Biosci. Biotechnol. Biochem., **75** (1), 159–161, 2011

Note

Characterization of an *Aspergillus oryzae* Cysteinyl Dipeptidase Expressed in *Escherichia coli*

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Received August 20, 2010; Accepted October 19, 2010; Online Publication, January 7, 2011
[doi:10.1271/bbb.100604]

Cysteinyl dipeptidase from *Aspergillus oryzae* (CdpA) was produced in *Escherichia coli* and purified. The enzyme showed activity specific toward cysteine-containing dipeptides, but its substrate specificity was distinct from those of other cysteinyl dipeptidases of the M20 family. It was optimally active at pH 7–8 and stable at pH 6–9 and at up to 40 °C.

Key words: *Aspergillus oryzae*; cysteinyl dipeptidase; metallopeptidase; characterization

Glutathione (gamma-glutamyl-cysteinylglycine) is a major thiol compound that plays various roles *in vivo*.[1)] Glutathione metabolism has been investigated because of its physiological importance, and it is thought to be conducted *via* the gamma-glutamyl cycle. The degradation of cysteinylglycine (Cys-Gly) is one of the steps in glutathione metabolism.[1)] To date, several enzymes have been reported to have hydrolyzing activity toward Cys-Gly. For example, Dug1p, which belongs to the metallopeptidase M20 family and has Cys-Gly hydrolyzing activity, was recently found in *Saccharomyces cerevisiae*.[2)] Other enzymes that are members of the M1, M17, and M19 families also have this function.[3–6)] Few studies have examined the degradation of glutathione, and there are no reports of Cys-Gly dipeptidase in filamentous fungi. In this study, we found that the CdpA of *A. oryzae* was active toward cysteine-containing dipeptides, and that its characteristics were distinct from those of other cysteinyl dipeptidases.

Using a BLAST search against the genome database of *A. oryzae* (http://www.bio.nite.go.jp/dogan/project/view/AO), we found an ortholog of *DUG1* and designated it *cdpA* (cysteinyl dipeptidase in *A. oryzae*) (AO090020000015). The length of the coding sequence was 1,437 bp, encoding 478 amino acids with a calculated molecular mass of 52.9 kDa. Expression plasmid pCold I (Takara Bio, Otsu, Japan) was used for the production of CdpA carrying an N-terminal His_6 tag in *E. coli* strain BL21 cells (Takara Bio). Expression plasmid pCold-cdpA was constructed as follows: *cdpA* cDNA was PCR amplified using primers 5′-GCTCGGTACCCTCGAGATGGCACCACAGCTGGAACCATTT-3′ and 5′-GCAGAGATTACCTATCTATGCCGCCACCATGGGCTCTTCT-3′. The underlined sequences are specific to the multi cloning site of pCold I. The cDNA pool synthesized from *A. oryzae* grown in YPD at 30 °C for 20 h[7)] was used as template. The amplified fragment was cloned into pCold I using an In-Fusion Advantage PCR Cloning Kit (Takara Bio). Cells of *E. coli* BL21 harboring pCold-cdpA were harvested by centrifugation, washed with buffer A (20 mM Tris–HCl buffer pH 7.5 containing 300 mM NaCl and 20 mM imidazole), and sonicated in the same buffer. The lysate was centrifuged, and the supernatant was absorbed into Ni-IMAC gel (Bio-Rad Japan, Tokyo, Japan). The resin was washed with buffer A, followed by elution of the protein with buffer B (20 mM Tris–HCl buffer pH 7.5 containing 300 mM NaCl and 100 mM imidazole). The purified protein was collected, concentrated, and desalted. The protein was confirmed to be CdpA by peptide mass fingerprinting (data not shown). SDS–PAGE analysis using 10% w/v polyacrylamide gels revealed that the molecular mass of the monomeric CdpA was 53 kDa. This is consistent with the size of the monomer as predicted from the cDNA sequence. The molecular mass of purified CdpA was determined using a Superose 12 gel filtration column (column size, 1.0 × 30 cm; flow rate, 0.5 mL/min) (GE Healthcare, Buckinghamshire, UK) pre-equilibrated with buffer D (20 mM Tris–HCl pH 7.5, 150 mM NaCl). A gel filtration calibration kit (GE Healthcare) was used for calibration. The gel filtration chromatography profile of CdpA showed a single peak at 109 kDa, suggesting that purified CdpA exists as a homodimer.

