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A new strategy for the selective determination of D-amino acids: Enzymatic and chemical modifications for pre-column derivatization

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Abstract

A new strategy for the selective determination of D-amino acids (DAAs) employing a pre-column derivatization was designed with concepts based on both enzymatic and chemical modifications. Selective determination of DAAs was accomplished by following: DAA was enzymatically modified with D-amino acid oxidase (DAAO: EC 1.4.3.3) to form an alpha-keto acid. Subsequently, resulting alpha-keto acid was detected by high-performance liquid chromatography (HPLC) after chemical modification with *o*-phenylenediamine (PDA) in the presence of 2-mercaptoethanol (2ME) to give the corresponding quinoxalinol derivative (PDA-alpha-keto acid derivative). After optimizing the pre-column derivatization and HPLC separation, five peaks corresponding to DAAs (D-alanine, D-leucine, D-methionine, D-phenylalanine, D-valine (as the standard mixture of DAAs in this paper) were separately eluted and monitored by means of a conventional HPLC system with a gradient elution on octadecyl silica gel (ODS) column and a fluorescence detector (Ex.: 341 nm, Em.: 413 nm), respectively. It was confirmed that the present method was incapable of detecting L-amino acids (LAA) when a sample solution consisting of both LAAs and DAAs was examined. The linearity of the peak-area responses to their concentration range of DAAs from 10 to 500 μM is 0.994–1.000, and their detection limits were 0.2–1 μM (signal/noise = 3). When this method was applied to a methanolic extract of short-necked clams, *Ruditapes philippinarum* (in Japanese, *Asari*), a big peak, corresponding to D-alanine was detected, corresponding to 2.9 mg/g D-alanine. In this paper, we present an example of pre-column derivatization method that was newly configured to take into account both the biological and chemical properties of the substances in question.

Keywords: Alpha-keto acid; D-Amino acid; D-Amino acid oxidase; *o*-Phenylenediamine; Pre-column derivatization

1. Introduction

Initially, it was generally believed that no D-amino acids (DAAs) of any kind existed in the bodies of mammals, even though there was evidence for their existence in microorganisms or proteins. Recently, however, several kinds of free DAAs were found to be present in mammalian bodies [1]. The biological role of free DAA in mammalian bodies is just now becoming clear. For example, free D-serine, which is confined predominantly to the mammalian forebrain structure, acts as a potent and selective agonist for the strychnine-insensitive glycine site of the *N*-methyl-D-aspartate (NMDA) receptor [2]. Free D-aspartate is likewise observed in the brain and in its periphery. Since the periods of maximal emergence of D-aspartate in the brain and periphery occur during critical periods of morphological and functional maturation of these organs, it may be the case that D-aspartate participates in the regulation of the developmental processes of the organs. Although no evidence for subacute toxicity of orally fed DAAs such as D-proline and D-aspartic acid were reported [3], free D-serine, D-alanine and D-proline were shown to be present in much higher concentration in human plasma from patients

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with renal diseases than from normal human subjects [4]. Actually, we believe that the major physiological functions of DAAs have not yet been adequately clarified. To boost such research, more accurate and convenient tools are needed, along with new ideas.

Under less equivocal circumstances, the first discovery of D-amino acid (DAA) in animal tissues was reported by Auclair and Patton in 1950 [5]. They detected free D-alanine by making two-dimensional paper-chromatograms of protein-free extracts of *Oncopeltus* blood. After developing the analytes, the paper was sprayed with a buffered solution of D-amino acid oxidase (DAAO: EC 1.4.3.3) to convert any DAAs present to alpha-keto acid, and, after an incubation, they were dried and treated with a solution of 2,4-dinitorophenylhydrazine (DNP) to create a visual chromophore of phenylhydrazone derivative, which served as the sign for positive detection. Although, at that time, this method was very popular among the scientists studying DAAs and related work, this method faded away as time passed because it took a long time to perform and had low detection sensitivity. In place of this method, enantiomeric separation techniques [6,7] and capillary electrophoresis (CE) [8] based on chiral-chromatography and physiological stereochemistry advanced steadily over the next 30 years. The most frequently applied techniques for determining the presence of DAAs used today first convert the DAAs into diastereoisomers with optically active reagents prior to their separation with either chiral or achiral stationary phased column chromatography [9–16]. Because there are over 40 kinds of amino acids, counting both L- and D-types, chromatographic techniques alone are not able to make accurate, reliable determinations.

