444	14.8×10^3
AdoMet[c]	0.226		972	43×10^2
AdoHcy		0.032		

[a] Replots of data from substrate interaction and product inhibition experiments were used to determine the value of the kinetic parameters.
[b] AdoMet with iodide.
[c] AdoMet with bisulfide.

the first substrate to bind and as a noncompetitive inhibitor to the second substrate. Since the inhibition by AdoHcy was competitive with respect to AdoMet (Fig. 7*A*) and noncompetitive with respect to the halide (Fig. 7*B*) or bisulfide (Fig. 7*C*) ion (29), AdoMet appears to be the first substrate to bind to the enzyme. The methylated product would be the first to be released and AdoHcy the last. This pattern is indicative of an Ordered Bi Bi mechanism whereby the product of the last substrate to bind to the enzyme is the first to be released (30).

To our knowledge, this is the first time that either a halide or a bisulfide methyltransferase enzyme has been purified to homogeneity from a plant. A functionally similar rat liver *S*-thiol methyltransferase was purified to homogeneity (18). However, we found no reports on any halide methyltransferase from animals. A methyl chloride transferase from *E. muricata* (15) and a thiol methyltransferase from *Pseudomonas fluorescens* (19) have been partially purified. Perhaps more importantly, this is the first report, for any organism, of a single enzyme with both halide and bisulfide methyltransferase activities. Our previous work (20) indicates that this dual function enzyme is common among higher plants and that it may also be present in a marine red alga *E. muricata*. This may be of environmental importance; on a fresh weight basis, the rate of halomethane production from *B. oleracea* extracts was at least 6-fold higher than that from extracts of *E. muricata* (15). The production of CH_3SH was of equal abundance to that of CH_3I in most cases tested.

Most studies on the production of halomethanes and CH_3SH have focused on their environmental or ecological significance. For instance, volatile halogenated organic compounds produced by algae have been considered as deterrents to herbivore feeding (31). However, such compounds may also have metabolic roles. Thus, CH_3Cl was found to be a methyl donor in the biosynthesis of veratryl alcohol, a secondary metabolite involved in the induction of the lignolytic system in many lignin-degrading fungi including *P. pomaceus* (32). Harper *et al.* (33) suggested that these compounds could play a similar role in other organisms, including higher plants. Our finding of the halide methyltransferase activity in a higher plant supports this suggestion.

Methanethiol is a product of sulfate reduction by microorganisms and plants (34). Many functions of sulfur emissions by plants have been suggested including antimicrobial effects, release of toxic compounds produced in cellular metabolism, and allelopathic effects in plant communities (9). Detoxification of sulfur compounds has been shown in mammals (18, 19) and bacteria (35), and H_2S (HS^- anion) was the simplest substrate of an *S*-methylating activity that produced CH_3SH (36). Recently, dimethyl sulfide was also reported as a product of sulfate reduction in some phototrophic bacteria (37).

The mammalian and bacterial thiol methyltransferases have been reported to methylate organic thiols in addition to HS^- ions. To determine whether such compounds can also serve as substrates for the cabbage enzyme, we tested the ability of 2-mercaptoethanol, 6-mercaptopurine, and thiobenzoic acid to inhibit the iodide-methylating activity in an assay containing 10 mM KI. CH_3I production was not inhibited by these thiols at concentrations up to 100 mM. The above thiol methyltransferases methylated CH_3SH to dimethyl sulfide. However, we did not detect any dimethyl sulfide using CH_3SH (15 mM) as the substrate in our assays. These preliminary results suggest that the cabbage enzyme may have a metabolic role different from those reported for the mammalian and bacterial enzymes. Further work to test the activity of this enzyme toward nonvolatile substrates in an appropriate phytochemical context is needed to confirm this possibility.

It is too early to attribute any specific role to the halide/bisulfide methyltransferase in *B. oleracea*, especially given its potential involvement in two different areas of metabolism. However, given the low rates of methylation and the high K_m value of the methyltransferase for the only physiologically important halide Cl^-, we suggest that this enzyme is more likely to function in sulfur metabolism than in that of halide ions. This role in sulfur metabolism is also favored because the Brassicaceae family is characterized by several sulfur-containing secondary metabolites, including non-protein sulfur-containing amino acids (38) and sulfur-containing glucosinolates (39, 40). Hydrolysis products of these latter compounds react with CH_3SH to produce a variety of phytoalexins (40) that are involved in defense responses of this family.

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