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Purification and characterization of a methylene urea-hydrolyzing enzyme from *Rhizobium radiobacter* (*Agrobacterium tumefaciens*)

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Abstract

Slow-release fertilizers are gaining acceptance to increase fertilizer use efficiency and reduce environmental impact. The release of nitrogen from methylene urea, a common slow release N fertilizer, is controlled by microbial decomposition. An enzyme hydrolyzing slow-release nitrogen fertilizer, methylene urea, was purified from *Rhizobium radiobacter* (*Agrobacterium tumefaciens*) to homogeneity using a four-step purification procedure with an overall yield of 3%. The active enzyme has a molecular mass of approximately 180 kDa determined by size exclusion chromatography, and the SDS page of the purified protein indicated three subunits of different sizes (62, 34 and 32 kDa). The N-terminal amino acid sequence of the 62 kDa fragment indicates identity with urease subunits from *Mycobacterium tuberculosis* (73%) and *Helicobacter pylori* (71%). However, for the internal amino acid sequences of the 62 kDa fragment no matches with known proteins were found. Some internal peptides in the smaller subunits (32 and 34 kDa) are homologous to urease subunits and unknown proteins in *Agrobacterium tumefaciens*. Based on the kinetic properties, substrate selectivity, and inhibition characteristics, the novel enzyme (MUase) is an intracellular enzyme complex with urease activity. The enzymatic mechanism of methylene urea breakdown was studied using a novel LC–MS method for MU analysis, which indicates that all cold-water soluble nitrogen forms of methylene urea are subjected to hydrolysis, and the hydrolysis proceeds via methylurea, urea and other yet unidentified hydrolysis-products, suggesting that the isolated enzyme complex performs a multistep hydrolysis. The microbiological and molecular data is useful in determining the soil factors affecting the efficacy of methylene urea as a slow release fertilizer in agricultural production systems.

Keywords: Soil enzymes; Methylene urea; Ureaformaldehyde; Slow-release fertilizer; Purification; *Rhizobium radiobacter*; *Agrobacterium tumefaciens*

1. Introduction

Methylene ureas (MU) are condensation products of urea and formaldehyde, which are used as slow-release fertilizers in agriculture, horticulture and forestry. The estimated use of methylene ureas is about 220,000 metric tons per year, mainly in US, Western Europe, and Japan and it is projected to increase in the future (Trenkel, 1997). The slow-release pattern of methylene urea depends on the degree of polymerization and the combination of different polymer chain lengths in the product. The shortest MU chains methylene diurea (MDU), dimethylene triurea (DMTU), and trimethylene tetraurea (TMTU) are completely soluble in cold water, whereas the longest ones are insoluble in boiling water and most other solvents. The release of available N from MU is directly correlated to chain length with the longer chains of MU showing the most slow-release properties.

In soil, methylene urea is degraded into soluble, plant available nitrogen forms, NH_4^+ and NO_3^-, by a specific microbial activity (Fuller and Clark, 1947; Corke and Robinson, 1966). Plant ureases have no activity towards MU but Jahns and co-workers have purified and characterized methylene urea-degrading enzymes (MDUases) from Gram-negative soil bacteria *Ochrobactrum anthropi* (Jahns et al., 1997) and *Ralstonia paucula* (Jahns and Kaltwasser, 2000). According to their results, these different types of MDUases were able to hydrolyze the cold water-soluble forms of methylene urea into ammonium, formaldehyde and

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urea in molar ratios of 2:1:1 (MDU), 4:2:1 (DMTU), and 6:3:1 (TMTU). However, little is known about the detailed mechanism of the hydrolysis and the research on MU degradation has been hampered by the low solubility of MU. Jahns et al. (1999) developed an HPLC method to identify methylene urea condensation products in different water-soluble fractions. This new method allowed them to detect condensates up to tetramethylene pentaurea (TMPU), which could not be detected by the earlier HPLC method (Christianson et al., 1988).

We have shown previously that a plant pathogenic bacterium, *Rhizobium radiobacter* (*A. tumefaciens*), isolated from a field soil in California was able to grow on methylene urea as a sole nitrogen source (Koivunen et al., 2003). The production of the cytoplasmic MU-hydrolyzing enzyme (MUase) was induced by using MU as a nitrogen source after which the bacteria could be transferred into a soluble N source for faster growth and higher specific MUase activity. In the present study, we purified the methylene urea-hydrolyzing enzyme from this microorganism to apparent homogeneity. We describe here the molecular and enzymatic properties of the purified enzyme, as well as partial N-terminal and internal amino acid sequences of the protein subunits. We also report a novel LC–MS method developed for detection of water-soluble methylene urea fragments during the enzyme assay.

2. Material and methods

2.1. Chemicals

All chemicals and reagents used in this study were at minimum analytical grade. Jack bean (*Canavalia ensiformis*) urease and formaldehyde dehydrogenase were purchased form INC Biomedicals Inc. (Aurora, OH). Most substrate analogs and inhibitors were from Sigma Chemical Company (St. Louis, MO) except hydantoin and acetohydroxamic acid, which were from Aldrich Chemical Company (Milwaukee, WI), and phenyl phosphorodiamidate, which was from Avocado Research Chemicals Ltd. (Heysham, Lancs, UK). The methylene urea substrate used in enzyme assays was prepared from commercial methylene urea (Enduro Long, Kemira Agro Oy, Finland). The preparation protocol was modified from the AOAC Official method 945.01 (AOAC, 1999), which involves fractionation based on water-solubility. The fraction used in enzyme assays contained the cold-water-soluble portion of nitrogen (CWSN) without free urea. This CWSN fraction was prepared by mixing 10 g of methylene urea in 500 ml of H_2O at 25 °C. After 30 min of mixing, the suspension was filtered, and the clear CWSN solution was evaporated to dryness. In order to remove excess free urea in the sample, the precipitate was dissolved in 50 ml of water, filtered and the filtrate discarded. The remaining precipitate (about 0.1 g) was dissolved in 100 ml of H_2O and filtered. The filtrate pH was adjusted to pH 8.0, and it was incubated with jack bean urease (INC Biomedicals Inc., Aurora, OH) for 24 h. After incubation, urease was removed by using a Centriplus YM30 micro filter concentrator (Amicon Inc., Beverly, MA). Distribution and abundance of different N containing compounds in the MU substrate presented in Fig. 1 was determined by using a Quattro Ultima triple quadrupole mass spectrometer (Micromass, Manchester, UK) run at unit resolution using positive mode electrospray ionization (capillary voltage 3.5 V; cone voltage 25 V). The sample was introduced by loop injection with a 100 μl min^{-1} flow of 100% H_2O.

