The simple and rapid quantification method for L-3,4-dihydroxyphenylalanine (L-DOPA) from plant sprout using liquid chromatography-mass spectrometry

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Abstract  L-3,4-dihydroxyphenylalanine (L-DOPA) is one of the important secondary metabolites of plants and has been used for various purposes, such as in clinical treatment for Parkinson’s disease and dopamine-responsive dystonia. In plants, L-DOPA is a precursor of many alkaloids, catecholamines, and melanin; the L-DOPA synthesis pathway is similar to that in mammals. L-DOPA acts as an allelochemical, has an important role in several biological processes, such as stress response and metabolism, in plants. L-DOPA is widely used in the clinical treatment as well as a dietary supplement or psychotropic drug, understanding of biosynthesis of L-DOPA in plant could lead to a stable supply of L-DOPA. This paper describes an improved method for simple and rapid quantification of L-DOPA content using liquid chromatography-tandem mass spectrometry. The standard quantitative methods for L-DOPA require multiple purification steps or relatively large amounts of plant material. In our improved method, quantification of L-DOPA was possible with extract of one–two pieces of cotyledon without any partitioning or column for purification. The endogenous L-DOPA (approximately 4,000 µg g⁻¹ FW) could be detected from the one pieces of cotyledon of the faba bean sprout using this method. This method was also effective for samples with low endogenous amounts of L-DOPA such as broccoli, Japanese white radish, pea, and red cabbage sprouts. Therefore, this improved method will allow to measurement of L-DOPA content easily and accurately from a small amount of plant tissue and contribute to understanding biosynthesis, catabolism, and transport of L-DOPA.

Key words: liquid chromatography-tandem mass spectrometry (LC-MS/MS), L-3,4-dihydroxyphenylalanine (L-DOPA), plant sprout.
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America (Bulduk 2020). Fujii et al. (1991) detected l-DOPA from Mucuna bean (Mucuna pruriens) and showed that l-DOPA content was 550.45 ± 11.34 mg in 100 g raw sprouts. Burbano et al. (1995) reported that the l-DOPA content in young seedlings of Vicia faba variety to be 60–67.5 mg g⁻¹ DW (dry weight) using high-performance liquid chromatography (HPLC). Etemadi et al. (2018) analyzed the distribution of l-DOPA in plant tissues of Faba bean and found that the highest concentration (15.3 mg g⁻¹ DW) was found in young seedlings, l-DOPA content in the mature plant was highest in leaves (10.5 mg g⁻¹ DW), followed by flowers, young pods, mature seeds, and roots. Oviedo-Silva et al. (2018) also showed l-DOPA concentrations (125 mg g⁻¹ DW) in faba bean sprouts using the HPTLC (high performance thin layer chromatography) -UV method. HPLC or HPTLC is common and widely used for quantifying l-DOPA (Hu et al. 2015; Saranya et al. 2021; Shetty et al. 2002). The quantitative NMR technique has also been used to quantify the l-DOPA of the sprouts (Fernandez-Pastor et al. 2019; Okumura et al. 2016).

The quantitative methods for l-DOPA described above require multiple purification steps or relatively large amounts of plant material, and it might lead to inaccurate quantification without clear identification in some cases. Therefore, in recent years, more accurate LC-MS-based analytical methods have been used for l-DOPA quantification (Pavón-Pérez et al. 2019). This paper describes an improved and simple method for rapid quantification of l-DOPA content using liquid chromatography-tandem mass spectrometry (LC-MS/MS). This method will allow to measurement of l-DOPA from Mucuna bean (Mucuna pruriens) and showed that l-DOPA content was 550.45 ± 11.34 mg in 100 g raw sprouts. Burbano et al. (1995) reported that the l-DOPA content in young seedlings of Vicia faba variety to be 60–67.5 mg g⁻¹ DW (dry weight) using high-performance liquid chromatography (HPLC). Etemadi et al. (2018) analyzed the distribution of l-DOPA in plant tissues of Faba bean and found that the highest concentration (15.3 mg g⁻¹ DW) was found in young seedlings, l-DOPA content in the mature plant was highest in leaves (10.5 mg g⁻¹ DW), followed by flowers, young pods, mature seeds, and roots. Oviedo-Silva et al. (2018) also showed l-DOPA concentrations (125 mg g⁻¹ DW) in faba bean sprouts using the HPTLC (high performance thin layer chromatography) -UV method. HPLC or HPTLC is common and widely used for quantifying l-DOPA (Hu et al. 2015; Saranya et al. 2021; Shetty et al. 2002). The quantitative NMR technique has also been used to quantify the l-DOPA of the sprouts (Fernandez-Pastor et al. 2019; Okumura et al. 2016).