It appears form a broad view of biological or biochemical tools for determination of naturally occurring substances, that radio immuno assay (RIA) techniques and enzyme-linked immuno sorbent assay (ELISA) were developed and widely used, because such methods make it easy to do high-throughput analysis. However, methods based on immunochemistry cannot be applied to molecules whose molecular weight is less than 1000, such as amino acids, because it is generally difficult to obtain a specific antibody for a given DAA.

Now, in our thinking regarding suitable for determining the presence in tissue of DAAs has returned back to an older concept mentioned above constructed from two ideas, one is enzyme-substrate specificity and the other is reagent-substrate chemical specificity. Based on these two ideas, we designed a method for the selective determination of DAAs by mean of a new pre-column derivatization method involving a two-step reaction, viz., an enzymatic reaction followed by a chemical reaction. When this method is applied to a common sample containing DAAs and LAAs, the number of peaks on the chromatogram is reduced by half in comparison with a standard chromatogram. Using this pre-column derivatization method, the determination of five kinds of DAAs was demonstrated by mean of reversed-phase HPLC equipped with a fluorescence detector, as discussed below in this paper.

2. Material and methods

2.1. Reagent and materials

DAAO (from hog kidney) was obtained form Boehringer Mannheim GmbH (Germany). DAAs, LAAs, PDAs and the other reagents were purchased from Wako Pure Chem. Co., Tokyo (Japan), and, being of the highest or HPLC grade commercially available, were used without further purification. All aqueous solutions were prepared using water purified with a Milli-Q purification system (Millipore, Milford, MA. USA). All amino acid solutions (DAAs, LAAs) were dissolved with 0.2 M Tris-HCl buffer (pH 8.3) to make adequate concentrations. The DAAO solution and a 1 mM PDA solution were prepared by dissolving with 0.2 M Tris-HCl and 3 M hydrogen chloride, respectively. The desired number of unit of DAAO was contained in a 10-μl portion by diluting the solution with 0.2 M Tris-HCl buffer (pH 8.3). In every experiment, the required solutions were prepared just before use.

2.2. Preparation of a "sea-food" sample

A seafood sample solution was prepared by removing a 5.0-g portion of the inside of a short-necked clam *Ruditapes philippinarum* (called *Asari* in Japanese) was removed from its shell and homogenized with 40 ml of methanol. After diluted with methanol to 50 ml as the final volume, a 300 μl of portion of the supernatant was transferred to a round-bottomed glass flask for drying in vaccuo. The resulting residue was subsequently reconstituted with 300 μl of 0.2 M Tris-HCl buffer (pH 8.3). As a precautionary note, proper care should be taken when handling PDA, 2ME, and other reagents or organic solvents, as they may be sources of hazard to one's health.

2.3. Apparatus

The HPLC system consisted of two intelligent PU-980 pumps (Jasco, Tokyo, Japan) used for high-pressure gradient-mode elution. Samples were loaded with a Rheodyne syringe loading valve (Reodyne, CA, USA) with an 10-μl of sample injection loop. The analytical column was Asahipak ODP-50 (150 × 6.0 i.d.; Showa Denko K.K., Tokyo, Japan). The column was maintained at 40 °C in a CO-965 column oven (Jasco). Column effluents were monitored with a 821-FP fluorescence detector (Jasco). The excitation and emission wavelengths were set at 341 and 413 nm, respectively. All mobile phases were degassed through a DG-980-51 on-line degasser (Jasco). The flow-rate of the eluent was 1.0 ml/min. All chromatographic data were printed with the intelligent data processor 807-IT (Jasco).

2.4. Assay procedure

A 300 μl of portion of sample solution of 0.2 M tris-HCl buffer (pH 8.3) and 10 μl of DAAO solution (50 units) were

transferred into a 1.6-ml polypropylene micro-centrifuge tube with the stopper, and mixed well. After incubation of the solution at 37 °C for 30 min, a 300 μl-PDA solution and a 5 μl-2-mercaptoethanol (2ME) were added, and heated at 100 °C for 20 min. Subsequently, the reaction mixture was extracted with 500 μl of ethylacetate four times. The combined extract was evaporated to dryness in vaccuo and then reconstituted with 500 μl of methanol. A 10-μl portion of the solution was directly applied onto the optimized HPLC apparatus. Then, the derivatives were separated with HPLC using either an isocratic or a binary gradient elution system, with "eluent A" and "eluent B" which consisted of 15% (v/v) aqueous acetonitrile and full-strength acetonitrile, respectively.

