Development of high-throughput quantitative analytical method for L-cysteine-containing dipeptides by LC–MS/MS toward its fermentative production

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Abstract

L-Cysteine (Cys) is metabolically fundamental sulfur compound and important components in various cellular factors. Interestingly, free-form Cys itself as a simple monomeric amino acid was recently shown to function in a novel antioxidative system (cysteine/cystine shuttle system) in Escherichia coli. However, as for Cys-containing dipeptides, the biological functions, effects, and even contents have still remained largely elusive. The potential functions should be a part of cellular redox system and important in basic and applied biology. For its progress, establishment of reliable quantitation method is the first. However, such accurate analysis is unexpectedly difficult even in Cys, because thiol compounds convert through disulfide-exchange and air oxidation during sample preparation. Addressing this problem, in this study, thiol molecules like Cys-containing dipeptides were derivatized by using monobromobimane (thiol-specific alkylating reagent) and detected as S-bimanyl derivatives by liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS). Sample separation was processed with a C18 column (2.1 mm × 150 mm, 1.7 μm) and with water-acetonitrile gradient mobile phase containing 0.1% (v/v) formic acid at flow rate of 0.25 ml/min. The mass spectrometer was operated in the multiple reaction monitoring in positive/negative mode with electrospray ionization. The derivatization could indeed avoid the unfavorable reactions, namely, developed the method reflecting their correct contents on sampling. Furthermore, the method was successfully applied to monitoring Cys-containing dipeptides in E. coli Cys producer overexpressing bacD gene. This is the first report of the quantitative analysis of Cys-containing dipeptides, which should be useful for further study of fermentative production of Cys-containing dipeptides.

Keywords: Cysteine-containing dipeptides, Escherichia coli, Sulfur metabolism, Ultraperformance liquid chromatography–tandem mass spectrometry

Introduction

For all organisms, sulfur is an essential element like carbon and nitrogen, although its elemental composition is basically much low (Ingenbleek and Kimura 2013). Sulfur-containing proteinogenic amino acids are L-cysteine (Cys) and L-methionine (Met). Unlike mammals, most microorganisms can biosynthesize Cys and Met from environmental inorganic sulfur sources like sulfate (SO₄²⁻), thiosulfate (S₂O₃²⁻), sulfite (SO₃²⁻), sulfide (S²⁻), etc. Many other organic sulfur compounds, most of which are metabolized from Cys, also play biologically critical roles especially in cellular redox reactions; e.g., catalytic residue of enzymes, iron-sulfur cluster, coenzymes, glutathione, etc. In addition to such well-understood roles as sulfur supplier, cellular Cys was recently found to function in a novel antioxidative system in Escherichia coli, designated as “cysteine/cystine shuttle system” (Ohtsu et al. 2010, 2015). In this system, Cys...
is exported from the cytoplasm to the periplasm by the membrane transporter YdeD, then detoxifies H$_2$O$_2$ by converting it to H$_2$O as reducing equivalents there. An alternatively generated oxidized product l-cystine (a Cys dimer via disulfide bond) is imported back to the cytoplasm by the membrane transporter FltY-YecSC to regenerate Cys for its continuous recycling without de novo Cys biosynthesis. In this way, free-form Cys itself as a simple monomeric amino acid exhibits a unique function in a cellular biology.

