Optimization of Reaction Conditions for \( \gamma \)-Glutamylcysteine Production from Glutathione Using a Phytochelatin Synthase-Like Enzyme from \textit{Nostoc} sp. Pasteur Culture Collection 7120

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\( \gamma \)-Glutamylcysteine (\( \gamma \)-EC) has antioxidant properties similar to those of glutathione (GSH) and acts as its precursor in mammals. There are a few procedures for the production of \( \gamma \)-EC, such as chemical synthesis or enzymatic synthesis from glutamate and cysteine; however, they are very costly and not suitable for industrial production. A phytochelatin synthase-like enzyme derived from \textit{Nostoc} sp. Pasteur Culture Collection 7120 (NsPCS) catalyzes the hydrolysis of GSH to \( \gamma \)-EC and glycine in the absence of ATP or other additives. Our research aims to establish an alternative \( \gamma \)-EC production procedure with low cost and high productivity. To this end, we optimized the reaction conditions of NsPCS and characterized its properties in this study. We found that 200 mM potassium phosphate buffer, pH 8.0, at 37 °C, had the highest NsPCS activity among the conditions we tested. Under these conditions, NsPCS had a \( K_m \) of 385 \( \mu \)M and a \( V_{\text{max}} \) of 26 mol/min/mg protein. In addition, NsPCS converted 100 mM GSH into \( \gamma \)-EC with high yields. These results suggest that the NsPCS reaction has great potential for the low-cost, industrial-scale production of \( \gamma \)-EC.

Key words \( \gamma \)-glutamylcysteine; glutathione; reaction conditions optimization; phytochelatin synthase-like enzyme

INTRODUCTION

Phytochelatin synthase (PCS, EC 2.3.2.15) is a glutamylcysteine-dipeptidyl-(trans)peptidase. PCSs are widespread in eukaryotes, including higher plants, algae, and fungi.1 In contrast, very few homologs of eukaryotic PCS genes have been reported in prokaryotes, such as cyanobacteria and bacteria.2) We previously characterized alr0975, a gene in \textit{Nostoc} sp. Pasteur Culture Collection (PCC) 7120 that encodes a PCS-like enzyme termed NsPCS. It contains the conserved N-terminal domain of eukaryotic PCSs, which shares 36% protein sequence identity with \textit{Arabidopsis thaliana} PCS1 (AtPCS1, Accession No. At5g44070).3) Eukaryotic PCSs commonly require heavy metals, particularly cadmium, and catalyze the synthesis of phytochelatin (PC) from glutathione (GSH) in a two-step reaction4) (Fig. 1a). In the first step, \( \gamma \)-glutamylcysteine \( (\gamma \text{-EC}) \) is formed by cleaving \( \text{L-} \)glycine (Gly) from GSH. In the second step, \( \gamma \text{-EC} \) is transferred to GSH to form \( (\gamma \text{-EC})_2 \text{-Gly (PC)}_2 \). This \( \gamma \text{-EC} \)-transfer reaction is subsequently repeated to produce \( \text{PC}_{n+1} \) from \( \text{PC}_n \). These reactions do not occur in the absence of heavy metals. In contrast, NsPCS catalyzes only the first reaction of eukaryotic PCSs, cleaving Gly from GSH to produce \( \gamma \text{-EC} \).5) Furthermore, NsPCS does not require heavy metals.5) GSH is the most abundant low-molecular-weight thiol and is widely found among both prokaryotes and eukaryotes. It plays significant roles in antioxidant defense and xenobiotic removal, and acts as an immune booster in higher eukaryotes.7–9) Specifically, it scavenges free radicals and reactive oxygen species directly and indirectly through enzymatic reactions. GSH is used as a pharmaceutical compound and can also be used in food additives and cosmetics. It is synthesized from \text{l-}cysteine (Cys) in two ATP-dependent steps \textit{in vivo}. In the first step, glutamate-cysteine ligase (GCL) catalyzes the formation of \( \gamma \text{-EC} \) from Cys and \text{l-}glutamate (Glu) (Fig. 1b). In the second reaction, glutathione synthetase (GS) catalyzes the formation of GSH from \( \gamma \text{-EC} \) and Gly.10)