3. Results and discussion

3.1. Optimization of the method for pre-column derivatization

To accomplish the selective determination of DAAs, the pre-column derivatization method was designed as shown in Fig. 1. The method was involved with two basic principles of biochemistry and chemistry. The first one is makes use of enzyme-substrate specificity. DAAO from pig kidney used in this study selectively catalyzes the oxidation of DAA to convert it into alpha-keto acid [17,18]. The second principle makes use of the selective chemical reaction between alpha-keto acid and PDA/2ME to give a fluorescent quinoxaline (PDA-alpha-keto acid) derivative [19]. To identify these resulting the PDA-alpha-keto acid derivatives, they are subsequently separated and detected via conventional chromatographic technique. As the result of these processes, only DAAs corresponding to their PDA-derivatives are observed on the chromatogram. Although each principle used in this assay scheme had been already developed separately, the combined method mentioned here is, the authors believe, the first time for their application to pre-column derivatization. Finally, these conditions were optimized and the performance and validity of this method were evaluated as followed.

3.2. Chemical reaction between alpha-keto acid and PDA

By referring to the conditions published already [19], the effect of the reaction time and temperature on the performance of chemical reaction between pyruvic acid (used in this study as an alpha-keto acid) and PDA were evaluated by means of measuring the peak-area obtained from HPLC with the isocratic elution of "eluent A" (15% acetonitrile). 300 μl of 0.1 mM of pyruvic acid solution in 0.2 M Tris-HCl buffer (pH 8.3), 300 μl of PDA solution in 3M hydrogen chloride and 5 μl of 2ME were mixed and heated over a tempera-

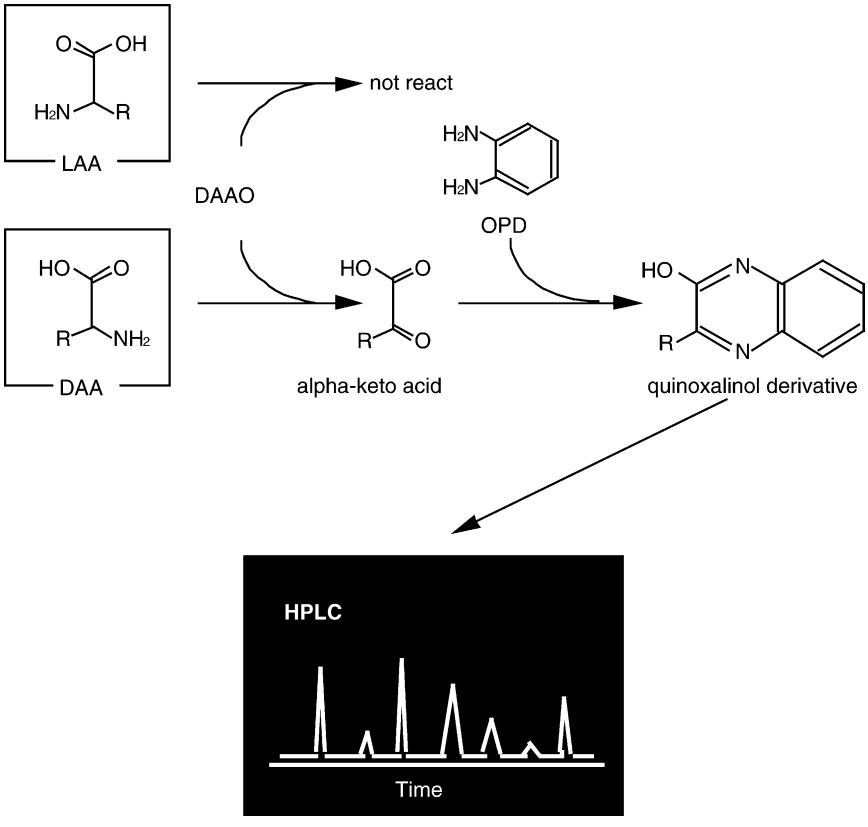

Fig. 1. Principle of pre-column derivatization of DAA using the DAAO digestion prior to PDA derivatization for the selective determination of DAAs.