2.2. Preparation of crude extract

Methylene urea-induced cultures of *R. radiobacter* were grown at 28 °C in a liquid mineral medium (Schlegel et al., 1961) with NH_4Cl as a nitrogen source. When the cultures were at a late exponential or an early stationary growth phase, the cells were harvested by centrifugation (3500 g for 15 min at 4 °C), and washed twice in 50 mM Na_2HPO_4 buffer (pH 7.5) containing 3 mM β-mercaptoethanol. Crude extracts were prepared from washed cells (0.2 g wet weight per ml buffer) by sonication on ice (30 W for 30 s ml^{-1}) with a Sonic Dismembrator 60 (Fisher Scientific, Pittsburg, PA). The broken-cell suspension was centrifuged (8500 g at 15 °C for 15 min), and the supernatant was used as crude extract for enzyme assays and protein purification.

2.3. Enzyme and protein assays

MUase enzyme assays were performed at pH 8.0 in 25 mM Na_2HPO_4 containing 1.5 mM β-mercaptoethanol and 0.5 mM EDTA. For analysis, 5 μl of enzyme solution was mixed with 80 μl of above-mentioned buffer and 20 μl of substrate (Fig. 1). The amount of ammonium or formaldehyde formed enzymatically from methylene urea was determined after a 1 h incubation at 30 °C. Ammonium was determined colorimetrically by using a modified Berthelot-reaction (Forster, 1995). In this method, the enzyme reaction was first stopped by adding an aliquot (20 μl) of assay medium to 0.5 ml of a salicylate–nitroprusside color reagent after which an alkaline oxidizer containing sodium hydroxide and sodium hypochlorite was added to a final volume of 1 ml. After a 15 min incubation at 30 °C, the intensity of the blue-green color was measured at 660 nm with a Shimadzu UV mini 1240 UV–VIS spectrophotometer (Shimadzu Corporation, Kyoto, Japan). Formaldehyde was determined using the protocol of Heinz (1984), in which formaldehyde is analyzed by measuring the formation of NADH in the presence of formaldehyde dehydrogenase and NAD. Enzyme activities are given in units (U), one unit corresponding to 1 μmol product formed from the substrate per minute. Protein was determined with the Bradford method (Bio-Rad microassay, Bio-Rad,

Compound	mM
Urea + Na	0.0
MDU + Na	0.3
DMTU + Na	3.0
TMTU + Na	0.8

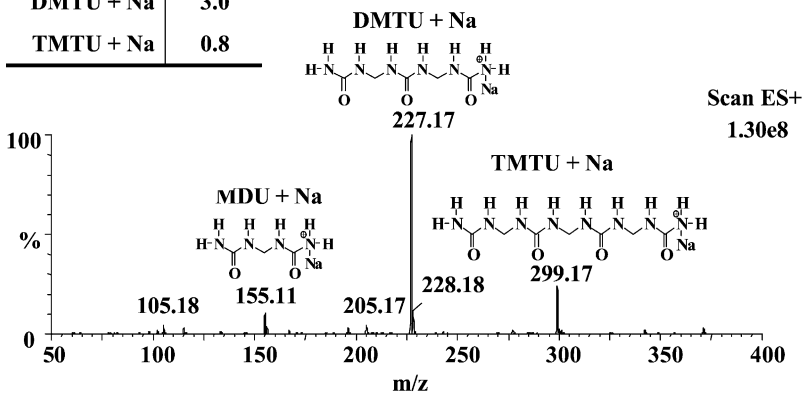

Fig. 1. LC–MS analysis of the cold-water-soluble MU used as a substrate in enzyme assays. The LC–MS spectrum presents the relative abundance of different polymers (detected as Na-substitutes) in the mixture. The table shows the molar concentration of each component in a solution of 1 mg MU ml^{-1}. A 5 μl aliquot of MU substrate was analyzed using a Quattro Ultima triple quadrupole mass spectrometer (Micromass, Manchester, UK) run at unit resolution using positive mode electrospray ionozation (capillary voltage = 3.5 V; cone voltage = 25 V). The sample was introduced by loop injection with a 100 μl min^{-1} flow of 100% H_2O.

Hercules, CA) by using BSA as a standard as previously described (Bradford, 1976).

