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Simultaneous determination of D-amino acids by the coupling method of D-amino acid oxidase with high-performance liquid chromatography☆

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ABSTRACT

An enzymatic assay system of D-amino acids was established using the D-amino acid oxidase of *Schizosaccharomyces pombe*. In this method, the enzyme converts the D-amino acids to the corresponding α-keto acids, which are then reacted with 1,2-diamino-4,5-methylenedioxybenzene (DMB) in an organic solvent. The resultant fluorescent compounds are separated and quantified by high-performance liquid chromatography (HPLC). Use of an organic solvent following the α-keto acid modification with DMB prevents the non-enzymatic deamination of L-amino acids, which are generally present at much higher concentrations than D-amino acids in biological samples. With this method, D-Glu, D-Asn, D-Gln, D-Ala, D-Val, D-Leu, D-Phe, and D-Ile can be quantified in the order of micromolar, and other D-amino acids except D-Asp can be assayed within a sensitivity range of 50–100 μM. The established enzymatic method was used to analyze the D-amino acid contents in human urine. The concentration of D-Ser obtained using this enzymatic method (223 μM) was in good agreement with that obtained using the conventional HPLC method (198 μM). The enzymatic method also demonstrated that the human urine contained 5.45 μM of D-Ala and 0.91 μM of D-Asn. Both D-amino acids were difficult to be identified using the conventional method, because the large signals from L-amino acids masked those from D-amino acids. The enzymatic method that we have developed can circumvent this problem.

1. Introduction

D-Amino acids had long been considered to have important functions only in bacteria, such as in the components of the peptidoglycan layer of bacterial cell walls. However, recent advances in analytical techniques have revealed that some D-amino acids, such as D-Ser and D-Asp, exist at high concentrations in eukaryotes, and play important physiological roles. For example, in mammalian brain, D-Ser is involved in higher functions such as memory and learning [1], and D-Asp is involved in functions such as cerebral hormone secretion [2]. D-Ala concentration fluctuates in the anterior pituitary gland, pancreas, and plasma, and the fluctuation seems to relate to hormone regulation [3]. Some of D-amino acids have been suggested to be related to diseases. It is known that D-Ser concentration in the blood serum and spinal fluid of schizophrenic patients is low [4], and that it is high in the glial cells of patients with amyotrophic lateral sclerosis [5]. Further, D-Asp concentration in the seminal plasma and spermatozoa of patients with teratospermia have been reported to be lower than that in healthy subjects [6]. The *in vivo* dynamics of D-amino acids are of acute interest, and a rapid and simple method for assaying D-amino acids would be useful for studying their physiological role and clinical importance.

D-Amino acids are generally assayed by HPLC after derivatization to fluorescent diastereomers [7]. This conventional method detects not only D-amino acids but also L-amino acids with the same fluorescence intensity. Although a variety of D-amino acids are present in biological samples such as human urine and blood serum, the quantities of most are much smaller than those of L-amino acids [8]. Therefore, on chromatograms, the signals of D-amino acids are often masked by the larger signals of L-amino acids, and are not easily identified (see Figs. 5 and 6). A method for specifically quantifying D-amino acids would be valuable for the analysis of such biological samples.

D-Amino acid oxidase (DAO: EC 1.4.3.3) catalyzes the oxidative deamination of D-amino acids to the corresponding α-keto acids, which is accompanied by the simultaneous formation of hydrogen peroxide and ammonia. Oguri et al. developed a method for assaying D-amino acids by using DAO [9]. The principle of their method is that α-keto acids produced from D-amino acids by DAO react with *o*-phenylenediamine (PDA) and 2-mercaptoethanol to form a fluorescent quinoxalinol derivative, which is separated and quantified by HPLC. Using this method, Oguri et al. determined the D-Ala content in shellfish [9]. However, this method has some drawbacks. In this method, the modification of α-keto acids in the presence

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of PDA is carried out in acidic aqueous conditions. Under these conditions, amino acids are non-enzymatically hydrolyzed to α-keto acids. If some amino acids are present at the stage of the α-keto acid modification, they are deaminated to α-keto acids and have an adverse effect on the accuracy of D-amino acid assay. This is often the case with biological samples, in which the concentration of L-amino acids is much larger than that of D-amino acids. Moreover, α-keto acids formed from D-Gln and D-Asn by the DAO reaction are hydrolyzed to α-ketoglutarate and oxaloacetate (oxaloacetate is then non-enzymatically decarboxylated to pyruvate), respectively, during the α-keto acid modification. In the present study, we addressed these problems by using an organic solvent instead of an aqueous solution at the stage of the α-keto acid modification. Using the *Schizosaccharomyces pombe* DAO with a broad substrate specificity and the reagent for the α-keto acid modification in the present study also made the enzymatic D-amino acid assay method more convenient.

