Quantification of Proline-containing Cyclic Dipeptides by LC-MS/MS

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Abstract

The Cyclic dipeptides, 2,5-diketopiperazines (DKPs), draw attention as bioactive and taste compounds. DKPs, especially those containing Proline (Pro), are contained in heated and fermented foods. Herein, we developed a method for the simultaneous quantitative analysis of Pro-containing DKPs using LC-MS/MS. After optimizing the LC-MS/MS conditions, the developed method was applied to quantify Pro-containing DKPs in Goishi tea, which is post-fermented tea produced by a two-step aerobic-anaerobic fermentation process. Consequently, 17 kinds of Pro-containing DKPs could be quantified, and the total amount of Pro-containing DKPs was 3.40 mg/L. The recovery in a spiked test was 93%–117%, which is satisfactory. We believe that the developed method can be used to elucidate the role of Pro-containing DKPs in food.

Keywords

Cyclic dipeptide, Pro-containing DKPs, LC-MS/MS, post-fermented tea
Introduction

2,5-diketopiperazines (DKPs) are cyclic dipeptides formed by a dehydration-condensation reaction of α-amino group on N-terminus and carboxyl group on C-terminus in linear peptide. So far, DKPs have been detected in various fermented and heated foods. Although DKPs were earlier recognized as flavor compounds in foods, they currently draw attention as bioactive compounds. However, there was no useful method for the simultaneous quantitative analysis of DKPs; hence the DKP content in food was not apparent. Previously, we developed a quantitative method for the simultaneous analysis of DKPs using LC-MS/MS, that enabled estimating on 31 kinds of DKPs. We selected 31 kinds of DKPs (including 9 kinds of Proline (Pro)-containing DKPs: cyclo(-His-Pro)(1), cyclo(-Gly-Pro)(4), cyclo(-Thr-Pro)(7), cyclo(-Ala-Pro)(9), cyclo(-Pro-Pro)(12), cyclo(-Tyr-Pro)(13), cyclo(-Val-Pro)(14), cyclo(-Leu-Pro)(17), and cyclo(-Phe-Pro)(19), Fig. 1 and Table 1) depending on their commercial availability and reported bioactivity. Thereafter, we determined the DKP contents in Pu-erh tea and dried bonito, which were fermented and heated, by the developed LC-MS/MS method. Both Pu-erh tea and dried bonito contained various types of DKPs and were especially rich in Pro-containing DKPs. The ratio of amount of Pro-containing DKPs (9 kinds) to total DKPs (31 kinds) in Pu-erh tea and dried bonito was 92.3% and 89.7%, respectively. We concluded that foods are rich in Pro-containing DKPs, which are preferentially formed over the other DKPs. Recently, we demonstrated the formation mechanism of Pro-containing DKPs in food as well. Cyclo(-Leu-Pro)(17) and cyclo(-Ile-Pro)(16) were known as bitterness compounds and it was reported that cyclo(-Asp-Pro)(6) exhibited high antimicrobial activity and cyclo(-Leu-Pro)(17) inhibited proliferation of the influenza A virus. Thus, Pro-containing DKPs could play an important role in the taste and physiological activity of food. The precise determination of Pro-containing DKPs holds a significant position in the quality control of
fermented and heated foods. Herein, we developed an LC-MS/MS analysis method for 20 kinds of Pro-containing DKPs and applied it to estimate the DKP content in a post-fermented tea. Specifically, in addition to LC-MS/MS conditions of 9 kinds of Pro-containing DKPs which whose LC-MS/MS conditions were reported in previous study\(^1\), those of other 11 kinds of Pro-containing DKPs (cyclo(-Lys-Pro)(2), Cyclo(-Asn-Pro)(3), cyclo(-Arg-Pro)(5), cyclo(-Asp-Pro)(6), cyclo(-Gln-Pro)(8), cyclo(-Glu-Pro)(10), cyclo(-Cys-Pro)(11), cyclo(-Met-Pro)(15), cyclo(-Ile-Pro)(16), cyclo(-Ser-Pro)(18), and cyclo(-Trp-Pro)(20)) were newly optimized and improved.