To shift a view to dimeric amino acid (dipeptide), many dipeptides itself have also been known to exert various biological effects such as antihypertensive effect (Arg-Phe, Ile-Trp) (Enari et al. 2008; Kagebayashi et al. 2012), anxiolytic-like effect (Tyr-Leu) (Kawano et al. 2017; Tanaka et al. 2019), sedative effect (Ser-His, Ile-His) (Tsuneyoshi et al. 2008), salt taste enhancing effect (Met-Gly, Pro-Gly) (Kino and Kino 2015; Kino et al. 2016), and so on. Focusing on Cys-containing dipeptides, those like Cys-Glu, Cys-Ser, Cys-Arg, etc. exert inhibitory effect against tyrosinase (Tseng et al. 2019; Yamada et al. 2019; Nakajima et al. 2019), metabolites including Cys (Kawano et al. 2017; Tanaka et al. 2019). In this method, thiol molecules like Cys, Cys-Ser, Cys-Arg, etc. in the sample are chemically derivatized by using dimethyl sulfoxide (No mBBr treatment) was added, into the solution, 10 μl of 1.3 mM monobromobimane (mBBr) in dimethyl sulfoxide (mBBr treatment) or only dimethyl sulfoxide (No mBBr treatment) was added, and 10 μl of 200 mM Tris–HCl pH 8.8 were mixed. Into the solution, 10 μl of 1.3 mM monobromobimane (mBBr) in dimethyl sulfoxide (mBBr treatment) or only dimethyl sulfoxide (No mBBr treatment) was added, and 1 μl of the solution was immediately subjected to analysis of LC–MS/MS (Nexera UHPLC system with
The sample was kept at 25 °C, and LC–MS/MS analysis was repeated every 20 min until 10 h, as previously reported (Kawano et al. 2015). The target quantity levels were determined from the peak area of mass chromatograms, monitoring each m/z characteristic to the individual target, and were represented as normalized peak area after normalization with that of the internal standard (CSA).

In the experiment in Fig. 2, each 70 μl of 100 μM authentic dipeptide (Cys-His, Cys-Ser, Cys-Thr, Cys-Val, Cys-Gly, Cys-Met, Cys-Ile, Cys-Leu, Cys-Cys, Cys-Phe, Cys-Trp, and Cys-Pro; Sigma Aldrich, custom peptide synthesis), 2 μl of 100 mM ammonium hydrogen carbonate, and 0.14 μl of 100 mM mBBr in dimethyl sulfoxide was mixed and incubated for 10 min at room temperature. Into the solution, 30 μl of 0.1% (v/v) formic acid in acetonitrile was added. As previously performed (Kawano et al. 2015), by utilizing this samples, auto-exploration program was carried out and determined the m/z transition parameters optimal for the detection of respective bimane-derivatives. Also, the retention time was experimentally investigated from the mass chromatography program monitoring the determined m/z in multiple-reaction monitoring mode, permitting the simultaneous detection of multiple targets.

SDS-PAGE analysis for confirmation of BacD expression

Escherichia coli strains of WT pQE85-BsBacD pCys<sup>HP</sup> and WT pQE85 pCys<sup>HP</sup> (Tanaka et al. 2019) was utilized. The cell cultures were started to exhibit OD<sub>562</sub> of 0.1 in Terrific Broth medium including 100 μg/ml ampicillin and 10 μg/ml tetracycline at 30 °C with vigorous shaking for 16 h. At 3 h, 0.1 mM IPTG (final conc.) was added to induce BacD expression. The cells were centrifugally collected and washed once with 100 mM Tris–HCl (pH 8.0) buffer. The cell pellet was resuspended with the same buffer. The cells were disrupted on ice with 10 cycles of 40-s sonication and 40-s interval (Handy Sonic model UR-20P, Tomy Seiko), whose output power was set to a maximum. The lysate was centrifuged at 15 krpm at 4 °C for 10 min, and the total protein concentration of the supernatant was determined by general Bradford assay. SDS-PAGE was performed by standard method (separation gel; 10%) applying the supernatants corresponding to 7 μg of the total protein.

Table 1 Bacterial strains and plasmids used

| Strains or plasmids | Genotype | References or sources |
|---------------------|----------|-----------------------|
| Escherichia coli BW25113 | mB3 ΔlacZ4787 hisDR514 Δ(araBAD)567 Δ(rhaBAD)568 rph-1 | Baba et al. (2006) |
| Bacillus subtilis 168 | trpC2 | Takagi et al. (1997) |
| Plasmids | | |
| pACYC184 | Tet<sup>R</sup>, Cm<sup>R</sup> | Nippon Gene |
| pCys<sup>HP</sup> | pACYC184 with serA (T410 Stop), ydeD and altered cysE (T167A, G203S, T234S, P252L, M256Q) genes under the control of the ompA promoter, Tet<sup>R</sup> | Lab strain<sup>a</sup> |
| pQE85 | Amp<sup>R</sup>, a derivative of pQE80L (Qiagen) | This study |
| pQE85-BsBacD | pQE85 with bacD gene from Bacillus subtilis | This study |