CdpA activity was determined using various peptide substrates. For the standard reaction, 1.5 mU CdpA (0.5 μg in a reaction volume of 150 μL) and 1 mM Cys-Gly were incubated in 20 mM Tris–HCl pH 7.0, for 15 min at 30 °C. The amount of L-cysteine was measured as described previously.[8)] L-Cysteine concentrations (measured by the optical density at 560 nm) were

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calculated from a standard curve produced with known amounts of L-cysteine. To determine the hydrolyzing activity of CdpA toward Ala-Gly and Gly-Ala, the reactions of other amino groups and ninhydrin were measured by color development at an absorbance of 570 nm. The enzyme activity was defined in terms of units (U), where 1 U was the amount of enzyme that released 1 μmol of L-cysteine per min. Because CdpA is an ortholog of Dug1p, it is possible that CdpA is involved in glutathione metabolism as a Cys-Gly dipeptidase in *A. oryzae*. Hence, we defined CdpA activity toward Cys-Gly as the standard. The specific activity, K_m value, and k_{cat} of the standard reaction were 3.04 ± 0.03 U/mg, 0.23 ± 0.10 mM, and 5.55 ± 0.91 s^{-1} respectively. When tested with peptide substrates, CdpA was most active toward Ala-Cys, followed by Leu-Cys, Cys-Gly, and Cys-Ala in decreasing order of activity. CdpA did not hydrolyze cysteinyl tripeptides (Table 1), and no activity toward cysteine-free dipeptides Ala-Gly and Gly-Ala was detected (data not shown). These results indicate that CdpA activity is specific to dipeptides containing cysteine. Among cysteinyl dipeptidases, Dug1p and CNDP2 (the human ortholog of Dug1p) showed highest activity toward Cys-Gly.[9] CdpA hydrolyzed Cys-Ala (this study), whereas Dug1p and CNDP2 does not.[9] Therefore, the substrate specific-

Table 1. Activity of CdpA toward Peptide Substrates

Substrate	Relative activity ± SD (%)[a]
Cys-Gly	100.0 ± 0.9
Cys-Ala	95.0 ± 4.2
Cys-Asp	81.6 ± 1.3
Gly-Cys	44.5 ± 7.2
Ala-Cys	161.0 ± 12.4
Leu-Cys	131.8 ± 5.8
Cys-Gly-Gly	ND[b]
Gly-Cys-Gly	ND
gamma-Glu-Cys-Gly	ND

[a]CdpA activity toward Cys-Gly was taken as 100%.
SD, standard deviation.
[b]ND, not detected.
Results represent the means for three independent experiments.

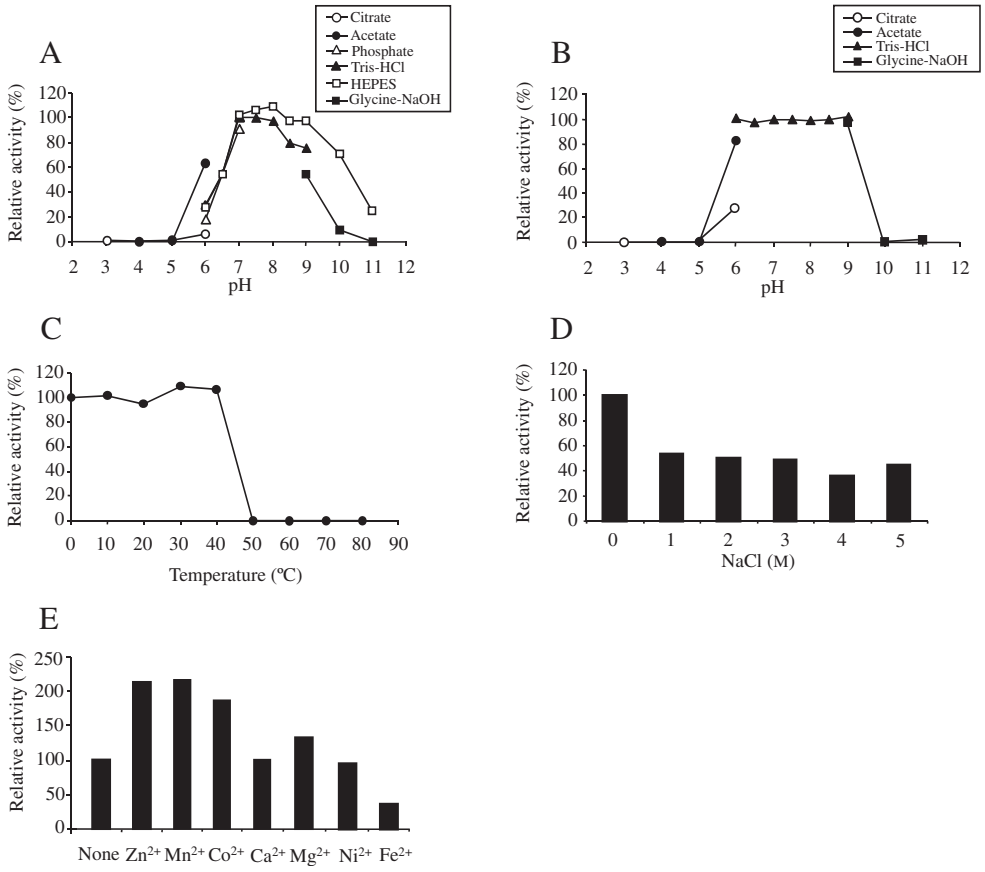

Fig. 1. Biochemical Characterization of CdpA.