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The plant sprouts used in this study (Broccoli, Brassica oleracea var. italica; Red cabbage, Brassica oleracea var. capitata; Pea, Pisum sativum; Japanese white radish, Raphanus sativus var. hortensis; Faba bean, Vicia faba) were purchased from a market in Utsunomiya, Tochigi, Japan. Cotyledon explants (1–2 pieces of cotyledon) were excised from each sprout and used for l-DOPA quantification.

Plant samples were homogenized with a micro pestle in 1 ml of 80% (v/v) aqueous methanol containing 10 µg [1H₃]-L-DOPA (Sigma-Aldrich, St. Louis, MO, USA) as internal standards. Homogenized samples were then briefly centrifuged (6,000 rpm, 1 min), and the supernatants were collected. Supernatants of 80% (v/v) methanol extracts were loaded onto a Bond Elut C-18 cartridge column (Bond Elut C-18, 100 mg 3 ml, Agilent Technologies, Palo Alto, CA, USA), which had been sequentially pre-washed with 3 ml each of MeOH and equilibrated with 3 ml 80% (v/v) aqueous MeOH. One µl of 10-fold (broccoli, Japanese white radish, red cabbage, and pea) or 1,000-fold (fava bean) dilution of 80% (v/v) MeOH extract (before column purification) or C-18 column flow-through fractions (after column purification) were subjected to combined LC-MS/MS comprising a quadrupole tandem mass spectrometer (Agilent 6460 Triple Quadrupole mass spectrometer, Agilent Technologies) with an electrospray ion source and an Agilent 1200 separation module equipped with Poroshell 120 PFP (3×150 mm; Agilent Technologies).

LC-MS/MS analysis was performed using the following conditions: Gas Temp: 300°C, Gas Flow: 51 ml min⁻¹, Nebulizer: 50 psi, Sheath Gas Temp: 400°C, Sheath Gas Flow: 121 ml min⁻¹, Capillary Vol: 2.500 V, Nozzle Vol: 0. The mobile phase consisted of 0.1% formic acid in water (solvent A) and 0.1% formic acid in MeOH (solvent B). The flow rate and the LC gradient conditions were 3% B from 0–5 min, at a flow rate of 0.2 ml min⁻¹, 98% B from 5–10 min at a flow rate of 0.3 ml min⁻¹, and 3% B from 10–25 min at a flow rate of 0.2 ml min⁻¹. L-DOPA was detected with high sensitivity in this condition, and quantification limits were 1 µg l⁻¹ (Figure 1). These quantification limits were much lower than those reported using other methods such as HPLC (approximately 30 µg to 2 mg l⁻¹; Baranowska and Plonka 2008; Singh et al. 2010), and the results were comparable with or better than those of other studies using LC-MS. Quantification of l-DOPA content was performed using Mass Hunter software (Agilent Technologies). l-DOPA was monitored using multiple reaction monitoring (MRM), at m/z 201 > 154 for [2H₃]-L-DOPA, 198 > 152 for L-DOPA. The endogenous level of L-DOPA was calculated by the ratio between the areas of the analyte and the stable isotope-labeled internal standard of a known concentration (Figures 2, 3). Experiments were performed using three independent plant materials. The statistical analysis between the before and after column purification was performed by Mann–Whitney U test.

In this study, we performed l-DOPA quantification by an improved simple and rapid method using LC-MS/MS method with stable isotope-labeled internal standards. In the standard l-DOPA analysis, such as by HPLC or quantitative NMR, plant samples are homogenized in liquid nitrogen or lyophilized and extracted by the organic solvent such as MeOH. Then samples are purified in several steps, such as partitioning or purification with solid-phase extraction columns. And then, the endogenous l-DOPA from a sample was detected using the spectrophotometric method, and quantification was performed compared to a calibration curve prepared using standard l-DOPA. These HPLC-based methods require multiple purification steps and a relatively large amount of plant material. Furthermore, inaccurate quantification might occur when some impurities are not completely removed.

l-DOPA could be analyzed easily from a low amount of sample by our improved method. The plant
material (approximately 20–100 mg FW) harvested were homogenized with a micro pestle in 1 ml of 80% MeOH containing internal standards. The supernatants of the extracts were diluted 10–1,000-fold and directly subjected to LC-MS/MS. Alternatively, MeOH extracts of plant materials were loaded onto a C-18 cartridge column for purification, and then flow-through fractions were diluted and subjected to LC-MS/MS.

Figure 1. Representative chromatograms from injection of the various amounts of L-DOPA standard. The different amount of authentic L-DOPA was monitored using MRM, at m/z 198>152. The signal-to-noise ratios (S/N) in each amount were shown, except for 0.01 and 0.1 µgL⁻¹ injection.