* The plasmid pDES (with serA [T410 Stop], ydeD and altered cysE [T167A] genes) from ajinomoto is first supplied (Wiriyathanawudhiwong et al. 2009). However, this plasmid was accidentally obtained in our laboratory during 10 years of stock.
strains) and of the Tris–HCl (pH8.0) was totally 100 mM. At each time point (0 [just after beginning reaction], 1, 3, and 5 min), 5-μl aliquot of the mixture was sampled and transferred to the derivatization buffer for enzyme inactivation and simultaneous bimane-derivatization of the thiol molecules—the derivatization buffer consists of 49.5 μl of methanol, 0.5 μl of 500 μM CSA, 5 μl of 2 M Tris–HCl (pH8.8), 1 μl of 100 mM mBBr in dimethyl sulfoxide, and 4 μl of dimethyl sulfoxide (totally 60 μl). The resulting solution was well mixed for 10 min. After centrifugation at 15 krpm at 4 °C for 3 min, 40 μl of the supernatant was mixed with 40 μl of Milli-Q water. After centrifuging again, 10 μl of the supernatant was applied for the analysis of LC–MS/MS developed as mentioned above.

**Results**

**Features of thiol derivatization by using monobromobimane in its quantitation**

In our recent study for measuring biological sulfur compounds, we are applying a method chemically modifying its thiol-group by using monobromobimane (mBBr) (Fig. 1a) before LC–MS/MS analysis. As its benefit, naturally occurring chemical redox reactions during metabolites extraction to a measurement, which is unsuitable in terms of quantitation, should be avoidable. To verify this effect experimentally, we examined time course of quantitative dynamics of Cys and its bimane-derivative, Cys-bimane, in the aqueous solution under atmospheric conditions (Fig. 1b). As the result, in the no mBBr treatment, Cys content was gradually decreased in a
Fig. 2  Mass chromatograms of cysteine-containing dipeptide authentic standards derivatized with monobromobimane. The y-axis (not shown) represents normalized intensity of ion count in the mass chromatogram monitoring m/z characteristic of individual target compounds, x-axis represents time in the analysis (0–20 min). The m/z transitions and structural formulae of each S-bimanyl derivative are indicated in the chromatogram.
time-dependent manner to the level less than 10% of the beginning after 8 h incubation (Fig. 2c), and cystine was inversely increased correspondingly (Fig. 2d). By contrast, in mBBR treatment, Cys-bimane and cystine kept almost constant level throughout the monitored period, respectively (Fig. 2c, d). These results show that bimane-derivatization method is effective to quantitate Cys and also other thiol molecules including Cys-containing dipeptides, probably because it can actually avoid oxidative reaction of Cys into cystine mediated by atmospheric oxygen in the sample preparation.

Development of analytical method for quantitating Cys-containing dipeptides by LC–MS/MS

Taking advantage of the derivatization to correctly quantitate thiol-compounds, we here attempted to develop quantitation method for Cys-containing dipeptides as its bimane-derivatives by LC–MS/MS. For this purpose, we first determined the \( m/z \) transition parameters of tandem MS/MS operation in the respective bimane-derivatized Cys-containing dipeptides (Fig. 2, structural formulae) prepared using the authentic compounds and mBBR. Compound registration to the mass analysis were performed according to the procedures that is recommended by the manufacturer. The LC condition (mobile phases, its gradient program, column, etc.) was fundamentally the same as the previous work to measure sulfur metabolites including bimane-derivatives like Cys-bimane, homocysteine-bimane, glutathione-bimane, sulfide-dibimane, sulfite-bimane, thiosulfate-bimane, etc. (Kawano et al. 2015). The retention time was actually investigated using the prepared bimane-derivatives as well. As a result, 12 species of Cys-X-bimane exhibited a single peak in their mass chromatogram monitoring the determined \( m/z \). By integrating each detection program, simultaneous detection method of Cys-containing dipeptide was successfully established for 20-min method. The peak of the chromatogram, retention time and \( m/z \) transition are shown in Fig. 2.