As shown in Fig. 1a and 1b, \( \gamma \text{-EC} \) is an intermediate structure in the synthesis of both PC and GSH. Since \( \gamma \text{-EC} \) consists of a \text{γ-glutamyl} conjugation and a Cys residue, this dipeptide is assumed to have a function similar to that of GSH.11) Although cultured fibroblasts from patients with GS deficiency have low levels of GSH, instead they accumulate \( \gamma \text{-EC} \) to compensate for GSH in the cellular defense against oxidative stress.12) Gamma-EC can also function as a source of GSH. It was reported that oral administration of \( \gamma \text{-EC} \) increased GSH concentrations in human lymphocytes.13) In addition, \( \gamma \text{-EC} \) was shown to have a neuroprotective function in the rat hippocampus after status epilepticus.14)

These studies suggest that \( \gamma \text{-EC} \) may not only act as a GSH source with comparative antioxidant properties, but also as a novel neuroprotective peptide in mammals. To identify such functions with useful applications, and to be able to produce \( \gamma \text{-EC} \) at similar industrial scales to those of GSH, stable and cost-effective \( \gamma \text{-EC} \) production procedures must be established because the current production of \( \gamma \text{-EC} \) is very costly.

Since the rate-limiting step of GSH synthesis is the reaction catalyzed by GCL, \( \gamma \text{-EC} \) is immediately used for GSH synthesis by GS and is rarely found in mammals. Therefore,
production by fermentation using yeast, normally used for the industrial production of GSH, is difficult.

There are a few procedures for the production of γ-EC other than fermentation. Gamma-EC is currently produced from Glu and Cys by a chemical method commonly used for peptide synthesis. However, this is costly and not suitable for industrial production since this method requires complex protection steps for the substrate to maintain the specific conjugating reaction, such that the production yield is very low. Consequently, chemically produced γ-EC is very expensive at a commercial level. The in vitro GCL-mediated production of γ-EC from Glu and Cys is possible. However, it is also assumed to be very costly, since GCL is an ATP-dependent ligase that not only requires the substrates, Cys and Glu but also ATP and Mg²⁺. In addition, GCL existing in animal and plants is known to consist of multi-subunits and its regulation is very complex.

On the contrary, NsPCS-mediated production is expected as an alternative enzymatic production of γ-EC, because NsPCS is a single protein peptidase that specifically hydrolyzes the Cys–Gly conjugation of GSH and requires only GSH as a substrate to produce γ-EC, without any regulation by other factors. In this study, our aim was to optimize NsPCS reaction conditions, and evaluate the feasibility of this reaction for stable and cost-effective production of γ-EC.

MATERIALS AND METHODS

Preparation of NsPCS The plasmid pET25b-alr0975, previously constructed from Tsuji et al., was used to express NsPCS in Escherichia coli. Recombinant NsPCS was expressed in E. coli C43 (DE3) (Lucigen, U.S.A.) transformed with pET25b-alr0975 at 20°C in Terrific Broth (TB) (Invitrogen, U.S.A.). Protein expression was induced at mid-log phase with pET25b-alr0975 at 20°C in Terrific Broth (TB) (Invitro) and expressed in C43 (DE3) (Lucigen, U.S.A.) transformed with pET25b-alr0975 at 20°C in Terrific Broth (TB) (Invitrogen, U.S.A.).

