

1 **2-Haloacrylate hydratase, a new class of flavoenzyme that catalyzes the addition of water**
2 **to the substrate for dehalogenation**

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17 **Running title:** Occurrence of 2-haloacrylate hydratase

18

19 **Abbreviations:**

20 2-CAA, 2-chloroacrylate; PAGE, polyacrylamide gel electrophoresis; KPB, potassium
21 phosphate buffer; DTT, dithiothreitol; ESI-MS, electrospray ionization mass spectrometry.

1 **Abstract**

2 Enzymes catalyzing the conversion of organohalogen compounds are useful in
3 chemical industry and environmental technology. We here report the occurrence of a new
4 FADH₂-dependent enzyme that catalyzes the removal of a halogen atom from an unsaturated
5 aliphatic organohalogen compound by the addition of a water molecule to the substrate. A soil
6 bacterium, *Pseudomonas* sp. YL, inducibly produced a protein named CAA67_YL when the
7 cells were grown on 2-chloroacrylate (2-CAA). The *caa67_YL* gene encoded a protein of 547
8 amino acid residues (*Mr* 59,301), which shared weak but significant sequence similarity with
9 various flavoenzymes and contained a nucleotide-binding motif. We found that 2-CAA is
10 converted into pyruvate when the reaction was carried out with purified CAA67_YL in the
11 presence of FAD and a reducing agent [NAD(P)H or sodium dithionite] under anaerobic
12 condition. The reducing agent was not stoichiometrically consumed during this reaction,
13 suggesting that FADH₂ is conserved by regeneration in the catalytic cycle. When the reaction
14 was carried out in the presence of H₂¹⁸O, [¹⁸O]-pyruvate was produced. These results indicate
15 that CAA67_YL catalyzes the hydration of 2-CAA to form 2-chloro-2-hydroxypropionate,
16 which is chemically unstable and probably spontaneously dechlorinated to form pyruvate.
17 2-Bromoacrylate, but not other 2-CAA analogs such as acrylate and methacrylate, served as the
18 substrate of CAA67_YL. Thus, we named this new enzyme 2-haloacrylate hydratase. The
19 enzyme is very unusual in that it requires the reduced form of FAD for hydration, which
20 involves no net change in redox state of the coenzyme or substrate.

21

1 **Introduction**

2 Dehalogenases catalyze the removal of halogen atoms from organohalogen compounds.
3 These enzymes have been attracting a great deal of attention partly because of their possible
4 applications to chemical industry and environmental technology. Several dehalogenases have
5 been discovered and characterized (6, 11, 14, 17, 22). Some of them act on unsaturated
6 aliphatic organohalogen compounds in which a halogen atom is bound to an sp^2 -hybridized
7 carbon atom. Examples are various corrinoid/iron-sulfur-cluster-containing reductive
8 dehalogenases (1, 7), *cis*- and *trans*-3-chloroacrylic acid dehalogenases (4, 19), and LinF
9 (meyleylacetate reductase), which acts on 2-chloromaleylacetate reductase (5).

10 In order to get more insight into the enzymatic dehalogenation of unsaturated aliphatic
11 organohalogen compounds, we searched for microorganisms that dissimilate 2-chloroacrylate
12 (2-CAA) as a sole source of carbon and energy (8). 2-CAA is a bacterial metabolite of
13 2-chloroallyl alcohol, an intermediate or byproduct in industrial synthesis of herbicides (26).
14 Rats treated orally with herbicides sulfallate, diallate, and triallate excrete urinary 2-CAA (16).
15 Various halogenated acrylic acids are produced by a red alga (27). We obtained three
16 2-CAA-utilizing bacteria as a result of screening (8). From one of them, *Burkholderia* sp. WS,
17 we previously discovered a new NADPH-dependent enzyme, 2-haloacrylate reductase (12)
18 (13). Although this enzyme does not directly remove a halogen atom from the substrate, it is
19 supposed to participate in the metabolism of 2-CAA by catalyzing the conversion of 2-CAA
20 into L-2-chloropropionate, which is subsequently dehalogenated by L-2-haloacid dehalogenase.

21 Another bacterium we obtained, *Pseudomonas* sp. YL, also dissimilates 2-CAA.
22 However, the metabolic fate of 2-CAA in this bacterium remains unclear. In the present study,
23 we analyzed proteins from 2-CAA- and lactate-grown cells of *Pseudomonas* sp. YL by
24 two-dimensional polyacrylamide gel electrophoresis (PAGE) and identified a
25 2-CAA-inducible protein. We found that the protein catalyzes the dehalogenation of 2-CAA by

1 the addition of a water molecule to the substrate, representing a new family of dehalogenases
2 that act on unsaturated aliphatic organohalogen compounds. Remarkably, the enzyme requires
3 FADH₂ for its activity although the reaction does not involve net change in redox state of the
4 coenzyme or substrate. We here describe the occurrence and characteristic of this unusual
5 flavoenzyme.

6

1 **Materials and Methods**

2 **Materials**

3 2-CAA was purchased from Lancaster Synthesis Ltd. (Lancashire, United Kingdom).
4 H₂¹⁸O (99atom%) was from Taiyo Nippon Sanso Corporation (Tokyo, Japan). All other
5 chemicals were of analytical grade.

7 **Microorganism and culture conditions**

8 *Pseudomonas* sp. YL isolated from soil as a 2-CAA-utilizing bacterium (8) was grown
9 at 28°C in a medium containing either 2-CAA or lactate as the sole carbon source as described
10 previously (12).

11

12 **Two-dimensional PAGE**

13 Proteins from 2-CAA- and lactate-grown cells were analyzed by two-dimensional
14 PAGE. The first-dimensional isoelectric focusing was performed with IPG ReadyStrip pH3-10
15 (Bio-Rad Laboratories, Inc., Hercules, CA), and the gel was subjected to the
16 second-dimensional SDS-PAGE.

17

18 **Determination of amino acid sequences**

19 The proteins in the two-dimensional PAGE gel were blotted onto an Immobilon-P^{SQ}
20 membrane (Millipore, Bedford, MA) and stained with Coomassie Brilliant Blue R-250. The
21 spot of CAA67_YL was excised, and the N-terminal amino acid sequence was determined with
22 a Shimadzu PPSQ-21 protein sequencer (Kyoto, Japan). Internal amino acid sequencing was
23 performed by the APRO Life Science Institute, Inc. (Naruto, Japan).