Experimental

Reagents and chemicals

The 20 kinds of Pro-containing DKPs used in this experiment are listed in Table 1. Cyclo(-His-Pro)(1), cyclo(-Lys-Pro)(2), cyclo(-Gly-Pro)(4), cyclo(-Arg-Pro)(5), cyclo(-Thr-Pro)(7), cyclo(-Ala-Pro)(9), cyclo(-Glu-Pro)(10), cyclo(-Tyr-Pro)(13), cyclo(-Val-Pro)(14), cyclo(-Met-Pro)(15), cyclo(-Leu-Pro)(17), cyclo(-Phe-Pro)(19), and cyclo(-Trp-Pro)(20) were purchased from Bachem AG (Bubendorf, Switzerland). Cyclo(-Asn-Pro)(3), cyclo(-Asp-Pro)(6), cyclo(-Gln-Pro)(8), cyclo(-Cys-Pro)(11), cyclo(-Ile-Pro)(16), and cyclo(-Ser-Pro)(18) were obtained from KNC Laboratories Co., Ltd. (Hyogo, Japan) and cyclo(-Pro-Pro)(12) was obtained from the Peptide Institute, Inc. (Osaka, Japan). Milli-Q water was obtained from Auto Pure WQ501 (Yamato Scientific Co., Ltd, Tokyo, Japan).

Preparation of DKP standard solution

Each DKP was dissolved in dimethyl sulfoxide to prepare a stock solution (200 mg/L).
Then, a standard solution that contained all DKPs (0–0.2 mg/L) was prepared using Milli-Q water. This standard solution was used for constructing calibration curves and performing spiked test.

**LC-MS/MS analysis**

LC-MS/MS conditions were those suggested by Yamamoto et al.\(^1\) but slightly modified. Mass spectra were recorded by a tandem mass spectrometry (ACQUITY TQD, Waters, MA, USA) apparatus coupled to a UPLC (ACQUITY UPLC, Waters) system equipped with an Atlantis T3 column (150×2.1 mm i.d., Waters) that was connected to an Atlantis T3 Sentry Guard cartridge (10×2.1 mm i.d., Waters). The eluent system was composed of 0.1% (v/v) formic acid/H\(_2\)O (A) and acetonitrile (B). The flow rate was 0.2 mL/min. The gradient system was as follows: 4% of eluent B at 0–5 min, 45% of eluent B at 25 min, and 98% of eluent B at 30–35 min. The mass spectrophotometer was operated in the positive electrospray ionization mode with a capillary voltage of 3 kV. The source temperature was 150ºC, and the desolvation temperature was 400ºC. The collision cell was operated in the presence of a collision gas (Ar). Multiple reaction monitoring (MRM) was carried out by setting [M+1]\(^+\) as a precursor ion (m/z) and fragment ion showing satisfactory detection sensitivity as a product ion. The cone energy (V) and collision energy (eV) were optimized for each Pro-containing DKP.

**Preparation of food sample**

Goishi tea, a post-fermented tea, was purchased from Otoyo Yutori Farm Co., Ltd. (Kochi, Japan). Dried tea leaves powder (0.5 g) was extracted with 50 mL of boiling water for 10 min, and then it was filtered by a cotton and filter membrane (pore size: 0.45 μm). This extract (10 mg d.w. eq./mL) was used for the spiked test.
**Spiked test**

The detection and determination limits of each DKP were calculated based on the signal-to-noise ratios, which were 3:1 and 10:1, respectively. Besides, the performance of our developed method was assessed by the spiked test. DKP standards at a final concentration of 0.05 ppm were added to the Goishi tea. Recovery percentage was calculated using the results of the spiked test.

**Results and Discussion**

**Optimization of MS/MS conditions for detecting Pro-containing DKPs**

We first selected the MS/MS conditions for the 20 kinds of Pro-containing DKPs. The precursor ion, product ion, cone energy, and collision energy are summarized in Table 2. The MRM chromatogram of the standard solution is shown in Fig. 2 (A), and it was possible to detect all Pro-containing DKPs in 18 min. Although the MS/MS conditions were already reported for cyclo(-His-Pro)(1), cyclo(-Gly-Pro)(4), cyclo(-Thr-Pro)(7), cyclo(-Ala-Pro)(9), cyclo(-Pro-Pro)(12), cyclo(-Tyr-Pro)(13), cyclo(-Val-Pro)(14), cyclo(-Leu-Pro)(17), and cyclo(-Phe-Pro)(19), re-optimization was attempted to obtain a better response.