2.4. Purification of MUase

Enzyme purification steps were performed at room temperature (21–22 °C) unless otherwise indicated. The crude extract from cultures (~300 ml) was first concentrated with a 50 kDa Minikros hollow fiber filter module (Spectrum Laboratories Inc., Laguna Hills, CA). The concentrate (~50 ml) was then loaded onto a Q-Sepharose column (Amersham Pharmacia Biotech, Inc., Uppsala, Sweden) equilibrated with 25 mM Na_2HPO_4 at pH 8.0. Using a linear gradient from 0 to 0.5 M NaCl the enzyme was eluted between 0.35 and 0.45 M NaCl. The active fractions were pooled and concentrated with Centriplus YM50 micro filters (Amicon Inc., Beverly, MA). The concentrate was adjusted to 2.0 M NaCl by adding 4.0 M NaCl, and loaded on an Octyl-Sepharose (Amersham-Pharmacia Biotech, Inc., Uppsala, Sweden) column equilibrated at pH 8.0 with 25 mM Na_2HPO_4 and 2.0 M NaCl. In a linear gradient from 1.2 to 0.9 M NaCl, the enzyme eluted at approximately 1.0 M NaCl. Pooled fractions were again concentrated by using Centriplus YM30 micro filters (Amicon Inc., Beverly, MA) and loaded on a Sephacryl S-300 HR (Sigma Chemical Company, St. Louis, MO) gel filtration column at 4 °C. The gel filtration column was equilibrated and the protein eluted with 50 mM Na_2HPO_4 at pH 7.5. Fractions showing MU-hydrolyzing activity were pooled, concentrated with Centriplus YM30 kDa micro filter, and loaded on a Novex 10% Tris–glycine electrophoresis gel (Invitrogen, Carlsbad, CA). A native-PAGE gel was run in a Novex Xcell II Minigel system for 2 h at 100 mV after which a small part of the gel corresponding to one sample lane was stained with 0.1% (w/v) Coomassie Brilliant Blue, and the rest was cut into 3 mm slices corresponding to the bands. Each gel slice was incubated overnight in an Eppendorf tube with 400 μl of 25 mM Na_2HPO_4 at pH 8.0 containing 1.5 mM β-mercaptoethanol and 0.5 mM EDTA. The sample with most activity was used for kinetic studies and sequencing. The enzyme preparation obtained from size exclusion chromatography was used for stability tests. All tests were run in at least three replicates, and the mean values of the replicates are reported here.

2.5. Electrophoresis and the molecular mass determination

After each purification step, the molecular sizes of the subunits were determined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) in a Novex XCell II Minigel system (Invitrogen, Carlsbad, California) according to Laemmli (1970). Gels were stained with a Silver Stain Plus kit (Bio-Rad, Hercules, CA). Isoelectrofocusing was performed with Novex 3-10 pH gradient gels (Invitrogen, Carlsbad, CA). Size exclusion chromatography (Sephacryl S-300 HR) was used for estimation of molecular mass of the native enzyme. A standard curve was created using the following proteins as known standards: β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa) and carbonic anhydrase (30 kDa).

2.6. Protein sequencing

The enzyme obtained from the last purification step was blotted from SDS-PAGE onto a small-pore (Immobilon PSQ, 0.1 μm) PVDF membrane (Millipore Corp., Bedford,

MA). The N-terminal sequences of the blotted bands were determined using standard Edman degradation chemistry performed on an ABI Procise HT protein sequencer (Applied Biosystems Inc., Foster City, CA) at the Molecular Structure Facility of UC Davis. The BLAST 2.2.1 algorithm (Altschul et al., 1997) at the National Center for Biotechnology Information (NCBI) was used for alignment of amino acid sequences with information from GenBank, EMBL, DDBJ, and PDB databases.

For the determination of internal peptides, the bands in the SDS-PAGE gel were excised, diced and digested overnight with sequencing grade trypsin. The resulting tryptic peptides where then extracted, reduced with DTT to break disulfide linkages and reacted with iodoacetamide. The peptides were then subjected to tandem mass spectrometry on an ABI Sciex Qstar hybrid triple-quadrupole time-of-flight mass spectrometer with a nano-electrospray source (Applied Biosystems Inc., Foster City, CA). Peptides were cleaned up by binding and washing on Poros resin prior to elution into the nanospray capillary with 50% methanol and 1% formic acid. Doubly and triply charged peaks were selected for MS/MS analysis and the resulting spectra were searched using MASCOT and MSDB databases for matching proteins. The internal protein sequencing was performed at the Molecular Structure Facility of UC Davis.

3. Results

3.1. Purification and molecular mass of MUase

A four-step procedure was used for the purification of a methylene urea-hydrolyzing enzyme (MUase) from the crude extract; ion exchange, hydrophobic interaction, size exclusion, and preparatory electrophoresis (native PAGE). In this purification process, the enzyme was purified about 2000-fold to apparent homogeneity, and to a specific activity of 100 U per mg protein with an overall 3% recovery in activity (Table 1). The most effective step in the purification was the ion exchange on Q-Sepharose. This resin proved to be much more efficient in terms of yield than the DEAE resin tested as an alternative. Hydrophobic interaction chromatography on Octyl-Sepharose was an effective purification step, but loss of activity in this step was quite high, probably due to high salt concentration. However, Octyl-Sepharose was chosen because the yield from another hydrophobic resin tested, Phenyl-Sepharose, was even lower due to poor binding of the protein onto the column. The gel filtration step yielded only minimal protein purification but this step allowed the direct molecular mass determination and an effective change of buffer. According to the size exclusion chromatography, the molecular mass of native MUase was 180 ± 20 kDa (data not shown). Native PAGE as the final purification step was essential and resulted in doubling the specific activity. The yield from this preparatory step was extremely low, which can be explained by either interference from the glycine running buffer or incomplete transfer of protein from the gel slices into the buffer.

When the high-molecular-weight band in a native PAGE gel with MUase activity was cut and loaded on the SDS-PAGE, three bands were revealed corresponding to approximate molecular masses of 62, 34, and 32 kDa (Fig. 2). Based on isoelectrofocusing, the pI of the MUase complex was estimated to be 4.8 (data not shown) at which point no MUase activity could be detected.

3.2. Sequence analysis

N-terminal amino acid sequencing resulted in 16 residues for the largest, 62 kDa band. Sequence for both low molecular weight bands contained only four residues each, and were not used for further analysis. In the BLAST analysis, the 62 kDa fragment N-terminal sequence (AKLLSRRQYVELYGPTTG) showed 73% homologies with the alpha subunit of urease from *M. tuberculosis* (Reyrat et al., 1995) and 71% similarity with the urease beta subunit from *H. pylori* (Akada et al., 2000).

