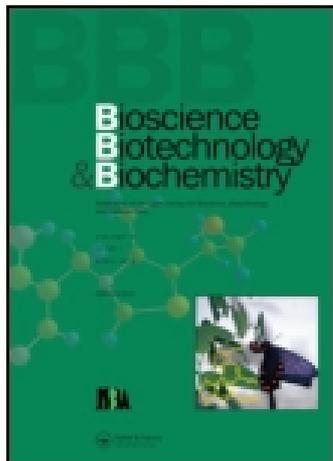


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Note

Synthesis of Hydroxymethylglutathione from Glutathione and L-Serine Catalyzed by Carboxypeptidase Y

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Hydroxymethylglutathione (γ -L-glutamyl-L-cysteinyl-L-serine; hmGSH) occurs in many species belonging to the family Gramineae, but the biosynthetic pathway for hmGSH has not been identified. We found that carboxypeptidase Y (CPY), but not carboxypeptidase A, catalyzed hmGSH synthesis from glutathione and L-serine *in vitro* at acidic pH. CPY also catalyzed methylglutathione synthesis from glutathione and L-alanine. These findings suggested that a carboxypeptidase-like enzyme may be involved in hmGSH synthesis *in vivo*.

Key words: aminolysis; carboxypeptidase Y; glutathione; hydroxymethylglutathione; methylglutathione

Hydroxymethylglutathione (γ -L-glutamyl-L-cysteinyl-L-serine; hmGSH) occurs in many species belonging to the family Gramineae, including rice, wheat, and barley.^{1,2)} The glycine residue in GSH is replaced with an L-serine residue in hmGSH. It was thought earlier that hmGSH is synthesized from γ -L-glutamyl-L-cysteine (γ -GluCys) and L-serine *via* a reaction similar to that for GSH synthesis, but there has been no evidence of the involvement of a GSH synthetase-like enzyme.

Carboxypeptidase Y (CPY, EC 3.4.16.5) is a yeast vacuolar carboxypeptidase that catalyzes the removal of the C-terminal amino acid from peptides.³⁾ In addition, CPY has aminolytic activity by replacing C-terminal amino acid of the peptide with a free amino acid.^{4,5)} Therefore, CPY seems to participate in the turnover and proteolytic processing of proteins.⁶⁾ Both hydrolysis and aminolysis involve enzyme-peptide complex formation and deacylation of the complex by such nucleophiles as water and free amino acids, respectively. Phytochelatins also are synthesized from GSH by a carboxypeptidase-catalyzed reaction.^{7,8)} We have attempted to synthesize hmGSH with CPY. In this communication, we describe hmGSH synthesis from GSH and L-serine

catalyzed by CPY.

hmGSH for use as the standard was synthesized by the solid-phase method from 9-fluorenylmethoxycarbonyl (Fmoc)-L-glutamate- α -*tert*-butylester, Fmoc-S-*p*-methoxybenzyl-L-cysteine, and Fmoc-*O*-*tert*-butyl-L-serine-Alko resin.⁹⁾ The synthesized hmGSH was examined by LC-MS (Shimadzu LCMS-2010A); the observed m/z (338) agreed with that of the protonated molecular ion ($[M+H]^+$) of hmGSH. hGSH was prepared by the solid-phase method also.¹⁰⁾ γ -GluCys was a product of Kohjin Co., Ltd. (Tokyo, Japan).