Fig. 1. Reaction Pathways of (a) Phytochelatin Synthase (PCS), Phytochelatin Synthase-Like Enzyme Derived from Nostoc sp. Pasteur Culture Collection 7120 (NsPCS), (b) Glutamate-Cysteine Ligase (GCL), and Glutathione Synthetase (GS)

disrupted by sonication in Buffer A (50 mM Tris–HCl buffer at pH 8.0, 100 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), and 1 mM diethylothreitol (DTT)). The supernatant obtained after clarification at 18000 × g for 30 min was filtered through a 0.22-μm membrane. The lysate was applied to a 5 mL HiTrap Q HP column (Cytiva, Japan) equilibrated with Buffer B (50 mM Tris–HCl buffer at pH 8.0, 10 mM NaCl, 1 mM EDTA, and 1 mM DTT). After washing twice with 10 mL Buffer B, elution was carried out with Buffer C (50 mM Tris–HCl buffer at pH 8.0, 1 mM NaCl, 1 mM EDTA, and 1 mM DTT). The flowthrough fraction was reapplied to the column four times. The flowthrough and wash fractions were concentrated using Amicon Ultra-15 Centrifugal Filter Units (Merck Millipore) and dialyzed against 50 mM Tris-HCl buffer at pH 8.0 containing 100 mM NaCl, 5 mM tris(2-carboxyethyl)phosphine (TCEP), and 25% glycerol. The volume of the supernatant obtained after centrifugation (18000 × g for 60 min) was 22 mL. The homogeneity of the purified protein was confirmed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis. The darkest band corresponded to 26 kDa, the predicted molecular weight of NsPCS. The purity of the purified protein was 50%, as estimated by the intensity of the band using bovine serum albumin as a standard.

Enzyme Assays The basal reaction conditions for AtPCS1 have been described by Owen et al. Here, unless indicated reactions were conducted under the conditions described below. Each 100-μL reaction contained 10 mM β-mercaptoethanol (β-ME), 200 mM Tris–HCl buffer at pH 8.0, 10 mM GSH, and 0.5 μg recombinant NsPCS. Reactions were incubated at 37°C for 60 min and terminated by the addition of 100 μL of 3.6 N HCl. The supernatant obtained after centrifugation was analyzed using HPLC. Concentrations of γ-EC produced per unit time are described as NsPCS activity. Temperature, use of reductant, and the concentration and pH of the buffer were the variables optimized in this study.

HPLC HPLC was conducted as described by Hirata et al. Reactions were centrifuged (15000 × g for 10 min at 4°C) and 20 μL of the supernatant was diluted with water to 200 μL. One hundred microliters of each sample was injected onto a reverse-phase column (Inertsil ODS-3, 5 μm, 4.6 × 250 mm, GL Sciences, Japan) attached to a modified HPLC post-column system (Hitachi, Japan) and equilibrated with Buffer A (5 mM sodium 1-octanesulfonate solution containing 0.02% trificluorooacetic acid (TFA)). Thiols were eluted with a gradient of Buffer B (30% acetonitrile solution containing 0.02% TFA) at a flow rate of 1.5 mL/min at 40°C. Buffer B was raised from 0 to 13% in 1 min, then to 70% in the following 19 min, and then to 100% in 1 min, which was kept for 7 min. Eluted thiols were mixed with 75.7 μM 5′-dithiobis(2-nitrobenzoic acid) (DTNB) in 50 mM potassium phosphate buffer (pH 8.0) containing 10% acetonitrile at 40°C and quantified by absorbance at 412 nm (Hitachi). GSH and γ-EC, purchased from Sigma-Aldrich, U.S.A., were used as standards for determining their respective concentrations.

Statistical Analysis Data are presented as means ± standard deviations (S.D.). In the evaluation of reductant effects, Tukey’s test was used to identify significant differences between groups. In the study on the effects of reaction buffer, unpaired Student’s t-test was performed between groups with different types of buffer salts at same pH and between groups with different concentrations of the same buffer salt at same
pH, and Bonferroni correction was conducted for multiple tests (total 13 combinations in the set). The threshold for significance was set at $p < 0.05$.

**RESULTS**

**Optimization of NsPCS Reaction** Based on the conditions used for the AtPCS1 reaction (200 mM Tris–HCl buffer solution at pH 8.0, 10 mM β-ME), reaction conditions were optimized in the following order: temperature, addition of reductant, and concentration and pH of the buffer solution. The NsPCS activities obtained under these conditions are shown relative (relative activity, RA) to those under basal conditions.