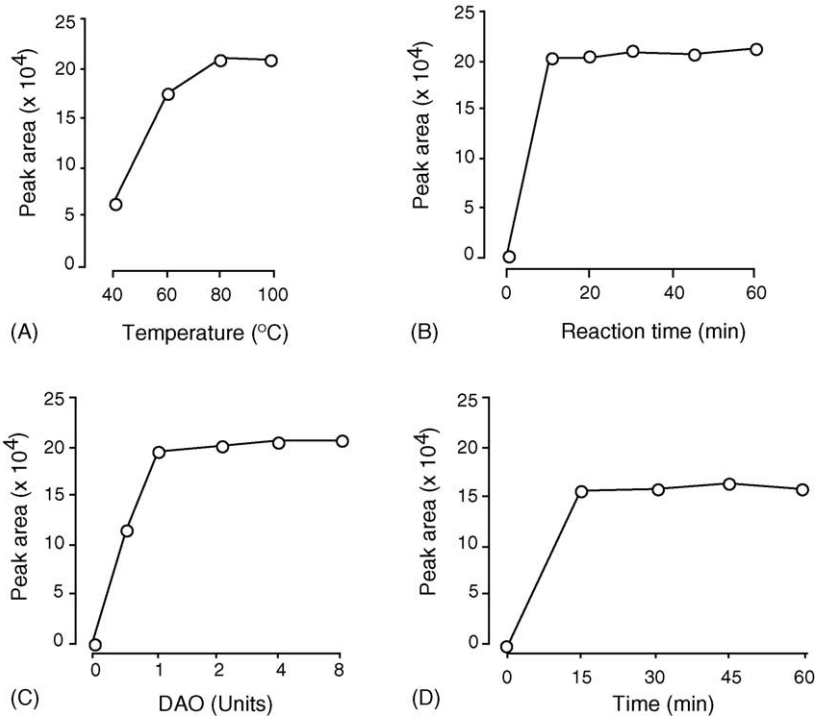

Fig. 2. Optimization of the pre-column derivatization with PDA (1 mM) chemical reaction and DAAO enzyme reaction by using 0.1 M pyruvic acid solution and D-alanine solution, respectively. The following conditions were optimized by means of measuring the peak-area counts obtained form HPLC in isocratic elution with "eluent A" (15% acetonitrile): (A) the effect of the reaction temperature (300 μl of pyruvic and 300 μl of PDA) for 20 min; (B) the effect of the reaction time (300 μl of pyruvic and 300 μl of PDA) at 100 °C; (C) the effect of the number of units (300 μl of D-alanine solution and 10 μl of DAAO solution) at 37 °C for 30 min and (D) the effect of digestion time at 37 °C (300 μl of D-alanine solution and 10 μl of DAAO solution; 2 units).

ture range from 40 to 100 °C for 20 min. The reaction was completed when the reaction temperature and time reached 100 °C after 20 min, as shown in Fig. 2A and B, respectively.

3.3. Enzyme reaction between DAA and DAAO

The stereospecificity of the reaction is absolute and is restricted to the D-isomers when both DAA and LAA are supplied as substrate. The reaction appears to be a simple second-order process [20]. DAAO in vitro displays broad substrate specificity, de-amminating several neutral and basic DAAs, however, the most efficient substrates are AAs with hydrophobic side chains. D-Aspartic acid and D-glutamic acid are not substrates for DAAO, but are oxidatively de-amminated by D-aspartate oxidase (D-AspO, EC 1.4.3.1).

The effect of the number of DAAO units on digestion time of D-alanine (as the standard DAA used in this study) was examined by means of measuring the resulting pyruvic acid under the conditions described above. 300 μl of 100 μM D-alanine solution of 0.2 M Tris-HCl buffer (pH 8.3) was digested with 300 μl of DAAO solution over a range of DAAO units from 0.5 to 8 at 37 °C for 30 min. The reaction was completed when over 1 DAAO unit was used, as shown in Fig. 2C. The effect of digestion time was further examined by varying the time at 37 °C, as shown in Fig. 2D. After considering these results, the number of units and digestion time were fixed at 1 unit and 30 min, respectively. Trace A and B of Fig. 3 show the chromatograms obtained from 100 μM L- and D-alanine, respectively. The peak corresponding to D-alanine appeared on trace B, but not on trace A. This confirmed that the present method can work selectively only for DAA, not for LAA.