The standard reaction mixture (final volume 100 μ l) contained 40 mM 2-morpholinoethanesulfonic acid (MES)-NaOH buffer (pH 5.5), 2 mM EDTA, 0.2 mM dithiothreitol, 1 mM GSH, 50 mM L-serine, and 3 μ g of CPY (Oriental Yeast Co., Ltd., Tokyo). After incubation of the reaction mixture for 30 min at 30°C, 220 μ l of 200 mM Tris-HCl (pH 8.0) and 15 μ l of 15 mM monobromobimane were added.¹¹⁾ Derivatization with monobromobimane was stopped by the addition of 310 μ l of 10% acetic acid after 15 min of incubation at 30°C. A portion (20 μ l) of the derivatized sample was injected into an HPLC equipped with a Wakopak Handy ODS column (4.6 \times 150 mm). Solution A (methanol/water/acetic acid, 10:90:0.25 by volume, pH 3.9) and solution B (methanol/water/acetic acid, 90:10:0.25 by volume, pH 3.9) were used as the standard mobile phase at a ratio of 98:2, and the flow rate was 1 ml/min. Fluorescence of the monobromobimane derivatives was detected at 480 nm with excitation at 380 nm. Standard curves were made with authentic γ -GluCys and hmGSH.

When GSH was incubated with a high concentration of L-serine in the presence of CPY, two new peaks, 1 and 2, appeared (Fig. 1). The retention times of peaks 1 and 2 were consistent with those of the derivatives of authentic γ -GluCys and hmGSH with monobromobimane, respectively. In addition, the retention times of the peaks predicted to be γ -GluCys

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Abbreviations: CPY, carboxypeptidase Y; γ -GluCys, γ -L-glutamyl-L-cysteine; Fmoc, 9-fluorenylmethoxycarbonyl; hGSH, homogluthathione; hmGSH, hydroxymethylglutathione; MES, 2-morpholinoethanesulfonic acid; mGSH, methylglutathione; PMSF, phenylmethylsulfonyl fluoride

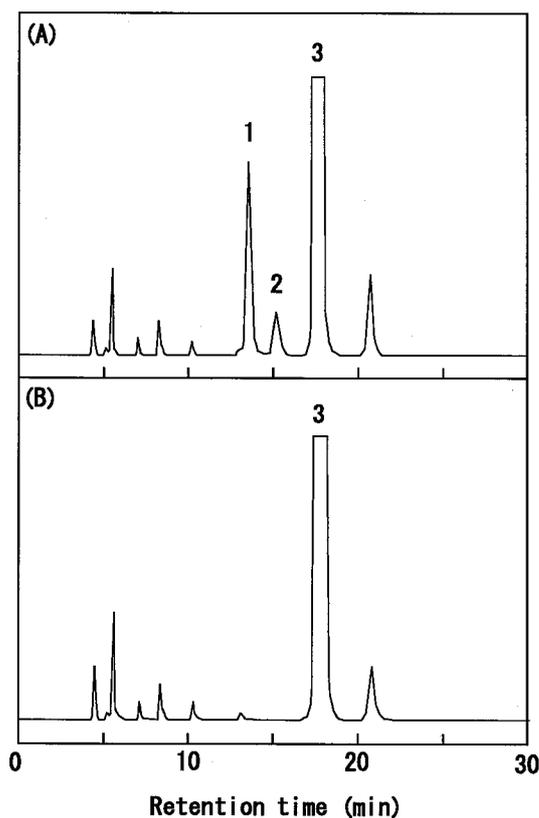


Fig. 1. HPLC Profiles of Reaction Products Obtained with Active (A) and Heat-denatured (B) CPY.

The standard reaction mixture described in the text was used for A and the active CPY was replaced with the heat-denatured CPY for B. Peak 1, γ -GluCys; peak 2, hmGSH; peak 3, GSH.

and hmGSH derivatives also were consistent with those of authentic γ -GluCys and hmGSH derivatives, respectively, when a gradient of 5–20% methanol in 20 mM Na_2HPO_4 adjusted to pH 6.0 with acetic acid was used as the mobile phase.¹⁾ However, the peaks corresponding to γ -GluCys and hmGSH derivatives did not appear when heat-denatured CPY was used, or when no CPY was used (not shown). When D-serine was the substrate, aminolysis catalyzed by CPY did not proceed. These findings indicate that hmGSH was synthesized stereospecifically from GSH and L-serine by the enzyme reaction catalyzed by CPY. In contrast, carboxypeptidase A (EC 3.4.17.1) from bovine pancreas did not have any activity causing hmGSH synthesis from GSH and L-serine at acidic or basic pH, although it catalyzed the hydrolysis of GSH into γ -GluCys.