2. Experimental

2.1. Materials and reagents

DAO from *S. pombe* (*Sp*DAO) [10] was used as a catalyst for conversion of D-amino acids to α-keto acids. HPLC-grade acetonitrile was purchased from Kanto Kagaku (Tokyo, Japan). *N*, *N*-Dimethylformamide (DMF), 2-mercaptoethanol, and trifluoroacetic acid (TFA) were from Sigma–Aldrich. 1,2-Diamino-4,5-methylene-dioxybenzene (DMB) was from Dojindo Laboratories (Kumamoto, Japan). D-Amino acids were from Wako Pure Chemicals Co. Ltd. (Osaka, Japan). Human urine sample was provided by one of the authors (healthy male subject; age, 28 years).

2.2. Enzymatic assay of D-amino acids with using SpDAO

2.2.1. Apparatus

The HPLC system used in the present study consisted of a degasser (DGU-20A$_5$), liquid chromatograph (LC-20AB), communications bus module (CBM-20A), auto sampler (SIL-20AC), column oven (CTO-20AC), and fluorescence detector (RF-10A$_{XL}$). All equipment was purchased from Shimadzu Co. (Kyoto, Japan).

2.2.2. Sample pretreatment

α-Keto acids, such as pyruvic acid, exist in various biological samples, like human urine and serum [11,12]. Since the intrinsic α-keto acids in biological samples would increase the background of the assay system, eliminating them from the sample is desirable. We attempted to eliminate the intrinsic α-keto acids from biological samples by sodium borohydride ($NaBH_4$) treatment as follows. After boiling for 10 min to remove proteins, the sample (human urine in the present experiment) was centrifuged at 20,000 × *g* for 15 min at 4 °C. We have confirmed that non-enzymatic racemization did not occur with 19 proteinous L-amino acids during boiling for 10 min under the weakly acidic condition (pH 6.0) mimicking the human urine. The supernatant (120 μL) was mixed with 1.2 μL of 1 M $NaBH_4$ and was incubated for 30 min at 30 °C for the reduction of endogenous α-keto acids. Then, the sample was mixed with 1.2 μL of 0.5 M HCl and incubated for 30 min at 30 °C to allow the decomposition of residual $NaBH_4$. The validity of the $NaBH_4$ treatment for pyruvate removal from biological samples has been confirmed using calf serum [13].

2.2.3. Conversion of D-amino acids to α-keto acids with SpDAO

The sample treated with $NaBH_4$ was mixed with 9.6 μL of 1 M potassium phosphate buffer (pH 8.0) and 12 μL of *Sp*DAO solution (10 mg mL^{-1}), and the mixture (final volume, 144 μL) was incubated at 30 °C for 1 h. Then, the reaction mixture was boiled for 10 min to inactivate *Sp*DAO and centrifuged at 20,000 × *g* at 4 °C for 15 min. After filtering the supernatant through Millex®-LG (0.2 (m; MILLIPORE, Billerica, MA), the filtrate (120 μL) was freeze-dried and stored at −30 °C until use.

2.2.4. Reaction of α-keto acids with DMB

DMB and 1,2-diamin-4,5-dimethoxy-benzene (DDB) are 1,2-diaminobenzene derivatives, which react with various α-keto acids under acidic conditions to produce fluorescent derivatives [14,15] and are used for the determination of α-keto acids in human urine and blood serum [16]. These compounds also react with sialic acids and are used for their detection in human or animal serum [17–19]. From the viewpoint of sensitivity of the α-keto acid detection, no significant difference was found between DMB and DDB (data not shown). Since the reaction conditions for α-keto acid-labeling with DMB are milder than those for DDB [14,15], DMB was used in the present study.

The freeze-dried samples were re-suspended in 100 μL of DMF by vigorous shaking for 30 min at 4 °C. The solution was mixed with an equal volume of DMF containing 0.7 mM DMB, 1 M 2-mercaptoethanol, and 20 mM TFA. The mixture was heated for 1 h at 80 °C and then cooled and filtrated. An aliquot of the filtrate was subjected to HPLC analysis.