24

25 **Sequencing of the gene coding for CAA67_YL**

1 A part of the CAA67_YL gene was amplified by degenerate PCR with a sense primer
2 (5'-ATGYTIGAYTTYTIGTIAC-3' or 5'-GAYTTYTIGTIACIGAYGT-3'), an antisense
3 primer (5'-GGIACYTGRTAIGCYTCIAT-3' or 5'-TTRTCIACIGGIACYTGRTA-3'), the
4 genomic DNA of *Pseudomonas* sp. YL, and TaKaRa LA *Taq* DNA polymerase (Takara Bio,
5 Otsu, Japan). PCR products of the predicted size were obtained with any set of the above
6 primers and used as sequencing templates. The flanking region of these PCR products was
7 amplified by inverse PCR (20) with the self-ligated AatII-digested genomic DNA as a template
8 and the following primers: 5'-CACGAAGGCTTCGATCGTGC-3' and
9 5'-GCCGTCTCCGAGCAAGATGA-3' for the first PCR and
10 5'-AAGCGATGTCGCGGACCACA-3' and 5'-ACCCTCCTCGCTCGCAGAAA-3' for the
11 second nested PCR. The size of the DNA obtained was 1.7 kbp for the first PCR and 1.6 kbp
12 for the second PCR. The product was sequenced, and the flanking region of this PCR product
13 was amplified by inverse PCR (20) with the self-ligated BamHI-digested genomic DNA as a
14 template and the following primers: 5'-GCAAAGCAGCGCAGCAAG-3' and
15 5'-TTCATCGACGAGACGCCT-3' for the first PCR and
16 5'-CGATCAAGCTGTCTGACGG-3' and 5'-TCGCTCGCAGAAAGGGCC-3' for the second
17 nested PCR. The size of the DNA obtained was 1.2 kbp for the first PCR and 0.7 kbp for the
18 second PCR. AatII and BamHI were used for inverse PCR because the recognition sites for
19 these restriction enzymes were not found in the partial sequence of the CAA67_YL gene
20 available before inverse PCR.

21

22 **Construction of a plasmid for overproduction of CAA67_YL**

23 The *caa67_YL* gene was amplified by PCR by using the total genomic DNA of
24 *Pseudomonas* sp. YL as a template, a forward primer,
25 5'-GGGAATTCCCATATGTTGGATTTTCTTGTAAC-3' (NdeI site is underlined), and a

1 reverse primer, 5'-CCGCCGCTCGAGCTAGACCGGGACGTCCTCGA-3' (XhoI site is
2 underlined). The PCR product was digested with NdeI and XhoI and inserted into pET-21a(+)
3 (Novagen, Darmstadt, Germany). The plasmid obtained was introduced into *Escherichia coli*
4 BL21(DE3).

5

6 **Expression and purification of CAA67_YL**

7 Recombinant *E. coli* cells were cultivated in 5-L Luria-Bertani medium containing 100
8 µg/ml ampicillin at 18°C until A_{600} reached 0.5. After addition of 50 µM
9 isopropyl-1-thio-β-D-galactopyranoside, the cells were cultured for 24 h at 18°C. The cells
10 were harvested, washed, and lysed by sonication in 70 ml ice-cold 5 mM potassium phosphate
11 buffer (KPB) (pH 7.1) containing 1 mM dithiothreitol (DTT). All the following purification
12 procedures were performed at 4°C. After centrifugation, the crude extract was treated with
13 streptomycin sulfate [1% (w/v)], and nucleic acids were removed by centrifugation. The
14 resulting supernatant was applied to a DEAE-Toyopearl 650M column (Tosoh, Tokyo, Japan)
15 equilibrated with 5 mM KPB (pH 7.1) containing 1 mM DTT. Unbound proteins were washed
16 out with 5 mM KPB (pH 7.1) containing 1 mM DTT. Chromatography was carried out with a
17 linear gradient of 5-60 mM KPB (pH 7.1) containing 1 mM DTT. Proteins in chromatographic
18 fractions were separated by SDS-PAGE and stained with Coomassie Brilliant Blue G-250.
19 CAA67_YL was eluted at about 30-60 mM KPB. The fractions containing CAA67_YL were
20 collected as the purified enzyme. The purified enzyme was dialyzed against 60 mM KPB (pH
21 7.1) containing 1 mM DTT, concentrated to 10 mg/ml, and stored at -80°C until use.

22

23 **Quantification of FAD bound to CAA67_YL**

24 Purified CAA67_YL (0.17 mM) was incubated with 2.9 mM FAD in 60 mM KPB (pH
25 7.1) for 12 h at 4°C. After removing excess FAD not bound to the protein by gel filtration with

1 a Bio-Spin 6 column (Bio-Rad Laboratories, Inc.), the protein was denatured by heating at
2 100°C for 10 min to release protein-bound FAD. FAD thus obtained was quantified by
3 measuring absorbance at 450 nm ($\epsilon_{450} = 11,300 \text{ M}^{-1}\text{cm}^{-1}$) (15). The content of FAD in the
4 purified protein not incubated with externally added FAD was also determined by the same
5 method.