The amount of hmGSH increased linearly with the reaction time for 1 h, followed by a slow increase up to 4 h (Fig. 2). After this time, the amount of hmGSH formed became constant. In contrast, the amount of γ -GluCys continued to increase linearly for 5 h. The ratio of hmGSH to γ -GluCys was approximately 1:5 under the reaction conditions used.

More hmGSH was synthesized at acidic than at basic pH; a small amount of hmGSH was detected at

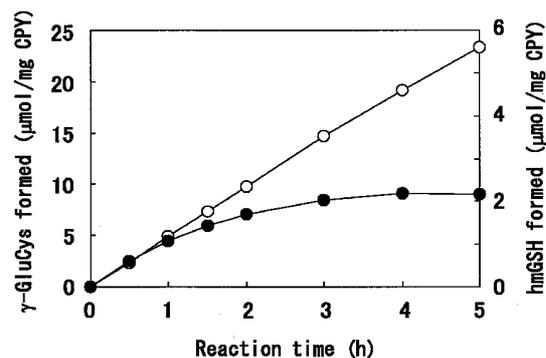


Fig. 2. Changes with Time in Formation of γ -GluCys and hmGSH Catalyzed by CPY.

The standard reaction mixture was incubated for the times indicated, and γ -GluCys and hmGSH were measured as described in the text. γ -GluCys (\circ); hmGSH (\bullet).

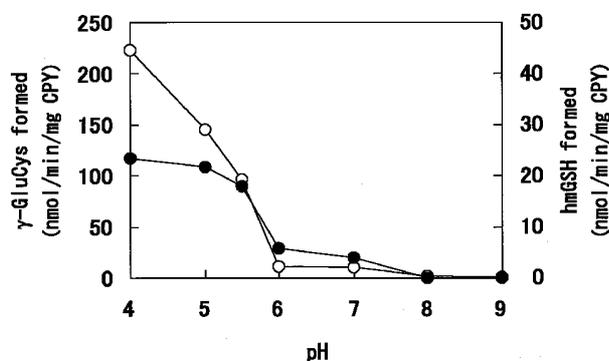


Fig. 3. Effects of pH on the Formation of γ -GluCys and hmGSH Catalyzed by CPY.

The following buffers were used instead of 40 mM MES-NaOH buffer (pH 5.5) in the standard reaction mixture; 40 mM acetate buffer (pH 4.0), 40 mM MES-NaOH buffer (pH 5–6), 40 mM HEPES-NaOH buffer (pH 7.0) and 40 mM Tris-HCl buffer (pH 8–9). γ -GluCys (\circ); hmGSH (\bullet).

pH 7.0 (Fig. 3). The amounts of hmGSH formed were almost constant at pH 5.5 or below. The amounts of γ -GluCys increased with decreasing pH. In general, CPY-catalyzed aminolysis proceeds at basic pH, but the optimum pH for the hydrolysis is 5.5–6.5.^{4,5,12)} However, our findings on hmGSH synthesis differ from those of other aminolytic reactions reported. Perhaps the amino group of the amino acid that would otherwise act as the nucleophile is protonated at acidic pH. A protonated amino group can not act as the nucleophile needed to attack the acyl-enzyme intermediate, so aminolysis seldom occurs at acidic pH. When the enzyme-substrate (CPY- γ -GluCys) complex forms, however, the three-dimensional structure of CPY may be changed, resulting in aminolysis regardless of the scarcity of a nonionized amino group of the amino acid as a nucleophile.