2.2.5. Chromatographic method

HPLC analysis was performed using a Cosmosil 5C$_{18}$-AR-II column (4.6 mm × 150 mm; Nacalai Tesque, Kyoto, Japan), and the column was equilibrated with 40 mM potassium phosphate buffer (pH 7.0) containing 10% acetonitrile. The derivatized α-keto acid was eluted at a flow rate of 0.8 mL/min by using an acetonitrile gradient (10–60%) in 40 mM potassium phosphate buffer (pH 7.0) as follows: 0–15 min, 10–17.5% acetonitrile; 15–45 min, 17.5–35% acetonitrile; 45–55 min, 35–40% acetonitrile; and 55–60 min, 40–60% acetonitrile. The eluate was monitored by measuring its fluorescence (367 nm excitation and 446 nm emission).

2.3. D-Amino acid quantification after derivatization of amino acids to fluorescent diastereomers

D-Amino acids in human urine were assayed using the conventional HPLC analysis; the amino acids were derivatized to their fluorescent diastereomers and separated and quantified by HPLC. Human urine sample was prepared as follows: After centrifugation at 20,000 × *g* for 15 min at 4 °C, the supernatant was mixed with 5% trichloroacetic acid (TCA) and centrifuged. After removal of TCA from the supernatant by extraction with diethyl ether, the amino acids in the aqueous solution were derivatized to fluorescent diastereomers using *o*-phthaldialdehyde and *N*-tert-butyloxycarbonyl-L-cysteine, as described previously [7], and analyzed by reverse phase HPLC. HPLC analysis was performed using a Cosmosil 5C$_{18}$-AR-II column (4.6 mm × 250 mm; Nacalai Tesque, Kyoto, Japan), and the column was equilibrated with 0.1 M acetate buffer (pH 6.0) containing 9% acetonitrile. The derivatized amino acids were eluted using a linear gradient of acetonitrile (9–50%). The eluate was monitored by measuring its fluorescence (344 nm excitation and 443 nm emission).

3. Results and discussion

3.1. Conversion of D-amino acids to a-keto acids with SpDAO and derivatization of α-keto acids to fluorescent compounds in an organic solvent

D-Amino acid oxidase shows broad substrate specificity, but generally does not act on acidic D-amino acids such as D-Glu or

D-Asp [20–22]. *Sp*DAO, an enzyme from *S. pombe*, catalyzes the oxidation of D-Glu but not D-Asp [10]. Thus, D-Glu can be assayed by using *Sp*DAO. If oxaloacetate is formed from D-Asp by the DAO reaction, it will be spontaneously decarboxylated to pyruvate during the DMB reaction. The resultant pyruvate cannot be distinguished from that formed from D-Ala. The lack of catalytic activity of *Sp*DAO against D-Asp is advantageous for an accurate D-Ala assay.

*Sp*DAO acts on various D-amino acids, and its specific activity for each D-amino acid differs. For example, D-Ala and D-Glu are the most and the least efficient substrates of *Sp*DAO, respectively. The specific activity of *Sp*DAO for D-Ala is about 8 times higher than that for D-Glu. To exclude the influence of the substrate specificity of *Sp*DAO, we carefully determined the amount of *Sp*DAO required for overcoming the difference in its catalytic efficiency. We confirmed that 1 mg/mL of *Sp*DAO and a 1-h reaction at 30 °C are enough to oxidize each D-amino acid at its maximum concentration (Table 1).

In the present assay method, α-keto acids are reacted with DMB. Generally, the DMB reaction is performed by heating under acidic aqueous conditions in the presence of HCl [15]. Amino acids are expected to be deaminated to α-keto acids under these conditions. As described in the Introduction section, the presence of amino acids is an obstacle to the D-amino acid assay, especially while using biological samples containing large amount of L-amino acids. We actually examined the influence of the amino acids on the assay system following the DMB reaction. L-Amino acids or L-amino acids plus D-amino acids were treated or not treated with *Sp*DAO, and then subjected to the DMB reaction, followed by HPLC analysis (Fig. 1). The concentrations of L-amino acids and D-amino acids were 100 and 1 μM, respectively. The difference in the concentrations of L- and D-amino acids was set to reflect the difference in the biological samples. No significant peaks were detected in the absence of amino acids (Fig. 1A). When L-Glu, L-Ser, and L-Ala were reacted with DMB, 3 peaks were observed on the chromatogram (Fig. 1B). These results suggest non-enzymatic formation of α-keto acids from L-amino acids during the DMB reactions. When the mixture of L-Glu, D-Glu, L-Ser, D-Ser, L-Ala, and D-Ala was reacted with DMB without *Sp*DAO treatment, 3 peaks

Table 1
Validation data of the present *Sp*DAO-HPLC coupling method for D-amino acid quantification.