Figure 2. Representative chromatograms of internal standards and endogenous L-DOPA. The chromatograms of internal standard ([²H₃]-L-DOPA) and endogenous L-DOPA of faba bean, before column purification (A) and after column purification (B). L-DOPA was monitored using MRM, at m/z 201>154 for [²H₃]-L-DOPA, 198>152 for L-DOPA. The levels of endogenous L-DOPA were calculated based on the ratio of the area between the analyte and the stable isotope-labeled internal standard of a known concentration.
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All internal standards and endogenous L-DOPA were detectable using our LC-MS/MS conditions as a single peak. Representative chromatograms of internal standards and endogenous L-DOPA are shown in Figures 2, 3. Previous studies reported that high concentrations of L-DOPA were detected in the sprout of faba bean (approximately 10–120 µg g\(^{-1}\) DW). Using an improved method, quantification of L-DOPA was possible with extract of approximately 20–100 mg FW plant material (1–2 pieces of cotyledon) without solvent partitioning (Figures 2, 3). Approximately 4,000 µg g\(^{-1}\) FW of L-DOPA were detected in the cotyledon of the faba bean sprout (Table 1). Although there is a difference in whether the unit is per DW or FW, these values are comparable to previous reports using other methods such as HPLC or quantitative NMR (Etemadi et al. 2018; Fernandez-Pastor et al. 2019; Okumura et al. 2016; Oviedo-Silva et al. 2018).

Furthermore, this improved method is also effective for samples with low endogenous amounts of L-DOPA, such as broccoli (Figure 3) or other sprouts. The endogenous amounts of L-DOPA in the cotyledon of broccoli, Japanese white radish, pea, and red cabbage sprouts were 0.32±0.04, 0.14±0.02, 0.43±0.03, and 0.29±0.07 µg g\(^{-1}\) FW.

Table 1. Quantification of L-DOPA contents in cotyledon of sprouts (µg g\(^{-1}\) FW).

| Plant materials | Before column purification | After column purification | \(p\) value |
|-----------------|-----------------------------|---------------------------|------------|
| Broccoli        | 0.32±0.04                   | 0.46±0.09                 | 0.42       |
| Faba bean       | 3987.2±176.51               | 4037.6±327.68             | 0.56       |
| Japanese white radish | 0.14±0.02        | 0.21±0.02                 | 0.12       |
| Pea             | 0.43±0.03                   | 0.47±0.09                 | 1.00       |
| Red Cabbage     | 0.29±0.07                   | 0.35±0.07                 | 0.66       |

One µl of 10-fold (broccoli, Japanese white radish, red cabbage and pea) or 1,000-fold dilution of 80% MeOH extract (before column purification) or C-18 column flow-through fractions (after column purification) were subjected to LC-MS/MS. The data represent an average of three independent plant materials with standard error. There were no significant differences between the before and after column purification (Mann–Whitney \(U\) test).
FW (before column purification) or 0.46±0.09, 0.21±
0.02, 0.47±0.09 and 0.35±0.07 µg g⁻¹ FW (after column
purification), respectively (Table 1). These results
indicated that accurate quantification from relatively
small amounts of material is possible using our improved
method. Furthermore, the extracts were diluted 10–1,000-
fold, indicating that a more small amount of sample
would theoretically be sufficient for the measurement
of l-DOPA. This value is a very small sample compared
to methods such as HPLC and quantitative NMR,
which require several or several dozen grams of sample.
In addition, there were no significant differences
between the before and after column purification (Table
1), indicating that it was not necessary to column
purification before LC-MS/MS analysis. On the other
hand, since some impurities could be removed during the
purification step, column purification and/or partitioning
would be useful in samples with various metabolites.

This study reported a simple and easy quantitative
method for analyzing l-DOPA from a small sample.
Since l-DOPA is widely used in the clinical treatment
of Parkinson’s disease and dopamine-responsive dystonia
and as a dietary supplement or psychotropic drug,
biochemical synthesis of l-DOPA could lead to a stable
supply of l-DOPA. In addition, fungi and bacteria are
also effective in the biological synthesis of l-DOPA (Patil
et al. 2013). Our simple and easy method reported in this
report will be useful for examining cultivation methods
to increase l-DOPA content and develop varieties
with high l-DOPA content in plants and for various
applications, including basic research and medical
research.

l-DOPA is one of the important secondary metabolites
of plants and has been used for various purposes and has
also been reported to play an important role, including
stress response and metabolism in plants (Soares et al.
2014). However, the physiological role and molecular
mechanisms of l-DOPA in a plant are still unclear. It is
well known that environmental and growth conditions
affect the accumulation of secondary metabolites in
plants, but there is limited information on their effects on
l-DOPA contents in plants. Our improved methods will
contribute to understanding biosynthesis, catabolism,
and transport of l-DOPA. This method will enable the
analysis of l-DOPA with the enhanced spatiotemporal
resolution, which will contribute to the understanding of
biosynthesis and metabolism, its regulatory
mechanisms in response to environmental signals, and
its physiological effects as allelochemicals of l-DOPA in
plants.

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