6

7 **Enzyme and protein assays**

8 Enzyme tests of CAA67_YL were carried out in a glove box at oxygen levels less than 2
9 ppm. For determination of the enzyme activity, halide ions released from 2-CAA were
10 measured according to the method of Iwasaki *et al.* (10). In addition, enzymatic conversion of
11 2-CAA and its analogs were monitored by electrospray ionization mass spectrometry (ESI-MS)
12 with a triple-quadrupole Sciex API3000 LC/MS/MS System (Applied Biosystems, Foster City,
13 CA). The standard assay mixture (100 μl) contained 3.5 mM 2-CAA, 3.5 mM NaOH (to
14 neutralize 2-CAA), 60 mM Tris-sulfate buffer (pH 9.0), purified CAA67_YL (50-100 μg), 0.1
15 mM FAD, and 10 mM NADH to reduce FAD. Such a high concentration of NADH was added
16 to the assay mixture for non-enzymatic reduction of FAD to produce FADH_2 (after 23). NADH
17 was replaced by 10 mM NADPH or 10 mM sodium dithionite when the cofactor requirement
18 was examined. For measurement of halide ions, the reaction was carried out at 35°C for 1-5
19 min and terminated by the addition of 11.1 μl of 1.5 M sulfuric acid. FAD in the assay mixture,
20 which interferes with the colorimetric assay of halide ions due to its yellow color, was removed
21 with charcoal powder. One unit of the enzyme activity was defined as the amount of enzyme
22 that catalyzes the dehalogenation of 1 μmol of the substrate per minute. The activity toward
23 other halogenated substrates, 2-bromoacrylate, 2-chloro-1-propene and 2-chloroacrylonitrile,
24 was assayed by the same method. Activities toward 2-fluoroacrylate, acrylate, methacrylate,
25 fumarate, phosphoenolpyruvate, 2-chloropropionate, and lactate were examined by ESI-MS.

1 Identity of the products obtained from 2-CAA and 2-bromoacrylate was also determined by
2 ESI-MS. For mass spectrometric analysis of the reaction, the standard assay mixture was
3 incubated for 240 min at 35°C. The reaction was terminated by the addition of 200 µl
4 acetonitrile, and the mixture was centrifuged, filtered, diluted with acetonitrile/10 mM
5 ammonium acetate (1:1), and then introduced into the mass spectrometer in negative ion mode
6 at 5 µl/min. For ¹⁸O incorporation experiment, the reaction was carried out in the standard
7 assay mixture containing 50% H₂¹⁸O (v/v).

8 Protein concentration was determined by the method of Bradford with bovine serum
9 albumin as a standard (3).

10

11 **Effects of pH and temperature on the enzyme stability and activity**

12 To examine the effect of pH on the stability of CAA67_YL, the enzyme was incubated
13 for 30 min at 30°C in the following buffers (60 mM): citrate-NaOH (pH 5.5-6.5); potassium
14 phosphate (pH 6.5-8.0); Tris sulfate (pH 8.0-9.0); and glycine-NaOH (pH 9.0-10.5). After
15 incubation, the incubation buffer was replaced by 60 mM KPB (pH 7.1) with a Microcon filter
16 device (Millipore), and the remaining activity was measured by the standard assay. To analyze
17 the effect of pH on the activity, the initial reaction velocities were measured with the standard
18 assay mixture containing the above buffers instead of the standard buffer. The effect of the
19 temperature on the stability of the enzyme was determined by incubating the enzyme at
20 different temperatures from 10 to 60°C for 30 min prior to the standard assay. The effect of the
21 temperature on the activity was examined by performing the standard assay at different
22 temperatures from 10 to 60°C. In all experiments, the reaction was started by the addition of the
23 enzyme after complete reduction of FAD to eliminate the effect of different temperatures and
24 pH values on the reduction of FAD by NADH.

25

1 **Molecular weight determination**

2 The subunit molecular weight of CAA67_YL was determined by SDS-PAGE and
3 ESI-MS. The molecular weight of the native enzyme was analyzed by gel filtration with an
4 ÄKTA Explorer 10S system (GE Healthcare UK Ltd., Buckinghamshire, United Kingdom)
5 equipped with a HiLoad 16/60 Superdex 200 pg column (GE Healthcare UK Ltd.).
6 MW-marker proteins (Oriental Yeast Co., Ltd., Tokyo, Japan) consisting of glutamate
7 dehydrogenase (290 kDa), lactate dehydrogenase (142 kDa), enolase (67 kDa), myokinase (32
8 kDa), and cytochrome *c* (12.4 kDa) were used as standards.

1 **Results**

2 **Identification of a protein inducibly synthesized in the 2-CAA-grown *Pseudomonas* sp.** 3 **YL and cloning of its gene**

4 To identify proteins inducibly synthesized in *Pseudomonas* sp. YL grown on 2-CAA,
5 proteins from 2-CAA-grown cells and lactate-grown cells were compared by two-dimensional
6 PAGE. One major protein (Fig. 1, arrowhead) was found only in the 2-CAA-grown cells,
7 suggesting its involvement in the metabolism of 2-CAA. This protein was named CAA67_YL.
8 The N-terminal amino acid sequence was MLDFLVTDVLLVGE, and the following internal
9 amino acid sequence was determined: EMAELIEAYQVPVDK. Degenerate primers were
10 designed based on the partial amino acid sequences of CAA67_YL, and the gene coding for the
11 protein was cloned as described in Materials and Methods. The *caa67_YL* gene (accession
12 number: AB519652) contained an open reading frame of 1,644 nucleotides coding for 547
13 amino acid residues (*Mr* 59,301). A putative Shine-Dalgarno sequence, AAGGAGG, was
14 found in the upstream region of the initiation codon of the *caa67_YL* gene.

15

16 **Structural characteristics of CAA67_YL**

17 A homology search revealed that CAA67_YL shares 84.6% sequence identity with a
18 2-CAA-inducible protein from *Burkholderia* sp. WS (CAA67_WS) (accession number:
19 BAD91550), whose function is unknown (12). Both proteins have a nucleotide-binding motif
20 (VXGXGXXGXXXA) probably involved in binding FAD or NAD(P) in the region from 13 to
21 18. In addition, CAA67_YL showed weak but significant sequence similarity to various
22 flavoproteins such as L-aspartate oxidase, succinate dehydrogenase flavoprotein subunit, and
23 thiol:fumarate reductase subunit A. The sequence identities to L-aspartate oxidase from
24 *Rhodopseudomonas palustris* BisA53 (accession number: YP_783305), succinate
25 dehydrogenase flavoprotein subunit from *Methanothermobacter thermautotrophicus* str. Δ H

1 (accession number: AAB85977) (21), and thiol:fumarate reductase subunit A from
2 *Methanothermobacter thermautotrophicus* str. Marburg (accession number: CAA04398) (9)
3 were 23.2%, 23.6%, and 22.9%, respectively. Sequence similarity to various flavoproteins and
4 occurrence of a nucleotide-binding motif suggested that CAA67_YL requires FAD for its
5 function.