Validity of quantitative analytical method of the cysteine-containing dipeptides by LC–MS/MS

We here assessed whether the constructed analytical method for Cys-containing dipeptides can be practically utilized in the experiments for biological research. To this end, we constructed \( E. \) coli mutant strain to biosynthesize Cys-containing dipeptides, harboring the two plasmids (Table 1). One is pQE85-BsBacD carrying \( \text{bacD} \) gene encoding L-amino acid \( \alpha \)-ligase derived from Bacillus subtilis. The enzyme is known to catalyze the formation of multiple combinations of dipeptide from the various kinds of monomeric amino acids in an ATP-dependent manner (Tabata et al. 2005). The other is \( \text{pCys}^{\text{HP}} \), which is shown to provide Cys overproduction ability (Tanaka et al. 2019).

We investigated expression level of BacD protein by SDS-PAGE, using the supernatant of the lysate of cells cultivated inducing BacD expression in various IPTG concentrations. As a result, in WT pQE85-BsBacD pCys\(^{\text{HP}} \) cells, BacD protein (52.3 kDa) was confirmed to be overproduced as soluble protein in the IPTG concentration dependent manner (Fig. 3, arrow). By contrast, the corresponding protein was not observed in WT pQE85 pCys\(^{\text{HP}} \) cells in all IPTG concentrations. Preparing the supernatant of the lysate of cells cultivated inducing BacD expression by IPTG addition, we carried out in vitro dipeptide formation assay of WT pQE85-BsBacD pCys\(^{\text{HP}} \) (Fig. 4). As the substrate combinations, Cys and 12 species of amino acids (including Cys) were tested. Consequently, in the 11 kinds of the combinations, time-dependent formation of bimane-derivatives of the corresponding Cys-containing dipeptides were observed successfully. In the case of substrate combination of Cys and Pro, its assuming product \( S \)-bimanyl-CysPro was not detected. On the other hand, in the same assay performed using the supernatant of the same WT pQE85 pCys\(^{\text{HP}} \) cells in all IPTG concentrations.
of WT pQE85 pCys\textsuperscript{HP} (BacD-absent negative control strain), no dipeptide was formed at all time points in all 12 combinations of substrates, strongly supporting a consistency of the developed assay.

**Discussion**

In this study, we developed high-throughput quantitative analysis for biogenic Cys-containing dipeptides by LC–MS/MS (Fig. 2). The method adopts a thiol-derivatization process prior to the measurement. It can exclude the chemical conversion of thiol compounds through redox reactions such as disulfide-exchange and oxidation by oxygen existing under the atmospheric conditions (Fig. 1) in the sample during the preparation for measurements. Namely, this method can provide contents correctly reflecting the state of biological sample on the sampling. We also illustrated that this method is indeed functional and useful for the experiment in the Cys-containing dipeptide formation (Fig. 4).

From results shown in Fig. 1c, another merit of S-bimanyl derivative method is considerable. Comparing the normalized peak area of S-bimanyl-Cys in the mBBr treatment with that of Cys in the no mBBr treatment at initial time point, the normalized peak area is approximately twofold higher in S-bimanyl-Cys than in Cys. This indicates that S-bimanyl derivative method is high sensitivity assay compared to direct detection method. This tendency is not limited to Cys but often confirmed in several other thiol compounds (not shown). The probable reason is that S-bimanyl derivatization enhances ionization efficiency in MS process, possibly due to a characteristic of S-bimanyl derivative moiety. In addition, S-bimanyl derivatization is favorable for the detection of too small molecules unsuitable for MS detection,
e.g., inorganic sulfur molecules, by increasing mass of the target molecule. Furthermore, although non-ionic and volatile compounds like (off-)flavor molecules consisting of thiol group is basically difficult to sensitively detect in electrospray ionization (ESI) mass spectrometry, the S-bimanyl derivatization should allow them to be detected by converting it to nonvolatile ones (not shown). Importantly, this S-bimanyl derivatization method is practical and useful method for many scientists, because it requires just widely used type of LC–MS machine (ESI-QqQ) and adopts well-established reverse-phase chromatography system giving easy-to-handle and stable measurements. This method will be effectively developed to increase targets of new sulfur metabolites. Such exploration and identification of unknown sulfur compounds are also developing as S-omics utilizing not widely prevalent but state-of-the-art LC–MS machine with a sophisticated strategy (Nakabayashi et al. 2017; Nakabayashi et al. 2013).