**Temperature** First, the effect of temperature on NsPCS activity was examined. As shown in Fig. 2a, the highest activities were observed at 30 and 37°C. Therefore, we used 37°C as the optimal temperature in subsequent experiments.

**Addition of Reductant** Since γ-EC is susceptible to oxidative degradation, an addition of reductant to NsPCS reactions is required to protect γ-EC from oxidative degradation. Therefore, the effect of reductant was examined. Since the AtPCS1 reaction requires β-ME and hardly occurs without it, NsPCS activity in the presence and absence of 10 mM β-ME was measured. Figure 2b shows that NsPCS activity was 10 times higher in the absence of β-ME than that with β-ME. When TCEP, commonly used as a non-thiol reductant, was added instead of β-ME, the activity was the same as that without TCEP addition. In subsequent experiments, 1 mM TCEP was added.

**Buffer Conditions** Lastly, the effects of the concentration and pH of the reaction buffer were examined. Two different concentrations, 10 and 200 mM, of sodium acetate buffer at pH 4.0, 5.0, and 6.0, potassium phosphate buffer at pH 6.0, 7.0, and 8.0, and Tris–HCl buffer at pH 8.0, 9.0, and 10.0 were tested. Figure 2c shows a tendency toward higher activities at 200 mM than at 10 mM in all buffers. The highest activity was obtained with 200 mM potassium phosphate buffer at pH 8.0. The optimal pH was not different from that for the AtPCS1 reaction, but activity was significantly higher in potassium phosphate buffer than in Tris–HCl buffer, the optimal buffer for AtPCS1.

**NsPCS Enzymatic Parameters** To evaluate the ability of NsPCS to convert GSH to γ-EC, enzymological characterization was conducted. As shown in Fig. 3, under the optimized conditions described above, crude NsPCS was reacted with GSH at 125, 250, 500, 750, 1000, 2500, and 5000 μM for 5 min. By plotting the initial reaction rate of γ-EC production at each GSH concentration, $K_m$ of 385 μM and a $V_{max}$ of 26 mol/min/mg-protein were obtained using the Lineweaver–Burk plot.

**Fig. 2. Phytochelatin Synthase-Like Enzyme Derived from Nostoc sp. Pasteur Culture Collection 7120 (NsPCS) Activities Obtained Under the Tested Conditions Relative to Those Under Basal Conditions**

(a) NsPCS activity as a function of temperature. The activity at 37°C was set as 100%, with activities at 4, 15, 25, 30, and 45°C presented as relative activities (RAs). Data are expressed as means ± S.D. (n = 3). (b) Effect of reducing agents on NsPCS activity. Activity in the presence of 10 mM β-mercaptoethanol (β-ME) was set as 100%, with activities without reductant (NONE), and in the presence of 1 and 10 mM tris(2-carboxyethyl)phosphine (1-TCEP and 10-TCEP) presented as RAs. Data are expressed as means ± S.D. (n = 3). Different letters indicate significant differences at $p < 0.05$. (c) Effect of buffer and pH on NsPCS activity. Production of γ-EC in 200 mM Tris–HCl buffer (200 TH) at pH 8.0 was set as 100%, with production in different buffers; 10 mM Tris–HCl buffer (10 TH), 200 and 10 mM potassium phosphate buffer (200 PP and 10 PP), 200 and 10 mM sodium acetate buffer (200 and 10 SA), as well as different pH values are presented as RAs. All of the S.D.s. (n = 3) were below 15. *$p < 0.00039$ after Bonferroni correction.

**Fig. 3. Initial Activity Rates of Crude Phytochelatin Synthase-Like Enzyme Derived from Nostoc sp. Pasteur Culture Collection 7120 (NsPCS)**

Different concentrations of substrate (glutathione, GSH) were incubated with crude NsPCS. Data are expressed as the mean values ± S.D. (n = 3).