7 **Reaction catalyzed by CAA67_YL**

8 We tested whether CAA67_YL catalyzes the degradation of 2-CAA in the presence of
9 FAD. CAA67_YL was overproduced in recombinant *E. coli* cells, and the crude extract was
10 incubated with 2-CAA. Under aerobic condition, 2-CAA was not degraded at all as judged by
11 ESI-MS analysis. In contrast, when the reaction was carried out under anaerobic condition in
12 the presence of high concentration of NADH (10 mM) for non-enzymatic reduction of FAD
13 (0.1 mM), the peaks of 2-CAA ($m/z = 105$ (2- ^{35}Cl]CAA) and 107 (2- ^{37}Cl]CAA)) disappeared,
14 and a new peak appeared at $m/z = 89$, which was likely due to the formation of lactate (data not
15 shown). Release of a chloride ion from 2-CAA was also observed by colorimetric assay under
16 the same condition. The reaction did not proceed when the cell-free extract from *E. coli*
17 harboring pET21a(+) without the *caa67_YL* gene was used.

18 We purified CAA67_YL from the recombinant *E. coli* cells overproducing CAA67_YL
19 by monitoring the CAA67_YL-dependent release of a chloride ion from 2-CAA (Table 1). The
20 protein was purified 2.2-fold with 43% recovery. The final preparation was shown to be
21 homogeneous by SDS-PAGE (Fig. 2). The specific activity of the purified enzyme was 0.96
22 units/mg.

23 Purified CAA67_YL contained an oxidized form of FAD as judged by its absorption
24 spectrum. The molar ratio of FAD to the protein was 0.25. The ratio increased to 0.91 after
25 incubation with externally added FAD as described in Materials and Methods. Despite the

1 presence of FAD in the purified protein, no enzyme activity was detected without reduction of
2 FAD.

3 To identify the product of the reaction catalyzed by CAA67_YL, the purified protein
4 was incubated with 2-CAA in the presence of 0.1 mM FAD and 10 mM NADH under anaerobic
5 condition. Reduction of FAD under this condition was confirmed by the disappearance of the
6 absorption peaks at 370 and 450 nm, characteristic of oxidized FAD. The reaction was
7 monitored by ESI-MS. We found that the peaks of 2-CAA disappeared and a new peak
8 appeared at $m/z = 87$ only in the presence of CAA67_YL (Fig. 3A and 3B). When lactate
9 dehydrogenase was added to the assay mixture, the peak at $m/z = 87$ disappeared and a new
10 peak appeared at $m/z = 89$ (data not shown). Judging from the molecular mass and the
11 reactivity with lactate dehydrogenase, the product of the CAA67_YL-catalyzed reaction was
12 concluded to be pyruvate. When the crude extract from the recombinant *E. coli* cells was used
13 instead of purified CAA67_YL in the above-mentioned experiment, pyruvate was probably
14 converted into lactate by endogenous lactate dehydrogenase.

15 CAA67_YL did not catalyze the conversion of 2-CAA unless the reaction mixture
16 contained a reduced form of FAD: the reaction proceeded when NADH was replaced by
17 NADPH or sodium dithionite, but lack of all these reducing agents completely abolished the
18 reaction. To investigate the dependence on FAD, we prepared the apoenzyme of CAA67_YL
19 by dialyzing the solution of the purified CAA67_YL against 2 M KBr. The UV-visible
20 spectrum of the apoenzyme did not show a peak around 450 nm, a characteristic absorption of
21 FAD, indicating that FAD was removed from CAA67_YL. The apoenzyme showed no activity
22 even when NADH, NADPH, or sodium dithionite was added to the reaction mixture. However,
23 the activity was restored to the original level when 0.1 mM FAD was added to the assay mixture
24 together with the reducing agent. When FAD was replaced with FMN (0.1 mM), the enzyme

1 did not recover its activity. Thus, CAA67_YL catalyzes the conversion of 2-CAA into
2 pyruvate in a reduced FAD-dependent manner.

3

4 **Substrate specificity of CAA67_YL**

5 Activities of CAA67_YL toward various 2-haloacrylates and their analogs were
6 determined. CAA67_YL catalyzed the conversion of 2-bromoacrylate into pyruvate.
7 However, 2-fluoroacrylate, methacrylate, acrylate, 2-chloroacrylonitrile, 2-chloro-1-propene,
8 fumarate, and phosphoenolpyruvate were inert as substrates. Also, D- and
9 L-2-chloropropionate and D- and L-lactate did not serve as the substrates. The velocity *versus*
10 substrate plot for 2-CAA and 2-bromoacrylate showed typical Michaelis-Menten kinetics when
11 the substrate concentration was low (2-CAA: <3.5 mM; 2-bromoacrylate: <4.0 mM). The
12 apparent K_m and V_{max} values for 2-CAA were 0.47 mM and 1.2 units/mg, respectively, and
13 those for 2-bromoacrylate were 1.3 mM and 1.6 units/mg, respectively. The enzyme activity
14 was inhibited by the high concentration of 2-CAA (>3.5 mM) and 2-bromoacrylate (>4.0 mM).

15

16 **Molecular weight and subunit structure of CAA67_YL**

17 The molecular weight of the purified CAA67_YL was estimated to be about 61,000 by
18 SDS-PAGE, which agrees well with the value (59,301) calculated from the deduced primary
19 structure of the enzyme. Mass spectrometric analysis showed two peaks for CAA67_YL: one
20 at 59,311 corresponding to the apoenzyme and the other at 60,099 probably due to the enzyme
21 binding to FAD. The molecular weight determined by gel filtration was 52,000, suggesting that
22 the enzyme is monomeric.

23

24 **Effects of pH and temperature on CAA67_YL**

1 The effect of pH on the activity of CAA67_YL was examined over the pH range from
2 5.5 to 10.5. The enzyme was relatively stable between pH 8.5 and 9.5 for 30 min at 30°C and
3 showed maximum activity at pH 9.0 (Fig. 4A and 4B). The optimum temperature was found to
4 be 35°C, and the enzyme was fairly stable at 20°C or less for 30 min (Fig. 4C and 4D).