On the average, 27–37 internal peptides per band were obtained (Table 2), but only a few meaningful matches were found when the identified peptides were compared with the known sequences in the SWISS PROT data base. For 62 kDa protein fragment, there were no matches. For the 34 kDa protein fragment 57 and 100% homologies were found between one peptide (IPEDIAFAESR) and urease alpha subunits from *A. tumefaciens* str. C58 (Goodner et al., 2001) and *Sinorhizobium meliloti* (Miksch et al., 1994), respectively. One peptide in the 32 kDa fragment

Table 1
Purification of MUase from *Rhizobium radiobacter*

Purification step	Total protein mg	Total activity (U)[a]	Specific activity (U mg^{-1} protein)	Recovery (%)	Purification factor
Crude extract	102	5.2	0.05	100	1
Q-Sepharose	3.26	4.5	1.4	86	27
Octyl-Sepharose	0.065	1	15.4	19	302
Sephacryl S300HR	0.011	0.8	72.7	15	1427
Native PAGE	0.001	0.1	100.0	3	1962

[a] One unit (U) of activity corresponds to formation of 1 μmol ammonium min^{-1}.

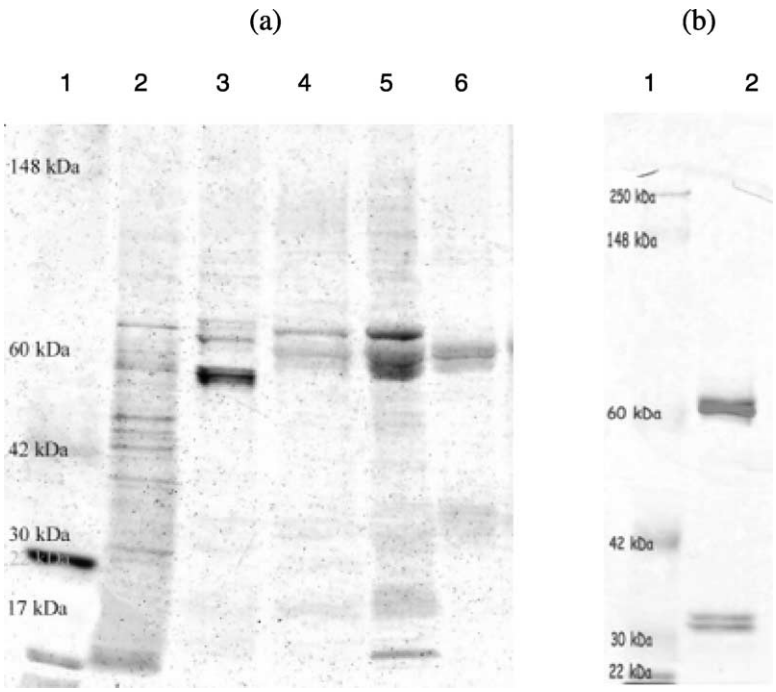

Fig. 2. SDS-PAGE of samples at each purification step (a) and the purified MUase (b). (a) 1. Standard, 2. Crude extract, 3. Q-Sepharose, 4. Octyl-Sepharose, 5. Sephacryl s300HR, 6. Native PAGE. (b) 1. Multimark® protein ladder (Invitrogen, Carlsbad, CA), 2. Protein obtained from a band in native-PAGE showing MUase activity.

(IAEDKNWPTGR) was 75% homologous to a hypothetical protein from *A. tumefaciens* str. C58 (Goodner et al., 2001).

3.3. Enzyme stability

The partially purified enzyme obtained from the gel filtration step was stable at room temperature. Only a small (2.5%) loss in activity was observed after a 30 min incubation at 50 °C, but the half-life of MUase at 50 °C was only 20% of that measured at 30 °C (Fig. 3). The decrease in stability was even more pronounced at 70 °C, where a 2 h incubation resulted in a complete loss of activity. The hydrolytic production of ammonium from MU was optimal at 50 °C (Fig. 4), at which temperature it followed the Arrhenius equation. Based on the Arrhenius plot the activation energy for the reaction was 15.9 kJ mol^{-1} (data not shown). Surprisingly, MUase retained some of its activity even at 0 °C. Optimum MUase activities were observed in a pH range of 8.0–8.5, and more than 90% of activity was lost below pH 6.5 and at pH 11 (Fig. 5). This indicates that for optimal conditions, the pH in enzyme activity measurements could have been higher (pH 8.5). Also, at this high pH another type of buffer (HEPES) would have offered a better buffering capacity than the 25 mM phosphate buffer used in this study. Low activity of MUase at pH 6 was obviously due to inactivation of the enzyme and as mentioned before, the enzyme had no activity at its pI 4.8.

3.4. Substrate selectivity and kinetic properties

MUase demonstrated high affinity for all major cold-water-soluble forms of methylene urea. According to the LC–MS analysis, MDU, DMTU, and TMTU were all partially degraded during ten hours of incubation with the enzyme. However, based on the change in the relative composition of different forms of methylene urea in the substrate, MDU was preferred over the longer-chain methylene ureas (Fig. 6). This could not be explained by better availability of MDU in the substrate, because the most abundant form of nitrogen in the substrate was dimethylene triurea (DMTU). Disappearance of MDU, DMTU, and TMTU in the substrate was accompanied by formation of urea and other unknown intermediates.