Amino acid	Calibration range (μM)	Correlation coefficient
D-Gln	0.5–10	0.9924
D-Asn	0.5–10	0.9844
D-Glu	1–10	0.9879
D-Ala	1–10	0.9886
D-Leu	1–10	0.9844
D-Val	1–10	0.9838
D-Phe	1–10	0.9910
D-Ile	1–10	0.9813
D-Ser	50–300	0.9869
D-Pro	50–200	0.9999
D-Arg	50–200	0.9994
D-Trp	50–200	0.9725
D-His	100–200	0.9908/0.9836[a]
D-Thr	100–200	0.9937/0.9507[a]
D-Lys	100–200	0.9978/0.9910[a]
D-Met	100–200	0.9657/0.9591[a]

[a] These amino acids were detected as two separated peaks, and therefore, two values of correlation coefficient corresponding to each peak were shown.

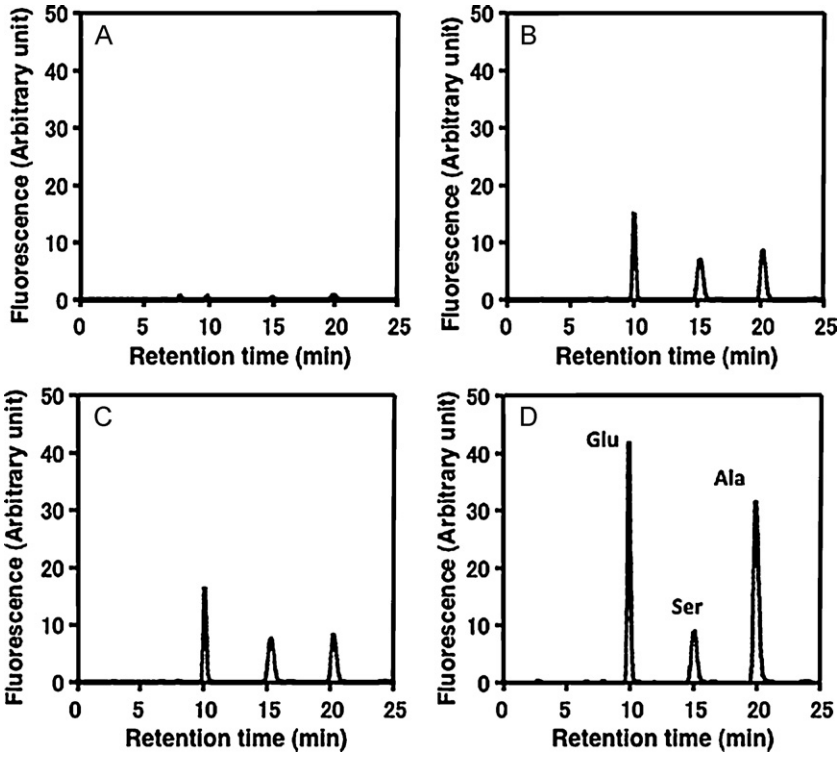

Fig. 1. Non-enzymatic deamination of amino acids during the DMB reaction in the aqueous solution. Mixture A contained 100 μM potassium phosphate buffer (pH 8.0), and 100 μg of *Sp*DAO. Mixture B contained the same buffer, L-Glu, L-Ser, and L-Ala. Mixture C contained the buffer, L-Glu, L-Ser, L-Ala, D-Glu, D-Ser, and D-Ala. Mixture D contained the same components as those in mixture C and 100 μg of *Sp*DAO. The volume of each mixture was 100 μL, and the amino acid concentrations of L-amino acids and D-amino acids were 100 and 1 μM, respectively. Each mixture was incubated at 30 °C for 1 h. *Sp*DAO was removed by boiling for 10 min, and the mixture was centrifuged. A 100-μL aliquot of the supernatant solution was mixed with an equal volume of a solution consisting of 0.7 mM DMB, 0.7 M HCl, 1.0 M 2-mercaptoethanol, and 28 mM $Na_2S_2O_4$, and incubated at 80 °C for 1 h. After this solution was cooled, a 20-μL aliquot was subjected to HPLC. HPLC analysis was performed using a Cosmosil $5C_{18}$-AR-II column (4.6 mm × 150 mm; Nacalai Tesque) at the flow rate of 0.8 mL/min. The column was equilibrated with 40 mM potassium phosphate buffer (pH 7.0), and the derivatized α-keto acids were eluted using a linear gradient of methanol (0–50%) in 40 mM potassium phosphate buffer (pH 7.0) for 25 min. The eluate was monitored by measuring its fluorescence (367 nm excitation; 446 nm emission).