5

6 **Identification of the substrate providing oxygen in the CAA67_YL-catalyzed reaction**

7 CAA67_YL catalyzes the conversion of 2-CAA and 2-bromoacrylate into pyruvate as
8 described above. In this reaction, an oxygen atom is incorporated into the substrate. To
9 determine the source of the oxygen atom, the reaction was carried out in the presence of 50%
10 H₂¹⁸O (v/v). Mass spectrometric analysis of the reaction mixture showed that two peaks
11 appeared after the reaction: one at $m/z = 87$ corresponding to unlabeled pyruvate and the other
12 at $m/z = 89$ corresponding to pyruvate containing ¹⁸O (Fig. 3C). The identity of the product as
13 pyruvate was confirmed by the addition of lactate dehydrogenase to the reaction mixture: two
14 peaks appeared at $m/z = 89$ and 91 corresponding to unlabeled and ¹⁸O-labeled lactate,
15 respectively (data not shown). The incorporation of ¹⁸O into pyruvate indicates that the oxygen
16 atom of a water molecule is introduced into the substrate in the CAA67_YL-catalyzed reaction.

17

18 **Conservation of the reducing agent in the CAA67_YL-catalyzed reaction**

19 A reducing agent is required for the reduction of FAD for the CAA67_YL-catalyzed
20 reaction. To examine whether the reduced form of FAD is consumed during the reaction, the
21 amount of the reducing agent (NADH) in the reaction mixture was monitored: an aliquot of the
22 reaction mixture was diluted, and the absorbance was measured at 340 nm for quantification of
23 NADH. We found that NADH did not decrease stoichiometrically for the release of chloride
24 ions from 2-CAA (Fig. 5), indicating that the reduced form of FAD is not consumed during the
25 reaction.

1 **Discussion**

2 **Occurrence of 2-haloacrylate hydratase**

3 We found a novel flavoenzyme that catalyzes the conversion of 2-CAA into pyruvate.
4 The ^{18}O -incorporation experiment showed that an oxygen atom of a water molecule is
5 introduced into the substrate, indicating that CAA67_YL catalyzes the hydration of the
6 substrate. 2-Chloro-2-hydroxypropionate, a geminal halohydrin produced by hydration of
7 2-CAA, is chemically unstable and probably spontaneously decomposes into pyruvate by the
8 removal of HCl (Fig. 6). We named this novel enzyme 2-haloacrylate hydratase because the
9 enzyme specifically acts on 2-CAA and 2-bromoacrylate.

10 2-Haloacrylate hydratase has an absolute requirement for the reduced form of FAD for
11 its catalytic reaction, which involves no net change in redox state of the coenzyme or substrate.
12 This cofactor requirement is notable because most flavoenzymes catalyze the net redox
13 reactions including oxidations, reductions, oxygenations, and electron transfers. A conceivable
14 mechanism is that 2-CAA is first reduced to form 2-chloropropionate, which is subsequently
15 hydrolyzed to form lactate, and lactate is further converted into pyruvate by oxidation.
16 Although this explains the production of pyruvate from 2-CAA and incorporation of an oxygen
17 atom of a water molecule into the product, the mechanism is unlikely because
18 2-chloropropionate and lactate did not serve as the substrate of the enzyme. Another possible
19 explanation for the FADH_2 requirement for 2-haloacrylate hydratase is that FADH_2 functions
20 as a general acid-base catalyst. A general acid-base role of the flavin was recently shown for
21 type 2 isopentenyl diphosphate isomerase (24, 25). FADH_2 may be involved in activation of a
22 water molecule that attacks the C-2 atom of 2-CAA or protonation of the C-3 position of
23 2-CAA. A more plausible mechanism is that FADH_2 acts as a radical catalyst. One electron
24 transfer from FADH_2 to 2-CAA and protonation at the C-3 position would produce
25 2-chloropropionate radical, which may be hydroxylated in the following step to produce

1 2-chloro-2-hydroxypropionate. Such a free radical redox role of the reduced flavin has been
2 reported for other flavoenzymes such as chorismate synthase (2, 18). Further studies such as
3 stopped-flow kinetic analysis and crystallographic analysis are required to elucidate the
4 reaction mechanism of this very unusual flavoenzyme.

5

6 **Metabolism of 2-CAA**

7 CAA67_YL is inducibly produced when the cells are grown on 2-CAA and catalyzes
8 the conversion of 2-CAA into pyruvate, suggesting that 2-CAA is metabolized by CAA67_YL
9 in *Pseudomonas* sp. YL. In contrast, another 2-CAA-utilizing bacterium, *Burkholderia* sp. WS,
10 inducibly produces two proteins named CAA43 and CAA67, which we call CAA67_WS in this
11 manuscript to avoid confusion, when the cells are grown on 2-CAA (12). We previously
12 reported that CAA43 catalyzes the reduction of 2-CAA to form L-2-chloropropionate by using
13 NADPH as a cosubstrate and named this enzyme 2-haloacrylate reductase.
14 L-2-Chloropropionate produced by 2-haloacrylate reductase is probably further metabolized to
15 D-lactate by L-2-haloacid dehalogenase occurring in this bacterium. On the other hand, the
16 function of CAA67_WS is unknown. Considering its high sequence similarity with
17 CAA67_YL, it is very likely that CAA67_WS also catalyzes the conversion of 2-CAA into
18 pyruvate. Contribution of CAA43 and CAA67_WS to the metabolism of 2-CAA in
19 *Burkholderia* sp. WS remains to be examined in future studies.

20 In contrast with *Burkholderia* sp. WS, *Pseudomonas* sp. YL does not produce
21 2-haloacrylate reductase when grown on 2-CAA as judged by the results of activity
22 measurement and two-dimensional PAGE analysis. The gene coding for CAA43 is located in
23 the immediate downstream of the gene coding for CAA67_WS on the genome of *Burkholderia*
24 sp. WS (12), whereas the gene coding for a CAA43 homolog was not found in the
25 corresponding region on the genome of *Pseudomonas* sp. YL (data not shown). Taken

1 together, 2-haloacrylate hydratase, but not 2-haloacrylate reductase, probably plays a principal
2 role in 2-CAA metabolism in *Pseudomonas* sp. YL.