The study of MUase kinetics under steady-state conditions at 30 °C indicated that the enzyme does not obey Michaelis–Menten kinetics in this inhomogeneous substrate. The binding isotherm for MUase was sigmoidal, and the Scatchard plot (v/[S] versus v, Fig. 7a) was concave downward indicating positive cooperation. The data suggest that the enzyme has multiple binding sites and binding of substrate molecule to the first site facilitates binding to the second one (Hammes, 1982). On the other hand, positive cooperation could also be explained by the inhomogeneous nature of the substrate if cleaving off one urea molecule from the long MU polymer would make the products more prone to hydrolysis. Also, the Hill plot ($\log[v/(V_s - v)]$

Table 2
Internal peptides characterized in the three bands (32, 34, and 62 kDa) of the purified MUase. Peptides are listed in the order of increasing mass

32 kDa	34 kDa	62 kDa
DIPAGTT	LNIPLDK	QMLGL
INLPEAR	FEPGDEK	LPEYW
GIGLNLAR	YTINPAR	ALAGGGDIR
LDNPENI	YNVSQPK	QIEEAIK
INNYYK	GLEVTTPK	TADQKIR
VASPDHAK	LATLDAPR	EKINNTD
GITLSVRP	VVYFSKK	YTMPPVK
AEVLSAPR	VTTGLAGPR	AVQEAVDK
VAQANSPR	GDTKILAR	AVAFSIPR
IVMEHLD	ATTQVAKR	LGKQFIR
VVYFSKK	TATKWIR	LDHNLEK
VTTGLAGPR	AGGGAGHEAGR	WAHFNAK
QEELQAR	ITITDSLDR	IKPEKMR
AEGHIEAGR	LDLQRLER	LEPYKPR
EDGLEINV	NNLPTDELK	LQQEEEK
IDLDEYFK	MRPAVDGGSR	VAALCDRR
LDLPVVMEK	EALAATAMLR	ALPPEPAAR
IPNITIMDK	YFFQKSTK	IAAGVWSVH
LSNNEMPIK	LYAVASRNR	LAGQTVVPR
LDAKFDPLK	LDAPSPEAPR	SFTAIGKVK
IAVACSGGGYR	LADALAARPR	AIFCDACGR
LAAGGGGGGGGAPR	IDAAIEPAVR	QLPEDSGR
SVSEYLGGER	DRSFEQQR	LKQNVEAEK
EGVSEVHEAR	VTDLVAATVAR	ETDRLTEETR
ADCLPFEYR	SLDLVGLSGQK	VVGTIVDRPG-KAK
VTDLFGVSDY	VTVGTAWGGNR	MTPPSFASRP-DQR
SDAASREILR	LVRPAMVVVPR	AVLITGASAGF-GEAMCR
IIVDATGSGGAIK	AIQELGEFCAR	
DPGEVGAGHLER	IAGQKPVVTVAR	
LADGPDEVHQR	IPEDIAFAESR	
LADSTGYEVKR	LADQVAEEVRR	
AIENTASVSEHK	LAELFHTRTAK	
IAEDKNWPTGR	AWGDSYASVPPDR	
QELDSGPHLEGAR	YAQAHEFLTIWR	
HFPSVNWLISYSK	MITVTEATATAG-ALQR	
QCSAPMTTYIGVLD	EVNFYIINATKLAR	
LDPMKIDDNSGYSS		

versus log[S], Fig. 7b) yielded a curve instead of a straight line, indicating that the binding isotherm for MUase is complex, and not possible to obtain conclusively.

Substrate selectivity was evaluated by substituting methylene urea with structurally similar compounds in the enzyme assay. The purified enzyme hydrolyzed urea slowly (~20% of the rate for MU), but did not hydrolyze any of the following structurally similar compounds: allantoin, allantoic acid, sarcosine, 1,1-dimethylurea, 1,3-dimethylurea, 1-phenyl 2-thiourea, phenylurea, benzoylurea, cyclohexylurea, *o*-methyl-isourea, phenylacetylurea, *N*-ethyl-*N*-phenylurea. Substituted urea herbicides monuron, diuron, siduron, and fenuron were not hydrolyzed by MUase, neither were hydantoin or octopine, which are unique and

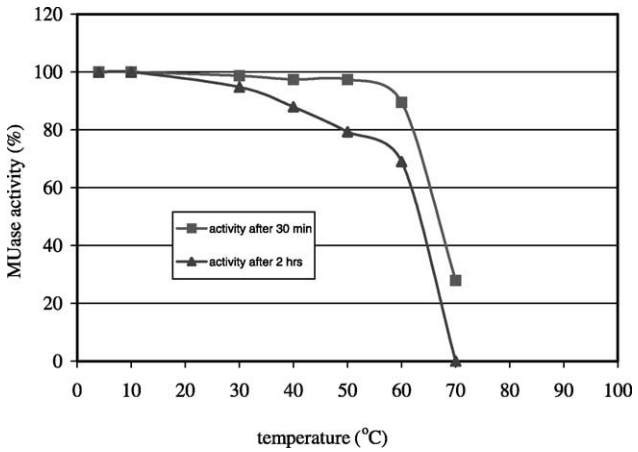

Fig. 3. Temperature stability of MUase activity. For temperature stability tests enzyme in 25 mM buffer was incubated at 4–70 °C for times specified in graph. Prior to the assay at 30 °C, the enzyme mixture was brought back to room temperature. Numbers represent the mean of three replicates with a CV < 11%.

important substrates for *R. radiobacter* (Syldatk et al., 1990; Dessaux et al., 1992).