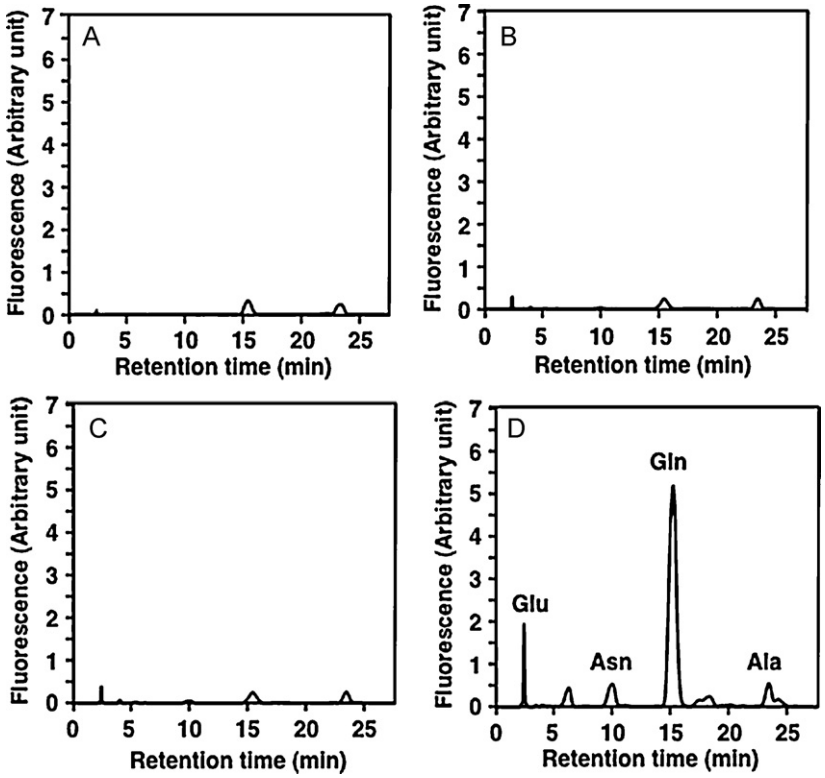

Fig. 2. Non-enzymatic deamination of amino acids during the DMB reaction in an organic solvent. The contents of the reaction mixtures and the procedure for the *Sp*DAO treatment were similar to those shown in Fig. 1, except that the amino acid concentrations of L-amino acids and D-amino acids were 200 and 3 μM, respectively. After the *Sp*DAO treatment, DMB reaction was performed in the DMF solution as described in the text. The derivatized α-keto acids were separated and quantified using HPLC. The elution was performed using a gradient of acetonitrile (10–17.5% for 0–15 min, 17.5–24.5% for 15–27 min) in 40 mM potassium phosphate buffer (pH 7.0).

similar to those shown in Fig. 1B were observed (Fig. 1C). The area of each peak in Fig. 1C was nearly the same as that of the corresponding peak in Fig. 1B. Treatment of the D- and L-amino acid mixtures with *Sp*DAO before the DMB reaction increased the area of each peak (Fig. 1D): this indicates that D-amino acids were converted to the corresponding α-keto acids in the presence of *Sp*DAO. These results suggest that the presence of the high concentrations of amino acids following the DMB reaction affects the D-amino acid assay. Since the L-amino acid content of most biological samples is much higher than the D-amino acid content, it is difficult to apply the enzymatic D-amino acid assay method using the DMB modification under acidic aqueous conditions in the case of biological samples.

To circumvent this problem, we attempted to perform the DMB reaction in an organic solvent (Fig. 2). The conditions of the experiments, as shown in Fig. 2, were similar to those shown in Fig. 1, except that the DMB reaction was carried out in a DMF solution instead of an aqueous solution. No significant peaks were obtained in the absence of amino acids (Fig. 2A). Also, no peaks appeared when the DMB reaction was carried out in the DMF solution either with L-amino acids (Fig. 2B) or with L- and D-amino acids (Fig. 2C) without the *Sp*DAO treatment. α-Keto acids were detected only in the presence of D- and L-amino acids, which were reacted with *Sp*DAO before the DMB reaction (Fig. 2 D). These results indicated that the DMB reaction carried out in the DMF solution successfully derivatized α-keto acids and caused no deamination of amino acids. This reaction also has another advantage: when it is performed in an aqueous solution, the α-keto acids formed from D-Asn and D-Gln by *Sp*DAO treatment were hydrolyzed to oxaloacetate (and then to pyruvate) and α-ketoglutarate, respectively. The DMB reaction carried out in the DMF solution did not induce the hydrolysis of these keto acid amides, and we could perform the D-Asn and D-Gln assays by using the present assay system (data not shown).