3

4 **Comparison with *cis*- and *trans*-3-chloroacrylic acid dehalogenases**

5 *cis*-3-Chloroacrylic acid dehalogenase (4) and *trans*-3-chloroacrylic acid dehalogenase
6 (19) catalyze the conversion of 3-chloroacrylate into 3-chloro-3-hydroxypropionic acid, which
7 is decomposed to malonate semialdehyde and a chloride ion (4, 19). They share low but
8 significant sequence similarity with each other and are supposed to evolve from a common
9 ancestor. 2-Haloacrylate hydratase discovered in the present study resembles these enzymes in
10 that it catalyzes dehalogenation by the addition of a water molecule to the substrate. However,
11 it is significantly different from 3-chloroacrylic acid dehalogenases not only in its substrate
12 specificity but also in its primary structure and cofactor requirement: 3-chloroacrylic acid
13 dehalogenases require no cofactor for their catalytic activities, whereas 2-haloacrylate
14 hydratase depends on a reduced form of FAD. Thus, 2-haloacrylate hydratase represents a new
15 class of dehalogenase that degrades unsaturated aliphatic organohalogen compounds.

16

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16

1 **Figure legends**

2

3 Fig. 1. Two-dimensional PAGE analysis of the proteins of 2-CAA- and lactate-grown
4 *Pseudomonas* sp. YL. Soluble proteins from 2-CAA-grown cells (A) and lactate-grown cells
5 (B) were analyzed. The arrowhead indicates the spot of a 2-CAA-inducible protein, which we
6 named CAA67_YL.

7

8 Fig. 2. SDS-PAGE analysis of CAA67_YL. Purified protein (10 μ g) was loaded onto the gel.

9

10 Fig. 3. Mass spectrometric monitoring of the conversion of 2-CAA with CAA67_YL. 2-CAA
11 was incubated with or without purified CAA67_YL in the presence of 0.1 mM FAD and 10 mM
12 NADH under anaerobic condition as described in Materials and Methods. The mixture was
13 analyzed after 4 h reaction without (A) and with (B) the addition of CAA67_YL by ESI-MS in
14 the negative ion mode. To determine the incorporation of an oxygen atom of a water molecule
15 into the substrate, the reaction was carried out in the presence of 50% H₂¹⁸O, and the solution
16 was analyzed after 4 h reaction (C). Because chlorine has two isotopes with a mass number of
17 35 and 37 in the ratio 3:1, 2-CAA has two peaks at 105 and 107. The peaks at 87 and 89 are due
18 to pyruvate and ¹⁸O-labeled pyruvate, respectively, as described in the text. The peak at 97 is
19 due to hydrogen sulfate in the reaction buffer and dihydrogen phosphate in the enzyme
20 preparation.

21

22 Fig. 4. Effects of pH and temperature on CAA67_YL. Effects of pH on the activity (A) and
23 stability (B) were determined by using the following buffers (60 mM): citrate-NaOH (closed
24 circles) (pH 5.5-6.5); potassium phosphate (closed squares) (pH 6.5-8.0); Tris sulfate (closed
25 triangles) (pH 8.0-9.0); and glycine-NaOH (closed diamonds) (pH 9.0-10.5). The enzyme

1 activity after treatment with Tris sulfate (pH 9.0) was taken as 100% in (A). Effects of
2 temperature on the activity (C) and stability (D) were determined as described in Materials and
3 Methods. The enzyme activity at 40°C was taken as 100% in (C).

4

5 Fig. 5. The amount of NADH consumed for dehalogenation of 2-CAA. 2-CAA was incubated
6 with CAA67_YL in the presence of 0.1 mM FAD and 10 mM NADH under anaerobic
7 condition, and consumption of NADH and formation of chloride ions were monitored. The
8 concentrations of NADH and chloride ions in the reaction mixture are indicated by closed
9 triangles and closed circles, respectively.

10

11 Fig. 6. Reaction catalyzed by 2-haloacrylate hydratase.

1 Table 1. Purification of CAA67_YL from recombinant *E. coli* cells. The enzyme activities
2 were determined by measuring halide ions released from 2-CAA.

3

Purification step	Total activity	Total protein	Specific activity	Yield	Purification
	units	mg	units/mg	%	-fold
Crude extract	51	120	0.43	100	1
DEAE-Toyopearl	22	23	0.96	43	2.2