3.5. Inhibitors

The effects of various heavy metal ions and known urease inhibitors on both ammonium ion and formaldehyde production were investigated in assays with the purified enzyme. Results presented in Table 3 show that all heavy metal ions had a strong inhibitory effect on formaldehyde production. Phenylmercuriacetate (PMA), a urease inhibitor, did not markedly reduce either ammonium or formaldehyde production. Another urease inhibitor, phenyl phosphorodiamidate, slightly inhibited the formation of both products. MU hydrolysis was completely inhibited by acetohydroxamate at a concentration of 200 μM. Moreover, boric acid had a dramatic effect on MUase activity; even at low concentrations it markedly reduced production of

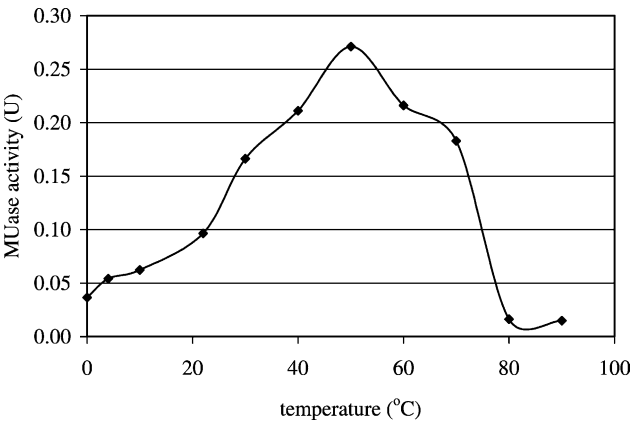

Fig. 4. Effect of temperature on the MUase activity. Assay was run for 30 min at temperatures (0–90 °C) indicated below. One unit of activity corresponds to 1 μmol NH_4^+ produced per minute. Numbers represent the mean of three replicates with a CV < 8%.

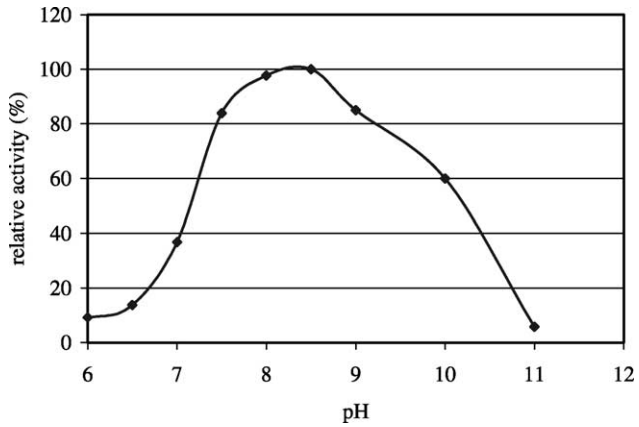

Fig. 5. MUase activity measured after 30 min incubation of enzyme in 25 mM sodium phosphate buffer at pH 6–11. After incubation, the enzyme assay was run at pH 8.0. Numbers represent the mean of three replicates with a CV < 15%.

ammonium, and totally inhibited production of formaldehyde from the substrate. It should be noted, however, that for all the other compounds tested, pH of the assay medium was in the optimal range (pH 7.5–8) except for boric acid, for which the assay was carried out at pH 4.0. EDTA at concentrations of 1 mM and 2 mM had a slight positive effect on MUase activity.

4. Discussion

According to the size exclusion chromatography, the methylene urea-degrading enzyme purified from *R. radiobacter* has a molecular mass of approximately 180 ± 20 kDa. Silver-stained SDS-PAGE gels of the purified enzyme revealed three bands, corresponding to subunits 62, 34 and 32 kDa. It is obvious that combining these three subunits does not give a molecular mass of 180 ± 20 kDa. Moreover, Fig. 2b suggests that the intensity of the 62 kDa band is much greater than those of the smaller bands. There is a possibility that the smaller bands represent a protelytic clip of the 62 kDa band. However, the sequence analysis of these three subunits revealed only minor similarities between the two small subunits, and there was no similarity between the large and small subunits.

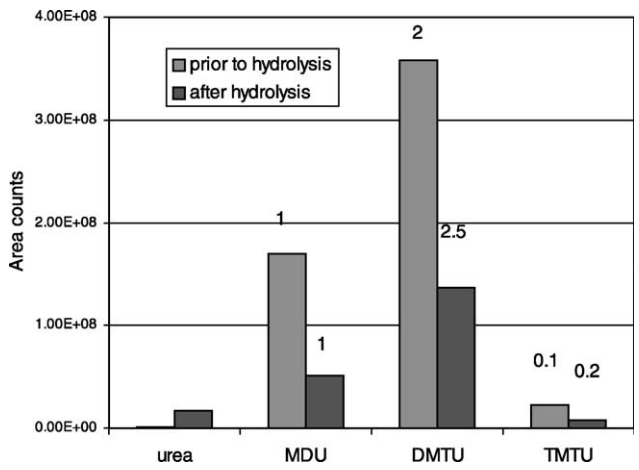

Fig. 6. Amount of different N forms in the cold-water soluble fraction of MU based on the area counts obtained in the LC–MS analysis. Relative abundance of each N form in the analyte before and after 10 h of incubation with the enzyme is presented as a molar ratio of MDU:DMTU:TMTU.

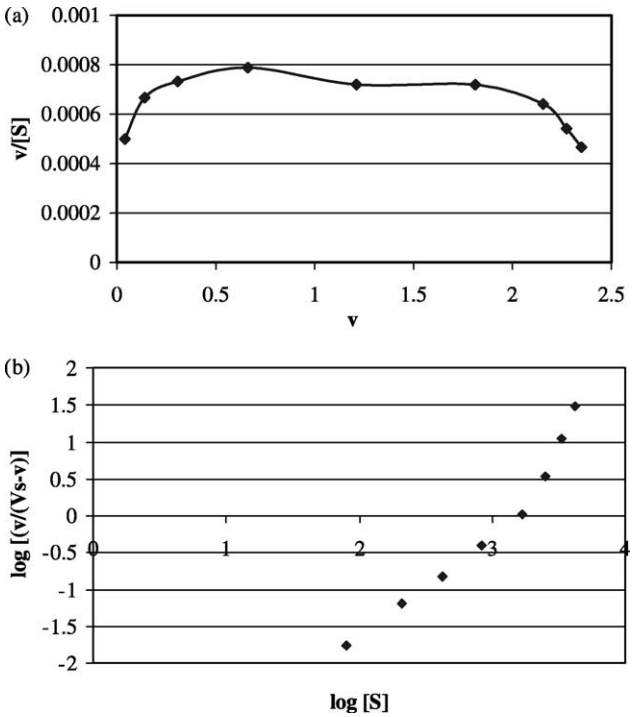

Fig. 7. Kinetic data suggesting positive cooperation of the enzyme system; v = reaction rate (μmol NH_4^+ min^{-1}), [S] = substrate concentration (μM MU), $V_s = v_{max}$. (a) Scatchard plot; v/[S] vs. v; (b) Hill plot; log[v/($V_s - v$)] vs. log[S].