In the previous literature with respect to BacD activities (Tabata et al. 2005), Cys-containing dipeptide formation for Cys-His, Cys-Ser, Cys-Thr, Cys-Met, Cys-Ile, Cys-Leu, Cys–Cys, Cys-Phe, and Cys-Trp was suggested, but Cys-Val, Cys-Gly, and Cys-Pro was not. Similarly, using the same enzyme of BacD, we also showed the activities for Cys-His, Cys-Ser, Cys-Thr, Cys-Val, Cys-Gly, Cys-Met, Cys-Ile, Cys-Leu, Cys–Cys, Cys-Phe, and Cys-Trp, but not for Cys-Pro (Fig. 4). Comparing these two results, of 12 Cys-containing dipeptides, 10 dipeptides were commonly confirmed to be formed (Cys-His, Cys-Ser, Cys-Thr, Cys-Met, Cys-Ile, Cys-Leu, Cys–Cys, Cys-Phe, Cys-Trp) or to be not formed (or formed but less than detection limit) (Cys-Pro). For other 2 dipeptides (Cys-Val and Cys-Gly), we here found to be formed for the first time. It is of note that the assay here just used the supernatant of cell lysate, while the previous report using cell lysate like this study, the activities of Cys-containing dipeptide formations were not detected, probably due to the sensitivity of the measurement and cellular dipeptide-degrading activity coexisting in the cell lysate.

In our method, although we could successfully detect the many Cys-containing dipeptide formations, in the most of them, the product level turned to decrease in the short time (1–5 min) (Fig. 4), and was almost abolished in ~1 h (data not shown). This also suggests the effect of strong cellular dipeptide-degrading activity. Altogether, our method is practically validated and steadily developed, basically consistent with the previous study.

Using the developed S-bimanyl derivatized quantitation method, we can monitor the correct contents of Cys-containing dipeptides in various biological samples, and it will progress the research with respect to physiological functions and valuable effects of Cys-containing dipeptides especially in aspect of new redox system. Also, it enables us to evaluate fermentative production of Cys-containing dipeptides in genetically engineered E. coli. In fact, we are trying this subject as a next step and could preliminary confirm Cys-Ser accumulation in E. coli cells of WT pQE85-BsBacD pCys$^{38}$ in the fermentative cultivation, in which Ser was added (not shown). The production (accumulation) was observed in the cells only just after IPTG induction, but undetected thereafter. This suggests a strong dipeptide-degradation activity in the cell, as mentioned. Therefore, it should be effective to construct the disruptant of multiple peptidase genes in E. coli. Also, it should be effective to explore l-amino acid α-ligases with various substrate specificities. In fact, several such enzymes were reported from diverse biological species to date (Arari et al. 2013; Kino et al. 2008, 2009). By using the identified genes and its genetically modified genes to the genetic engineering in E. coli overproducing Cys by pCys$^{38}$, we will develop a fermentative production of Cys-containing dipeptides with cost-effectiveness to achieve the market availability in accordance with a philosophy of applied microbiology and biotechnology. Its attainment will promote the research of its physiological functions and beneficial effects.

**Abbreviations**  
E. coli: Escherichia coli; Cys: l-cysteine; LC–MS/MS: liquid chromatography coupled to tandem mass spectrometry; Met: l-methionine; WT: wildtype; DNA: deoxyribonucleic acid; PCR: polymerase chain reaction; IPTG: isopropyl β-d-thiogalactopyranoside; mBBr: monobromobimane; CSA: α-camphor-10-sulfonic acid sodium salt; SDS-PAGE: sodium dodecyl sulfate–polyacrylamide gel electrophoresis; Tris: tris(hydroxymethyl)aminomethane; ATP: adenosine triphosphate; ESI: electrospray ionization; QqQ: triple quadrupole.

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**Authors' contributions**  
KK, YAS, and IO designed the study. YK and MS performed experiments. YK and IO wrote the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials
The datasets supporting the conclusions of this article are included within the article.

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Not applicable.

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Competing interests
The authors declare that they have no competing interests.

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