**Repeatability and linearity for determination of Pro-containing DKPs**

The RSD (%: n = 3) and linearity for the determination of Pro-containing DKPs are shown in Table 3. RSD was calculated at 0.05 mg/L, and a calibration curve was constructed for 0–0.2 mg/L (6 points of 0, 0.01, 0.025, 0.05, 0.1, and 0.2 mg/L). The repeatability was satisfactory since the RSD was below 6%. The RSD for cyclo(-His-Pro)(1), cyclo(-Gly-Pro)(4),
cyclo(-Thr-Pro)(7), cyclo(-Ala-Pro)(9), cyclo(-Pro-Pro)(12), cyclo(-Tyr-Pro)(13),
cyclo(-Val-Pro)(14), cyclo(-Leu-Pro)(17), and cyclo(-Phe-Pro)(19) was improved as compared
with the previous report. The linearity of the calibration curve was 0.995 or more for all
Pro-containing DKPs.

Determination of Pro-containing DKPs in Goishi tea and spiked test

DKPs were known to be present in heated and fermented foods. We attempted to
determine Pro-containing DKPs in Goishi tea using the developed LC-MS/MS method. Goishi
tea is post-fermented tea, which is produced by a two-step aerobic-anaerobic fermentation, and
its physiological activities (such as protection against influenza infection and improvement of
blood lipid profiles) have been recently revealed in the clinical studies.\(^8\)\(^,\)\(^9\) The MRM
chromatogram of Pro-containing DKPs in Goishi tea extract (10 mg d.w. eq./mL) is shown in
Fig. 2 (B). In the spiked test, Pro-containing DKPs were added to Goishi tea extract (10 mg d.w.
eq./mL) at a final concentration of 0.05 mg/L. The detection limit, determination limit,
concentration of Pro-containing DKPs in Goishi tea, recovery (%), and RSD (%) are listed in
Table 4. RSD (%) was calculated for the concentration of Pro-containing DKPs in Goishi tea \(n = 3\).

Consequently, 17 kinds of Pro-containing DKPs, excluding cyclo(-Cys-Pro)(11),
cyclo(Met-Pro)(15), and cyclo(-Ser-Pro)(18), could be determined in Goishi tea, and their
concentration ranged from 0.013 to 0.80 mg/L. Additionally, the total DKP content was 3.40
mg/L. In Goishi tea, Cyclo(-Asp-Pro)(6) was the predominant DKP among the 20 tested
Pro-containing DKPs. Furthermore, cyclo(-Asn-Pro)(3) which could be a precursor of
cyclo(-Asp-Pro)(6), cyclo(-Thr-Pro)(7), and cyclo(-Leu-Pro)(17) were mainly detected in
Goishi tea. The composition ratio of these four Pro-containing DKPs was more than 50% of all
the tested DKPs. As described above, it was noted that cyclo(-Asp-Pro)(6) exhibited high
antimicrobial activity\(^8\) and cyclo(-Leu-Pro)(17) inhibited proliferation of the influenza A virus\(^9\).

These results indicated that some Pro-containing DKPs in Goishi tea could contribute to the functions as bioactive components.

Additionally, the satisfactory level of RSD was established between 0.08\%-1.66\%. In the spiked test, recovery was 93–117\%. From this result, 19 kinds of Pro-containing DKPs, excluding cyclo(-Cys-Pro)(11), could be determined in foods by the developed LC-MS/MS method. It was difficult to determine cyclo(-Cys-Pro)(11) because of the instability of the Cys residue and the effect of other food components.

Thus, we established the simultaneous analysis of 19 kinds of Pro-containing DKPs to a practical level. The present method could be applied to various heated and fermented foods, and it was useful in investigating the relationship between Pro-containing DKPs and various physiological functions.

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Table 1  Pro-containing DKPs used in this study