Higher L-serine concentrations in the reaction mixture led to more hmGSH synthesized (Fig. 4), but the amount of γ -GluCys formed was roughly con-

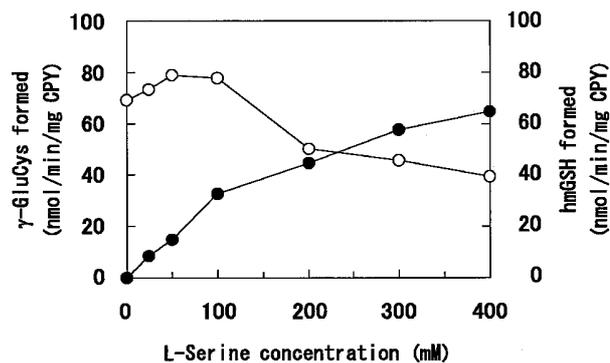


Fig. 4. Effects of L-Serine Concentration on Formation of γ -GluCys and hmGSH Catalyzed by CPY.

L-Serine concentrations in the reaction mixture were in the range of 0 to 400 mM. γ -GluCys (\circ); hmGSH (\bullet).

stant in the range of 0–100 mM L-serine and decreased at higher concentrations of L-serine. At L-serine concentrations higher than 200 mM, the amount of hmGSH formed exceeded that of γ -GluCys. However, the K_m for L-serine was 330 mM, which was fairly high value. This high K_m may be a reason why yeast cells do not synthesize hmGSH.

When CPY (50 μ g in 100 μ l) was incubated with 1 mM phenylmethylsulfonyl fluoride (PMSF) for 30 min at 30°C and then excess PMSF was removed by gel filtration with Sephadex G-25, the enzyme activity of CPY was completely inhibited. This finding indicates that the serine residue in CPY was essential for aminolysis. CPY catalyzed the synthesis of other tripeptides (γ -GluCys-amino acids) from GSH and free amino acids. When L-alanine was used, methylglutathione (γ -L-glutamyl-L-cysteinyl-L-alanine; mGSH) was synthesized. In contrast, D-alanine did not act as a substrate. The synthesis of mGSH was confirmed by LC-MS analysis; m/z of the monobromobimane derivative of mGSH was 534 ($[M+Na]^+$) and 556 ($[M+2Na-H]^+$). The K_m for L-alanine was 800 mM, which accounts for the absence of this tripeptide in yeast cells. When L-threonine and L-glutamic acid were added to the reaction mixture instead of L-serine, novel peaks with a thiol group were found by HPLC, which peaks were presumably γ -L-glutamyl-L-cysteinyl-L-threonine and γ -L-glutamyl-L-cysteinyl-L-glutamic acid, respectively. On the other hand, D-isomers of threonine and glutamic acid did not give the desired reaction products. Nor did β -alanine act as a substrate for hGSH synthesis. Therefore, CPY can catalyze the synthesis of some GSH homologues from GSH and L-forms of α -amino acids.

Table 1 shows the relative retention times and fluorescence intensities of the monobromobimane derivatives of GSH and related compounds including hmGSH under the HPLC conditions used here. We could easily measure the retention times of the monobromobimane derivatives of GSH, hGSH, and

Table 1. Retention Times and Fluorescence Intensities of Monobromobimane Derivatives of GSH and Related Compounds

Compound	Relative retention time	Relative intensity
γ -GluCys	0.77	1.09
hmGSH	0.86	0.93
GSH	1.00	1.00
mGSH	1.25	— ^{a)}
hGSH	1.62	0.95

^{a)} not determined.

hmGSH, all of which occur in the plant kingdom.

Gramineous plants such as barley, wheat, and rice have multiple forms of serine-type carboxypeptidase.^{13–16} When we measured the enzyme activity toward hmGSH synthesis using a crude extract of rice leaves, hmGSH was formed from GSH and L-serine, probably by an aminolysis reaction of carboxypeptidase (not shown). These findings suggested that carboxypeptidase or a carboxypeptidase-like enzyme may be involved in hmGSH synthesis *in vivo*.

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