Table 3
Effect of inhibitors on MUase activity of *Rhizobium radiobacter*. Relative activity (%) for production of ammonium ion (A) and formaldehyde (F)

Concentration	mM	A (%)	F (%)
$ZnSO_4$	0.2	39 ± 4	0
$CuSO_4$	0.2	33 ± 3	0
	0.4	42 ± 5	0
$CdSO_4$	0.2	43 ± 2	0
$NiCl_2$	0.2	33 ± 4	0
Acetohydroxamate	0.2	0	0
Phenylphosphorodiamidate	0.2	24 ± 2	38 ± 4
Phenylmercuriacetate	0.075	88 ± 5	75 ± 5
	0.15	79 ± 3	75 ± 4
	0.3	64 ± 4	50 ± 5
EDTA	1.0	147 ± 2	100 ± 1
	2.0	143 ± 5	125 ± 3
Tetraborate	20	100 ± 1	100 ± 2
	40	87 ± 2	75 ± 4
	80	79 ± 2	75 ± 2
Boric acid	10	27 ± 5	0
	20	8 ± 3	0
	40	2 ± 1	0

The data strongly suggests that the MUase of *R. radiobacter* is different from the enzyme of *O. anthropi* with a homotetrameric structure (Jahns et al., 1997). However, the purified enzyme had some similarities with the MDUase isolated from *R. paucula* (Jahns and Kaltwasser, 2000). Specific activities for the two enzymes were around 100 U mg protein^{-1}, and in both cases the enzyme lost its activity close to its pI of pH 4.8. The biggest difference between the two enzymes is the number of subunits and the fact that MUase from *R. radiobacter* was induced by methylene urea, whereas the one from *R. paucula* was regulated by repression and derepression. The large 62 kDa subunit of the purified MUase showed similarities with a 62 kDa-subunit from *M. tuberculosis* urease (Clemens et al., 1995). Another resemblance between MUase and the *M. tuberculosis* urease was the number and size-distribution of subunits; both consisted of three subunits, two of which were of very similar size as determined by electrophoretic techniques.

The N-terminal and some internal peptide sequences of the purified protein from *R. radiobacter* had similarities with bacterial ureases from the family Rhizobiaceae. Most ureases (urea amidohydrolase; EC 3.5.1.5) isolated from Gram-negative microbes have three subunits, α/β/γ or UreC/UreB/UreA (Mobley et al., 1995). The primary amino acid sequence of each of the bacterial urease subunits displays a high degree of similarity to the primary amino acid sequence of the jack bean urease subunit (Collins and D'Orazio, 1993). Therefore, although the enzymes differ in the number of subunits, the amino acid composition in plant and bacterial ureases is highly conserved. Homologies between MUase and bacterial ureases were expected because urea is an intermediate of the hydrolysis. However, this does not prove that the protein purified from *R. radiobacter* is simply a urease because there is also evidence for substantial homology in the N-terminal sequences of ureases and the cyclic amidases hydantoinase and allantoinase (May et al., 1998). Interestingly, there is hardly any sequence information available on purified urease of *R. radiobacter* even though the whole 5.67 Mb genome of *A. tumefaciens* is sequenced (Goodner et al., 2001), and urease is constitutively expressed in all species of *Agrobacterium* sp.

Jahns and his co-workers (Jahns et al., 1997) suggested a pathway for methylene diurea (MDU) hydrolysis, in which the enzyme first hydrolyses the substrate into ammonium, carbon dioxide and a postulated unstable intermediate, aminated methylurea ($NH_2-CO-NH-CH_2-NH_2$). This compound is assumed to decompose non-enzymatically to *N*-hydroxymethylurea and ammonium. *N*-hydroxymethylurea in turn, is known to be unstable and to decompose non-enzymatically to formaldehyde and urea (Winsor et al., 1958; Bulygina et al., 1987). Jahns et al. (1997) showed that the MDUase from *O. anthropi* was hydrolyzing MDU, DMTU, and TMTU to ammonium ion, formaldehyde, and urea in molar ratios of 2:1:1. In our study, only ammonium ion and formaldehyde production were used to quantify the hydrolysis. Upon the MU hydrolysis by the enzyme from *R. radiobacter*, ammonium and formaldehyde were produced from the substrate in a molar ratio of 2:1, which corresponds to the ratio reported earlier for MDUases from *O. anthropi* (Jahns et al., 1997) and *R. paucula* (Jahns et al., 1999). According to the LC–MS analysis of the methylene urea substrate before and after hydrolysis, MDU, DMTU and TMTU were all partially degraded during ten hours of incubation with the enzyme. In the LC–MS analysis, several hydrolysis intermediates including urea and methylurea were detected in the assay medium. However, no accumulation of aminated methylurea or *N*-hydroxymethylurea was detected.