4

Figure 1

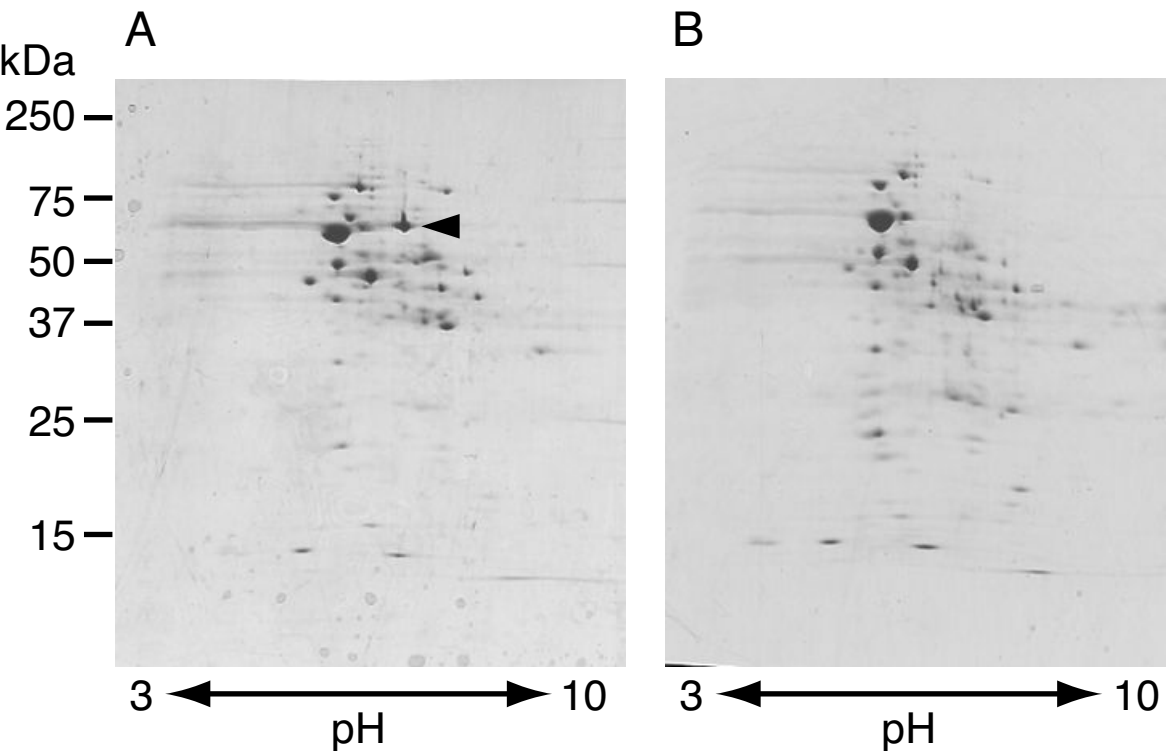


Fig. 1. Two-dimensional PAGE analysis of the proteins of 2-CAA- and lactate-grown *Pseudomonas* sp. YL. Soluble proteins from 2-CAA-grown cells (A) and lactate-grown cells (B) were analyzed. The arrowhead indicates the spot of a 2-CAA-inducible protein, which we named CAA67_YL.

Figure 2

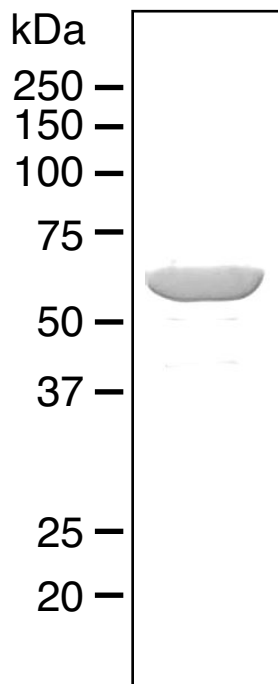


Fig. 2. SDS-PAGE analysis of CAA67_YL. Purified protein (10 μ g) was loaded onto the gel.

Figure 3

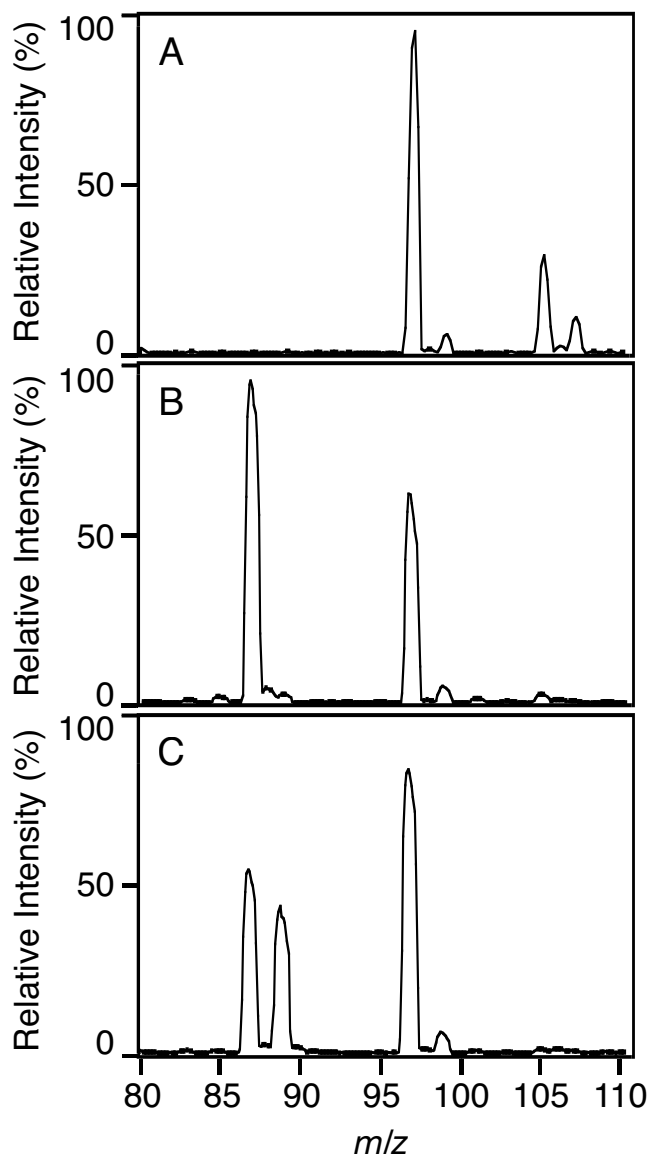


Fig. 3. Mass spectrometric monitoring of the conversion of 2-CAA with CAA67_YL. 2-CAA was incubated with or without purified CAA67_YL in the presence of 0.1 mM FAD and 10 mM NADH under anaerobic condition as described in Materials and Methods. The mixture was analyzed after 4 h reaction without (A) and with (B) the addition of CAA67_YL by ESI-MS in the negative ion mode. To determine the incorporation of an oxygen atom of a water molecule into the substrate, the reaction was carried out in the presence of 50% H_2^{18}O , and the solution was analyzed after 4 h reaction (C). Because chlorine has two isotopes with a mass number of 35 and 37 in the ratio 3:1, 2-CAA has two peaks at 105 and 107. The peaks at 87 and 89 are due to pyruvate and ^{18}O -labeled pyruvate, respectively, as described in the text. The peak at 97 is due to hydrogen sulfate in the reaction buffer and dihydrogen phosphate in the enzyme preparation.