A, The pH optimum was determined in 20 mM citrate buffer (pH 3–6), 20 mM acetate buffer (pH 4–6), 20 mM phosphate buffer (pH 6–7), 20 mM Tris–HCl buffer (pH 6–9), 20 mM HEPES buffer (pH 6–11), or 20 mM glycine-NaOH buffer (pH 9–11) at 30 °C for 15 min. Relative activity was calculated relative to the standard reaction (20 mM Tris–HCl pH 7.0, for 15 min at 30 °C). B, The pH stability of CdpA was determined by preincubation of the enzyme in 20 mM citrate buffer, 20 mM acetate buffer, 20 mM Tris–HCl buffer, or 20 mM glycine-NaOH buffer at 30 °C for 30 min, followed by the standard enzyme reaction. Relative activity was calculated relative to a sample maintained at pH 7.0. C, The thermal stability of CdpA was determined by preincubation of the enzyme at 0–80 °C for 30 min, followed by the standard enzyme reaction. Relative activity was calculated relative to a sample preincubated at 0 °C. D, The effect of NaCl on CdpA dipeptidase activity was determined by preincubating the purified enzyme in 20 mM Tris–HCl buffer (pH 7.0) containing 0–5 M NaCl for 30 min at 30 °C, followed by the standard enzyme reaction. Relative activity was calculated relative to a sample preincubated at 0 M NaCl. E, The effects of metal ion salts ($FeCl_2$, $NiCl_2$, $MnCl_2$, $CaCl_2$, $MgCl_2$, and $ZnCl_2$) on enzyme activity were measured in 20 mM Tris–HCl pH 7.0. The enzyme was preincubated with the various metal ion salts at 0.1 mM for 30 min at 30 °C, and its activity was measured for 15 min at 30 °C using a standard enzyme assay in the presence of the metal ion salt at 0.1 mM. Relative activity was calculated relative to a sample in the absence of metal ions. The results shown in A–E are the means of three independent experiments.

ity of CdpA is distinct from those of other known Cys-Gly dipeptidases in the M20 family. Aminoacyl-*p*-nitroanilide-hydrolyzing activities were determined by the method of Exterkate.[10] CdpA hydrolyzed only Leu-*p*NA (5.11 ± 0.32 mU/mg) among the tested synthetic substrates. Some aminopeptidases have been reported to have activity toward Cys-Gly,[11] and some leucine aminopeptidases in the M17 family have high activity toward Cys-Gly.[5,12]

The optimal pH range of CdpA for hydrolyzing Cys-Gly was pH 7–8 (Fig. 1A), and CdpA was stable in a pH range of 6–9 (Fig. 1B). CdpA remained stable at temperatures up to 40 °C (Fig. 1C). When the effect of salt concentration on CdpA activity was examined, CdpA activity with 1 M NaCl was half the level without NaCl, and the activity in a range of 1–5 M NaCl was nearly constant (Fig. 1D).

Next, we examined the influence of metal ions on the activity of CdpA (Fig. 1E). Metal ions Mn^{2+}, Zn^{2+}, and Co^{2+} increased CdpA activity relative to control (no ion added). Mg^{2+}, Ca^{2+}, and Ni^{2+} influenced CdpA activity only slightly. Fe^{2+} reduced activity.

The effect of inhibitors on enzyme activity was measured in 20 mM Tris–HCl (pH 7.0). The purified enzyme was incubated with each compound for 30 min at 30 °C, and its activity was measured by standard enzyme assay containing each compound. CdpA activity was not detected in the presence of 10 mM ethylenediaminetetraacetic acid (EDTA). Bestatin (10 μM) reduced the activity to $50.2 \pm 16.5\%$. While pepstatin A (10 μM) did not reduce the activity, leupeptin (40 μM), E-64 (25 μM), and benzamidine (1 mM) reduced it to $61.1 \pm 5.0\%$, $71.3 \pm 1.0\%$, and $79.8 \pm 6.1\%$ of control respectively.

The complete inhibition of enzyme activity by EDTA clearly indicates a metal ion requirement for CdpA activity. Serine protease inhibitor and cysteine protease inhibitor somewhat reduced the activity.

The results of our study suggest that CdpA is a metallopeptidase with a substrate specificity different from other cysteinyl dipeptidases in the M20 family.

Acknowledgments

We greatly appreciate the help of Dr. Hiroto Morita of Tokyo University of Agriculture and Technology in peptide-mass finger printing. This study was supported by the Program for Promotion of Basic Research Activities for Innovative Biosciences (PROBRAIN).

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