The methylene urea-hydrolyzing enzyme (MUase) purified from *R. radiobacter* was selective for methylene urea and urea degradation. None of the structural analogs of methylene urea used in this study were hydrolyzed by MUase, neither were hydantoin and octopine which are unique carbon and nitrogen sources utilized in soil only by strains of *Rhizobium* sp. (Olivieri et al., 1981; Tempe and Petit, 1982). A structural analog of methylene urea, allantoic acid, is a purine degradation intermediate which is further hydrolyzed by specific enzymes, allantoicase (EC 3.5.3.4) and allantoate amidohydrolase (EC 3.5.3.9), to yield one mole of urea and ureidoglycolic acid (Vogels and van der Drift, 1976). According to Jahns et al. (1997) the methylene urea-degrading enzyme from *O. anthropi* was able to degrade allantoate at 5% of its MDU-degarding activity. However, the enzyme purified from *R. radiobacter* hydrolyzed urea at a slow rate, but could not hydrolyze allantoin or allantoic acid, showing this enzyme to be catalytically distinct as well as structurally unique.

Measuring the effect of known urease inhibitors on the ammonium and formaldehyde production tested the importance of the urease moiety in the MUase enzyme complex. Several classes of urease inhibitors are known to date; substrate analogs, hydroxyurea, hydroxamic acids, phosphoroamides, boric acid, thiols, and heavy metal ions (Mobley and Hausinger, 1989). Hydroxamic acids are good metal chelators, so the mechanism of action is assumed to involve binding to the active site nickel ion (Kobashi et al., 1962; Dixon et al., 1980). The inhibitory effect of boric acid is based on its binding to the urease metal center or covalent binding to other active site groups to form a tetrahedral complex (Breitenbach and Hausinger, 1988). Of the inhibitors tested in this study, only acetohydroxamate and boric acid at the concentration of 40 mM were able to block production of ammonium and formaldehyde completely. The inhibitory effect of boric acid on the formation of formaldehyde could possibly be explained by the non-enzymatic breakdown of MDU into urea and subsequent depletion of substrate at pH lower than 5.5 suggested by Jahns and Kaltwasser (2000). Since the formation of ammonium was not as dramatically affected by boric

acid, it is possible that the urease moiety of MUase was still partially active in the acidic medium.

Phenyl phosphorodiamidate and phenyl mercuriacetate, which are very potent soil urease inhibitors (Bremner and Douglas, 1971; Byrnes et al., 1983; Martens and Bremner, 1984a,b; Rao and Ghai, 1986) exhibited only partial inhibition of MUase. In comparison, Jahns et al. (1997) showed a nearly complete inhibition of *O. anthropi* MDUase by acetohydroxamate, boric acid and copper or mercury salts. In our study, heavy metal ions inhibited the production of formaldehyde completely but the adverse effect on ammonium production was only partial. This could be explained by the EDTA in the assay medium interfering with the availability of the heavy metal ions to the enzyme active sites but this does not explain the differences compared with the results by Jahns et al. (1997) who also were using 0.5 M EDTA in the buffer. Based on the effect of well-known urease inhibitors on the activity of the MUase from *R. radiobacter*, it seems obvious that the MUase holoenzyme contains a urease moiety. However, the data suggest that there is more than one type of enzyme involved in the hydrolytic action of MUase, and this enzyme complex theory is supported by the kinetic data indicating an allosteric effect with positive cooperation even though the non-Michaelis–Menten type of kinetics could also be a result from a use of an inhomogeneous substrate.

Our earlier studies showed that MUase from *R. radiobacter* is a cytosolic enzyme induced by using methylene urea as a sole nitrogen source in the growth medium (Koivunen et al., in preparation). Also, MUase was found in cells in extremely small quantities, and for characterization a high level of purification (2000-fold) was needed. Regardless of the small quantities of MUase in the cytosol, the hydrolytic activity seems to be high enough to support bacterial cell growth on methylene urea as its sole nitrogen source. This feature makes the ecological significance of methylene urea-hydrolyzing enzyme in the soil very interesting. Obviously, the function of MUase is connected to the cycling of organic nitrogen in the rhizosphere, and the plant pathogenic bacterium *R. radiobacter* is already known to be able to utilize unique carbon and nitrogen sources in the soil (Syldatk et al., 1990; Dessaux et al., 1992; Yadzi et al., 2000). According to Vissers et al. (1986) it can use arginine as the sole nitrogen source, and by doing this it is able to regulate futile urea cycle in cells by inhibiting ornithine carbamoyltransferase activity. It is also known for its ability to transport non-metabolizable opines into the cell via an active cellular mechanism (Krishnan et al., 1991). This so-called arginine transport pathway in *R. radiobacter* suggests that similar mechanisms might exist for transporting long-chain MU molecules into the cytosol.

The characterization of an MU-hydrolyzing enzyme purified from a soil microorganism, *R. radiobacter*, indicates that it is a novel holoenzyme consisting of moieties that resemble urease. However, its kinetic properties and high substrate selectivity make it unique among other ureases. The fact that MUase from *R. radiobacter* is different from the MU-hydrolyzing enzyme purified and characterized from *O. anthropi* is fascinating, and indicates that the enzyme responsible for MU degradation can be different depending on the location, ecosystem and microbial species present. Whether the use of MU can selectively enhance the growth of plant pathogenic *R. radiobacter* in the rhizosphere is an interesting question, which should be looked into in the future.

In the past, studies on methylene urea have been hampered by the low solubility of the polymers as well as the lack of pure substances and chemical methods for MU analysis. The novel mass spectrophotometer method for methylene urea analysis allows us to follow the hydrolysis of MU and to better understand the mechanism of action of this highly specific hydrolytic enzyme in the soil environment. The peptide-sequencing results will help us design molecular tools for cloning of this interesting enzyme. Finally, these results show the complex processes involved in regulating the release of N from a polymeric organic compound. Understanding the regulation of MUase activity in soil is very important in order to maximize the agronomic and environmental benefits of this slow-release nitrogen fertilizer.

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