**Fig. 4. Concentration of Substrate (Glutathione, GSH) and Product (γ-Glutamylcysteine, γ-EC) in the Reaction Catalyzed by Phytochelatin Synthase-Like Enzyme Derived from Nostoc sp. Pasteur Culture Collection 7120 (NsPCS)**

Crude NsPCS (2.0 μg) was added to 100 μL reactions with 100 mM GSH. Data are expressed as means ± S.D. (n = 3).
γ-EC Production from at High GSH Concentration

To evaluate the ability as a γ-EC production procedure, the NsPCS reaction was conducted at a GSH concentration of 100 mM, which is close to its solubility in water. As shown in Fig. 4, during the first 2 h GSH decreased linearly and γ-EC increased proportionally, with 100 mM GSH completely disappearing after 4 h without any product inhibition. The yield of γ-EC was approximately 90%.

DISCUSSION

NsPCS is one of the prokaryotic PCS-like enzymes. Compared with eukaryotic PCSs, NsPCS does not require heavy metals and catalyzes only the first of the two reactions required for PC synthesis. Several studies have recently shown that the antioxidant capacity of γ-EC may be comparable to that of GSH; moreover, it may act as a neuroprotective peptide in mammals. However, in chemical synthesis, the current production procedure of γ-EC from Cys and Glu, and GCL-mediated enzymatic synthesis are not practically suitable for industrial production. In this study, our aim was to optimize the conditions of the reaction whereby NsPCS synthesizes γ-EC from GSH to establish an alternative production procedure with lower cost and higher productivity. Toward that aim, we optimized the temperature, addition of reductant, and buffer concentration/pH starting from the optimal reaction conditions for AtPCS1, which is derived from A. thaliana and commonly used as a model in PCS studies.

We found that the optimal reaction conditions for NsPCS were: 200 mM potassium phosphate buffer solution, pH 8.0, at 37°C. In addition, we found that a reductant is not necessary for the reaction to occur, and that β-ME suppressed NsPCS activity, while TCEP did not have any effect on its activity. Crude NsPCS showed a $K_{m}$ of 385 μM and a $V_{max}$ of 26 mol/min/mg-protein, although the actual $V_{max}$ may be up to two-fold higher because the NsPCS was estimated to be 50% pure. Finally, we found that when the reaction was carried out at the optimal conditions, high yields of γ-EC were obtained.

The optimal temperature and the concentration and pH of the buffer solution did not differ from those used in the AtPCS1 reaction. However, the activity of NsPCS was much higher in the absence of β-ME than in its presence, and in potassium phosphate buffer solution than in Tris–HCl. AtPCS1 requires β-ME to synthesize γ-EC during the first step of PC synthesis. In contrast, we had previously reported that NsPCS does not require it. In this study, NsPCS activity was suppressed by β-ME. Because TCEP, which does not have a thiol group, did not affect activity, it is possible that the thiol group promotes the AtPCS1 reaction by stabilizing the GSH–heavy metal complex. In contrast, β-ME may not be necessary in the NsPCS reaction because heavy metals are not required for its activity.

To examine the production of γ-EC by NsPCS, we carried out the reaction under the optimized conditions using 100 mM GSH, which is close to solubility of GSH in water. GSH completely disappeared after 4 h, with a γ-EC yield of approximately 90%. According to the $V_{max}$ value obtained, the production rate can be controlled by the concentration of NsPCS, with higher rates obtained at higher concentrations of NsPCS. We have achieved continuous and stable production of γ-EC from GSH for more than 1 week in preliminary experiments using a small-scale column reactor packed with immobilized NsPCS (data not shown). Since we observed that GSH completely disappeared in the reactant, purification of γ-EC was not required. Therefore, immobilized NsPCS will be available to the industrial production of γ-EC when GSH can be supplied stably at low cost. Although the NsPCS reaction results in the equivalent production of Gly together with γ-EC, Gly can easily be separated by ion-exchange chromatography from γ-EC and used as a food additive and in cosmetics. Thus, Gly production may increase the industrial value of the NsPCS reaction.

In conclusion, the results of this study suggest that the NsPCS reaction is a promising alternative procedure for industrial-scale production of γ-EC.

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Conflict of Interest The authors declare no conflict of interest.

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