| No. | DKPs            | Mw   |
|-----|-----------------|------|
| 1   | cyclo(-His-Pro) | 243.25 |
| 2   | cyclo(-Lys-Pro) | 225.29 |
| 3   | cyclo(-Asn-Pro) | 211.22 |
| 4   | cyclo(-Gly-Pro) | 154.70 |
| 5   | cyclo(-Arg-Pro) | 253.30 |
| 6   | cyclo(-Asp-Pro) | 212.20 |
| 7   | cyclo(-Thr-Pro) | 198.22 |
| 8   | cyclo(-Gln-Pro) | 225.24 |
| 9   | cyclo(-Ala-Pro) | 168.20 |
| 10  | cyclo(-Glu-Pro) | 226.23 |
| 11  | cyclo(-Cys-Pro) | 200.26 |
| 12  | cyclo(-Pro-Pro) | 194.23 |
| 13  | cyclo(-Tyr-Pro) | 260.29 |
| 14  | cyclo(-Val-Pro) | 196.25 |
| 15  | cyclo(-Met-Pro) | 228.31 |
| 16  | cyclo(-Ile-Pro) | 210.27 |
| 17  | cyclo(-Leu-Pro) | 210.27 |
| 18  | cyclo(-Ser-Pro) | 184.19 |
| 19  | cyclo(-Phe-Pro) | 244.29 |
| 20  | cyclo(-Trp-Pro) | 283.33 |
| No. | DKPs            | Precursor ion (m/z) | Product ion (m/z) | Cone energy (V) | Collision energy (V) |
|-----|----------------|---------------------|-------------------|-----------------|----------------------|
| 1   | cyclo(-His-Pro)| 235.03              | 109.99            | 36              | 28                   |
| 2   | cyclo(-Lys-Pro)| 226.00              | 124.97            | 34              | 24                   |
| 3   | cyclo(-Asn-Pro)| 211.87              | 125.02            | 22              | 28                   |
| 4   | cyclo(-Gly-Pro)| 154.84              | 69.96             | 34              | 22                   |
| 5   | cyclo(-Arg-Pro)| 254.08              | 194.99            | 38              | 22                   |
| 6   | cyclo(-Asp-Pro)| 212.91              | 125.02            | 32              | 26                   |
| 7   | cyclo(-Thr-Pro)| 198.93              | 69.96             | 32              | 24                   |
| 8   | cyclo(-Gln-Pro)| 225.95              | 69.96             | 24              | 20                   |
| 9   | cyclo(-Ala-Pro)| 268.78              | 70.03             | 38              | 16                   |
| 10  | cyclo(-Glu-Pro)| 227.01              | 69.96             | 26              | 18                   |
| 11  | cyclo(-Cys-Pro)| 200.91              | 69.96             | 24              | 20                   |
| 12  | cyclo(-Pro-Pro)| 194.94              | 69.95             | 32              | 18                   |
| 13  | cyclo(-Tyr-Pro)| 261.00              | 69.95             | 34              | 18                   |
| 14  | cyclo(-Val-Pro)| 196.86              | 69.64             | 38              | 24                   |
| 15  | cyclo(-Met-Pro)| 228.96              | 180.98            | 24              | 10                   |
| 16  | cyclo(-Ile-Pro)| 211.05              | 69.96             | 32              | 28                   |
| 17  | cyclo(-Leu-Pro)| 210.98              | 70.02             | 34              | 18                   |
| 18  | cyclo(-Ser-Pro)| 184.84              | 69.95             | 34              | 20                   |
| 19  | cyclo(-Phe-Pro)| 244.94              | 69.96             | 34              | 16                   |
| 20  | cyclo(-Trp-Pro)| 284.04              | 129.51            | 32              | 14                   |
Table 3  Retention time, RSD, linearity of Pro-containing DKPs

| No. | DKPs          | Retention time (min) | RSD (%) (n = 3) | Linearity |
|-----|---------------|----------------------|-----------------|-----------|
| 1   | cyclo(-His-Pro) | 4.17                 | 0.48            | 1.000     |
| 2   | cyclo(-Lys-Pro) | 5.28                 | 1.69            | 1.000     |
| 3   | cyclo(-Asn-Pro) | 6.02                 | 0.75            | 0.999     |
| 4   | cyclo(-Gly-Pro) | 6.66                 | 0.58            | 0.999     |
| 5   | cyclo(-Arg-Pro) | 7.16                 | 1.22            | 0.996     |
| 6   | cyclo(-Asp-Pro) | 7.49                 | 1.21            | 1.000     |
| 7   | cyclo(-Thr-Pro) | 7.76                 | 1.35            | 1.000     |
| 8   | cyclo(-Gln-Pro) | 8.38                 | 2.07            | 1.000     |
| 9   | cyclo(-Ala-Pro) | 8.93                 | 4.20            | 1.000     |
| 10  | cyclo(-Glu-Pro) | 9.76                 | 3.79            | 1.000     |
| 11  | cyclo(-Cys-Pro) | 10.54                | 5.81            | 1.000     |
| 12  | cyclo(-Pro-Pro) | 10.70                | 0.21            | 1.000     |
| 13  | cyclo(-Tyr-Pro) | 12.61                | 0.54            | 1.000     |
| 14  | cyclo(-Val-Pro) | 12.63                | 1.19            | 1.000     |
| 15  | cyclo(-Met-Pro) | 13.84                | 1.46            | 0.995     |
| 16  | cyclo(-Ile-Pro) | 15.04                | 0.84            | 1.000     |
| 17  | cyclo(-Leu-Pro) | 15.78                | 0.69            | 1.000     |
| 18  | cyclo(-Ser-Pro) | 15.83                | 1.51            | 1.000     |
| 19  | cyclo(-Phe-Pro) | 17.04                | 0.28            | 1.000     |
| 20  | cyclo(-Trp-Pro) | 17.90                | 0.94            | 1.000     |
## Table 4  Analytical result and recovery of DKPs in Goishi tea.