3.2. Sensitivity of the enzymatic D-amino acid assay using SpDAO

Derivatized α-keto acids were separated and quantified by HPLC. Eighteen D-amino acids (5 μM), which are enantiomers of the proteinous L-amino acids except for L-Asp, were assayed using the enzymatic assay system. Only 8 peaks corresponding to D-Glu, D-Asn, D-Gln, D-Ala, D-Val, D-Leu, D-Phe, and D-Ile were found on the chromatogram (Fig. 3). For the detection of the other 10 D-amino acids, much higher concentrations were required. This is probably because of the difference in the fluorescence intensities among the α-keto acid derivatives [14,15]. The calibration curve of each D-amino acid standard was generated by plotting the fluorescent intensity versus the concentration of each D-amino acid. The calibration curves for D-Asn (Fig. 4A) and D-Phe (Fig. 4B) are shown as examples. The effective concentration range of each D-amino acid, as determined by our assay method, is summarized in Table 1. The concentrations of D-Gln and D-Asn could be accurately quantified in the range of 0.5–10 μM. D-Glu, D-Ala, D-Val, D-Leu, D-Phe, and D-Ile could be quantified in the range of 1–10 μM, and D-Ser in the range of 50–300 μM. D-Pro, D-Arg and D-Trp could be quantified in the range of 50–200 μM. By the DAO reaction, D-proline is converted to an imino acid, which is equilibrated with an α-keto form (α-keto-δ-aminovaleric acid) [23]. As the α-keto form reacts with DMB, D-proline can be determined by the present method. D-His, D-Thr, D-Lys, and D-Met were detected as 2 separate peaks, but could be quantified in the range of 100–200 μM. D-Tyr and D-Cys could be detected if their concentration was above 100 μM. These 2 D-amino acids gave 3 and 4 separate peaks, respectively, on the chromatogram (data not shown). We found that this method is not

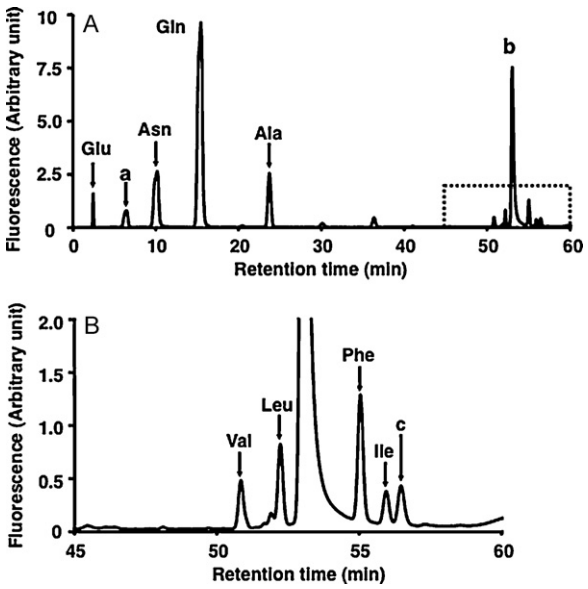

Fig. 3. Chromatogram of α-keto acid derivatives with 18 D-amino acid standards (5 μM) (A). Eight peaks derived from D-Glu, D-Asn, D-Gln, D-Ala, D-Val, D-Leu, D-Phe, and D-Ile could be identified under the set conditions. For the detection of the other 10 amino acids, higher concentrations of amino acids were needed, as described in the text. Peaks a, b, and c are unknown peaks that appeared after the *Sp*DAO, DMB, and DMB reactions, respectively. (B) Enlarged view of the boxed region of panel A.

suitable for the D-Tyr or D-Cys assay. We were interested in the reason why several amino acids gave more than one signals. If the HPLC analyses were performed with acetate buffer (pH 5.0) instead of potassium phosphate buffer (pH 7.0), D-His and D-Tyr gave a single peak (data not shown). These results suggest that multiple peaks reflect the difference in the ionization states of the side chain of the keto acid derivative formed from each amino acid. However, under the same conditions, D-Thr, D-Lys, D-Met, and D-Cys still gave the multiple signals. The reason for the multiple signals observed with these D-amino acids remains unclear. We speculate upon the possibility that the side chain of D-Met and D-Cys were partially oxidized during the DMB reactions.