Figure 4

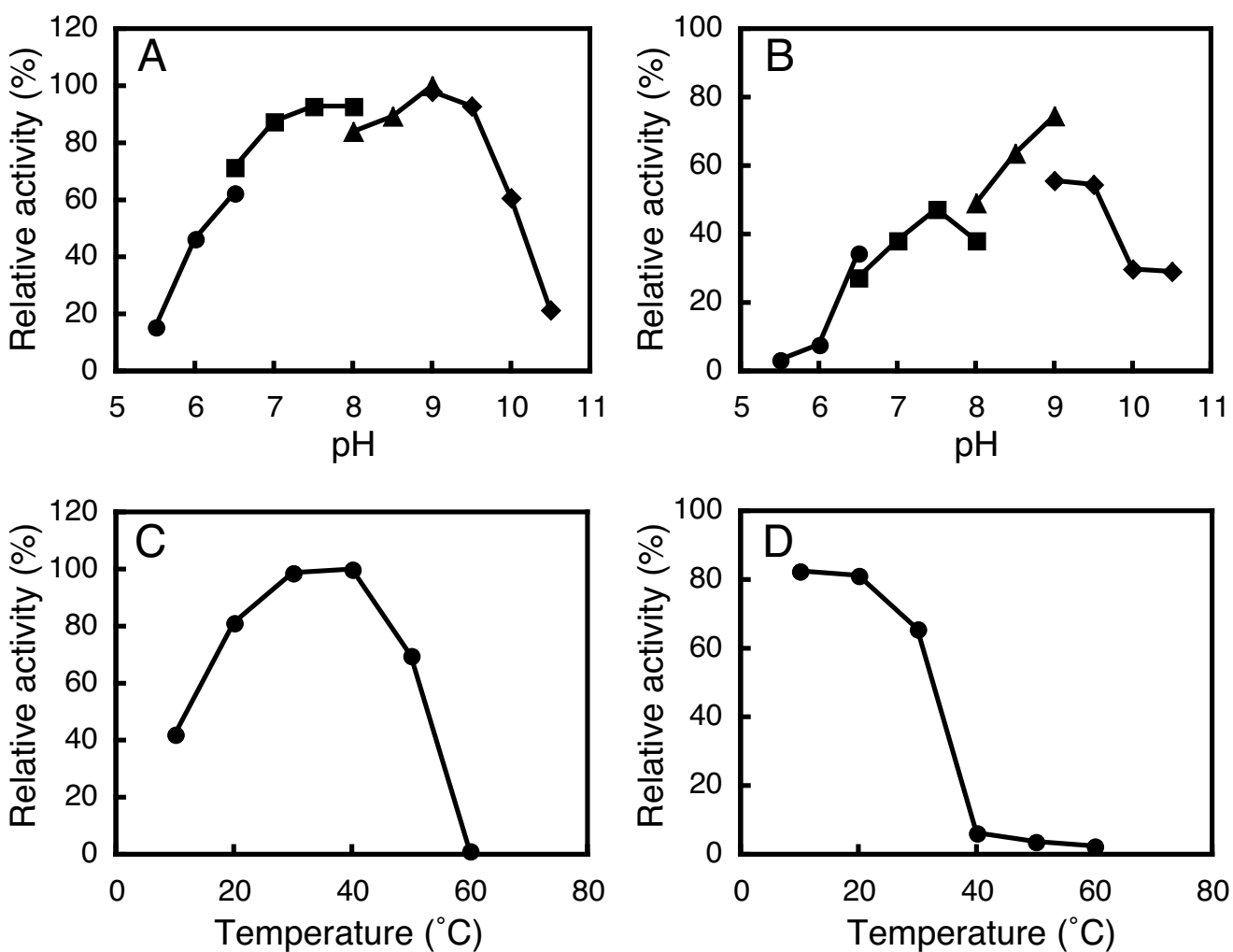


Fig. 4. Effects of pH and temperature on CAA67_YL. Effects of pH on the activity (A) and stability (B) were determined by using the following buffers (60 mM): citrate-NaOH (closed circles) (pH 5.5-6.5); potassium phosphate (closed squares) (pH 6.5-8.0); Tris sulfate (closed triangles) (pH 8.0-9.0); and glycine-NaOH (closed diamonds) (pH 9.0-10.5). The enzyme activity after treatment with Tris sulfate (pH 9.0) was taken as 100% in (A). Effects of temperature on the activity (C) and stability (D) were determined as described in Materials and Methods. The enzyme activity at 40°C was taken as 100% in (C).

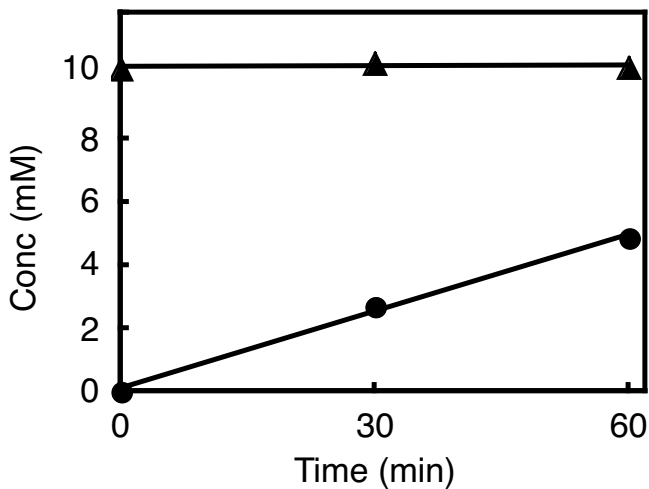


Fig. 5. The amount of NADH consumed for dehalogenation of 2-CAA. 2-CAA was incubated with CAA67_YL in the presence of 0.1 mM FAD and 10 mM NADH under anaerobic condition, and consumption of NADH and formation of chloride ions were monitored. The concentrations of NADH and chloride ions in the reaction mixture are indicated by closed triangles and closed circles, respectively.

Figure 6

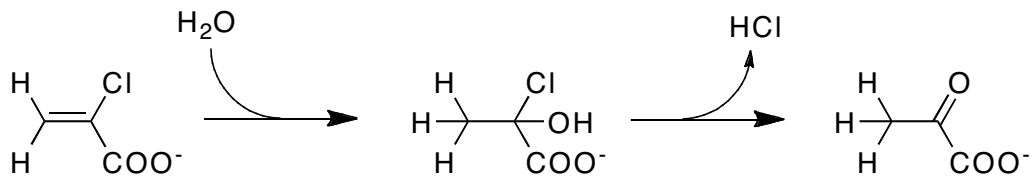


Fig. 6. Reaction catalyzed by 2-haloacrylate hydratase.