| No. | DKPs          | Detection limit (mg/L) | Determination limit (mg/L) | Concentration (mg/L) | Recovery (%) | RSD (%) (n = 3) |
|-----|---------------|------------------------|----------------------------|----------------------|--------------|-----------------|
| 1   | cyclo(His-Pro)| 0.002                  | 0.005                      | 0.080                | 95           | 0.53            |
| 2   | cyclo(Lys-Pro)| 0.003                  | 0.01                       | 0.15                 | 93           | 0.92            |
| 3   | cyclo(Asn-Pro)| 0.006                  | 0.02                       | 0.39                 | 106          | 0.30            |
| 4   | cyclo(Gly-Pro)| 0.01                   | 0.05                       | 0.10                 | 108          | 0.48            |
| 5   | cyclo(Arg-Pro)| 0.001                  | 0.003                      | 0.078                | 95           | 0.31            |
| 6   | cyclo(Asp-Pro)| 0.01                   | 0.05                       | 0.80                 | 100          | 0.68            |
| 7   | cyclo(Thr-Pro)| 0.02                   | 0.06                       | 0.50                 | 99           | 0.39            |
| 8   | cyclo(Gln-Pro)| 0.01                   | 0.04                       | 0.078                | 117          | 1.6             |
| 9   | cyclo(Ala-Pro)| 0.008                  | 0.02                       | 0.081                | 95           | 0.78            |
| 10  | cyclo(Glu-Pro)| 0.009                  | 0.03                       | 0.22                 | 104          | 0.18            |
| 11  | cyclo(Cys-Pro)| 0.03                   | 0.1                        | [0.039]*             | 35           | 1.7             |
| 12  | cyclo(Pro-Pro)| 0.004                  | 0.01                       | 0.13                 | 97           | 0.56            |
| 13  | cyclo(Tyr-Pro)| 0.008                  | 0.03                       | 0.086                | 95           | 1.1             |
| 14  | cyclo(Val-Pro)| 0.02                   | 0.06                       | 0.14                 | 97           | 0.076           |
| 15  | cyclo(Met-Pro)| 0.01                   | 0.03                       | n.d.                 | 99           | 0.42            |
| 16  | cyclo(Ile-Pro)| 0.008                  | 0.03                       | 0.14                 | 97           | 0.70            |
| 17  | cyclo(Leu-Pro)| 0.01                   | 0.03                       | 0.25                 | 98           | 0.55            |
| 18  | cyclo(Ser-Pro)| 0.0004                 | 0.00                       | n.d.                 | 106          | 1.3             |
| 19  | cyclo(Phe-Pro)| 0.004                  | 0.01                       | 0.16                 | 103          | 0.84            |
| 20  | cyclo(Trp-Pro)| 0.002                  | 0.005                      | 0.013                | 111          | 1.4             |

n.d.: Not detected

*Not determined
Figure Captions

Fig. 1. Structure of Pro-containing DKPs.

Fig. 2. MRM chromatogram of the Pro-containing DKPs: (A) 20 kinds of Pro-containing DKPs listed in Table 1, (B) Pro-containing DKPs in Goishi tea. Number of peak corresponded to the Pro-containing DKPs listed in Table 1.
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Graphical Index

LC-MS/MS analysis

MRM chromatogram of Pro-containing DKPs in Goishi tea.