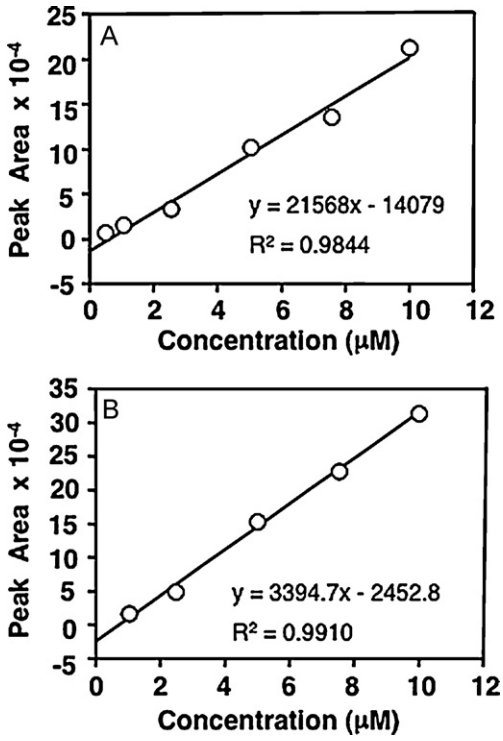

Fig. 4. The calibration curves for D-Asn (A) and D-Phe (B). Detailed conditions are described in the text.

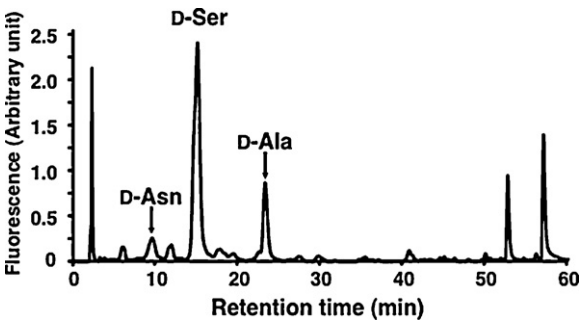

Fig. 5. Chromatogram of the derivatized α-keto acids obtained upon the D-amino acid assay of human urine by the enzymatic assay method. Detailed conditions are described in text.

Table 2
D-Amino acids concentrations in human urine sample analyzed by the enzymatic and conventional assay methods.

Amino acid	Enzymatic method (μM)	Conventional method (μM)
D-Ala	5.45 ± 1.13	n.d.[a]
D-Asn	0.91 ± 0.24	n.d.[a]
D-Ser	223 ± 36	198 ± 17

Each value is the average of triplicate measurements.
[a] n.d., not determined.

3.3. Application of the enzymatic assay method to the analysis of D-amino acids in human urine

To evaluate the validity of this enzymatic assay method for biological samples, we quantified the D-amino acids in human urine. Human urine contains D-amino acids such as D-Ser and D-Ala, but the physiological meaning of these amino acids remains unclear [8]. With the enzymatic assay method, 3 D-amino acids, namely D-Ser, D-Asn, and D-Ala, were detected in the human urine (Fig. 5). The concentrations of D-Ser, D-Asn, and D-Ala were calculated to be 223, 0.91, and 5.45 μM, respectively (Table 2).

The human urine sample was also analyzed by the conventional HPLC method after the amino acids were derivatized to the fluorescent diastereomers, as described in Section 2.3 (Fig. 6). Although the chromatogram had numerous peaks, D-Ser could be identified by performing a co-chromatography with a D-Ser standard, and its concentration was estimated to be 198 μM (Fig. 6, Table 2).

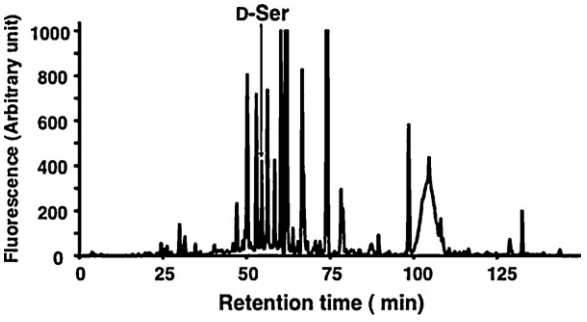

Fig. 6. Chromatogram of the fluorescent D- and L-amino acid derivatives obtained upon the D-amino acid assay of human urine by the conventional HPLC assay. D-Amino acids and L-amino acids in human urine were analyzed after they were derivatized to their fluorescent diastereomers, as described in the text.

The D-Ser concentrations determined using the 2 methods were in good agreement with each other, which suggests that the present enzymatic assay method can be used to analyze human urine samples. It was difficult to find the peaks corresponding to D-Asn and D-Ala among the many peaks of the chromatogram by using the conventional HPLC method (Fig. 6).

The conventional HPLC method is very sensitive and has the advantage of being able to determine the concentrations of multiple D- and L-amino acids simultaneously. However, as shown in Fig. 6, the method has a disadvantage in the analysis of biological samples. As shown in Fig. 6, due to the relatively greater abundance of L-amino acids compared to that of D-amino acids in biological samples, the signals from L-amino acids often mask those from D-amino acids. The present enzymatic assay system contributes to solving this problem. Recently, it has been shown that 2-dimensional HPLC (2D-HPLC) can circumvent this problem by first separating the mixture of D- and L-serine and then separating each enantiomer [24]. However, 2D-HPLC is currently expensive. The present enzymatic D-serine assay can be performed if an ordinary HPLC system is available. However, the large difference in the sensitivity of this method for different D-amino acids probably narrows the application of the present method. We are now trying to improve the sensitivity by altering the reagent used to modify α-keto acids.

4. Conclusions

We developed an enzymatic assay method for D-amino acids that involves the use of *Sp*DAO. In this method, α-keto acids formed from D-amino acids in the presence of *Sp*DAO were reacted with DMB in DMF, and the resultant fluorescent derivatives were separated and quantified using HPLC. Using DMF instead of an aqueous solution suppresses the non-enzymatic deamination of amino acids that otherwise interferes with assay results. This enzymatic assay method was able to detect at least 0.5–1 μM of D-Glu, D-Asn, D-Gln, D-Ala, D-Val, D-Leu, D-Phe, and D-Ile. Other 10 kinds of D-amino acids except D-Asp could be detected and quantified if their concentrations were higher than 50–100 μM. We confirmed that this assay method could be applied for the quantification of D-amino acids in human urine.

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References

[1] J.S. Bains, S.H.R. Oliet, Trends Neurosci. 30 (2007) 417.
[2] A. D'Aniello, Brain Res. Rev. 53 (2007) 215.
[3] K. Hamase, A. Morikawa, S. Etoh, Y. Tojo, Y. Miyoshi, K. Zaitsu, Anal. Sci. 25 (2009) 961.
[4] S. Kumashiro, A. Hashimoto, T. Nishikawa, Brain Res. 681 (1995) 117.
[5] J. Sasabe, T. Chiba, M. Yamada, K. Okamoto, I. Nishimoto, M. Matsuoka, S. Aiso, EMBO J. 26 (2008) 4149.
[6] G. D'Aniello, S. Ronsini, F. Guida, P. Spinelli, A. D'Aniello, Fertil. Steril. 84 (2005) 1444.
[7] R.H. Buck, K. Krummen, J. Chromatogr. 387 (1987) 255.
[8] H. Brückner, A. Schieber, Biomed. Chromatogr. 15 (2001) 166.
[9] S. Oguri, M. Nomura, Y. Fujita, J. Chromatogr. A 1078 (2005) 51.
[10] T. Yoshimura, Vitamins 79 (2005) 277.
[11] K. Koike, M. Koike, Anal. Biochem. 141 (1984) 481.
[12] K. Pailla, F. Blonde-Cynober, C. Aussel, J.P. De Bandt, L. Cynober, Clin. Chem. 46 (2000) 848.
[13] T. Naka, T. Ito, H. Hemmi, T. Yoshimura, J. Mol. Catal. B 67 (2010) 150.
[14] S. Hara, Y. Takemori, T. Iwata, M. Yamaguchi, M. Nakamura, Y. Ohkura, Anal. Chim. Acta 172 (1985) 167.
[15] N. Nakamura, S. Hara, M. Yamaguchi, Y. Takemori, Y. Ohkura, Chem. Pharm. Bull. 35 (1987) 687.
[16] S. Hara, Y. Takemori, M. Yamaguchi, M. Nakamura, Y. Ohkura, J. Chromatogr. 344 (1985) 33.
[17] S. Hara, M. Yamaguchi, Y. Takemori, M. Nakamura, Y. Ohkura, J. Chromatogr. 377 (1986) 111.
[18] S. Hara, Y. Takemori, M. Yamaguchi, M. Nakamura, Y. Ohkura, Anal. Biochem. 164 (1987) 138.
[19] S. Hara, M. Yamaguchi, Y. Takemori, K. Furuhata, Y. Ohkura, M. Nakamura, Anal. Biochem. 179 (1989) 162.
[20] M.P. Simonetta, M.A. Vanoni, B. Curti, FEMS Microbiol. Lett. 15 (1982) 27.
[21] B. Geueke, A. Weckbecker, W. Hummel, Appl. Microbiol. Biotechnol. 74 (2007) 1240.
[22] Y.H. Lee, W.S. Chu, Process Biochem. 33 (1998) 461.
[23] J. Cabello, B. Leon, V. Prajoux, M. Plaza, Arch. Biochem. Biophys. 107 (1964) 51.
[24] Y. Miyoshi, K. Hamase, Y. Tojo, M. Mita, R. Konno, K. Zaitsu, J. Chromatogr. B 877 (2009) 256.