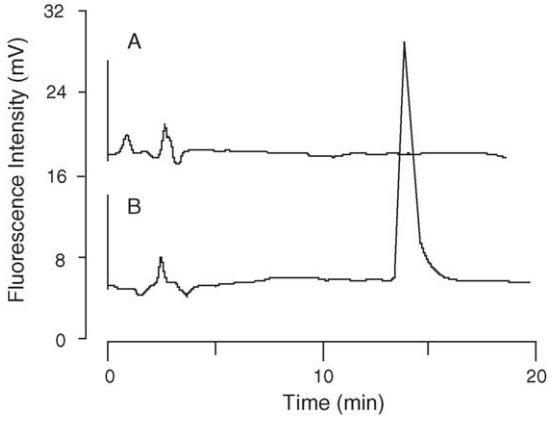

Fig. 3. Comparison of chromatograms obtained from pre-column derivatization of (A) L-alanine and (B) D-alanine. The digestion of 0.1 M alanine (L or D form) with DAAO (1 unit) was carried out at 37 °C for 30 min. Subsequently, the derivatization of resulting pyruvic acid with PDA (300 μl) was done at 100 °C for 20 min. HPLC with the isocratic elution of "eluent A" (15% acetonitrile). Refer to the Experimental Section for other HPLC conditions.

3.4. DAAO inhibition in the presence of LAAs

Because we hypothesized that LAA would interfere with the activity of DAAO, which would cause assay result to show a lesser amount of DAA than the actually present, we tested this hypothesis by examining the digestion of DAA with DAAO in the presence of LAAs or not. In Fig. 4, as the reference, the peak-area counts obtained from the digestion of each D-alanine solution (10, 100, 500 and 1000 μM) with 5 units DAAO are shown as indicated by "open circles". Each peak-area count obtained from the same D-alanine solutions digested with 5, 25 and 50 units of DAAO in the presence of a five-component LAA solution (L-alanine, L-leucine, L-methionine, L-phenylalanine and L-valine; 500 μM each) is also indicated on the same figure by "closed circles", "open triangles", and "closed triangles", respectively. By comparing each peak-area count obtained from D-alanine solutions, either in the presence of the five-component LAA solution or not, the interference of DAAO's activity by LAA was observed. In the case of the 1000 μM-D-alanine solution in the absence of LAA, the peak-area counts treated with 5 units DAAO reached 19.4×10^4. The peak-area counts, however, obtained from the D-alanine solution treated under the same conditions as above in the presence of the five-component LAA solution decreased by about 10%. Under these conditions, the peak-area counts subsequently increased with increasing of the number of units of DAA, and almost the same counts as above was given when 50 units of DAAO was used. Although the optimized number of units of DAAO had been fixed at 1 unit in the previous section, it proved necessary to use about 10–50 units of DAAO when more than 100 μM DAAs sample having LAAs was digested with DAAO to prevent the interference or inhibition by LAAs.

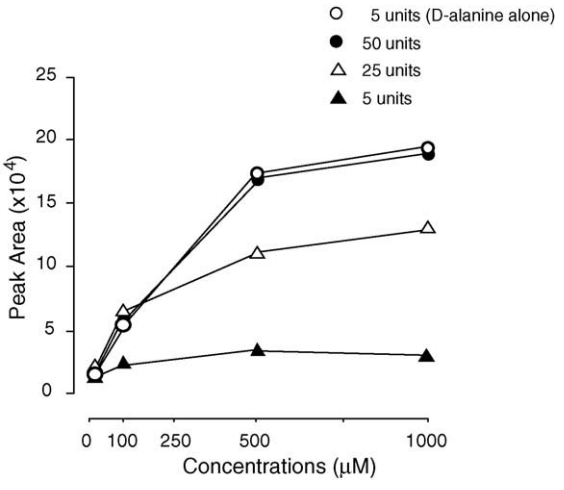

Fig. 4. Effect of LAAs on the performance of DAA pre-column derivatization when the number of units of DAAO varied from 5 to 50. D-alanine (10–1000 μM) was digested with 5 units of DAAO (○). D-alanine (10–1000 μM) in the presence of LAAs (L-alanine, L-leucine, L-methionine, L-phenylalanine and L-valine; 500 μM each) was digested with 5 units (△), 25 units (▲) and 50 units (●) of DAAO. The other conditions were the same as in Fig. 3.

Therefore, 50-units of DAAO was chosen for the present method.

3.5. Separation of quinoxaline derivatives using HPLC

As the determination and measurement of alpha-keto acids is important in various clinical situations, a number of convenient and precise methods had been developed using reverse-phase HPLC. Determination of the alpha-keto acids occurs after derivatization into fluorescent quinoxalines by reaction with PDA [21–25]. Koike's group [25] developed a procedure for rapid separation and micro-quantitative determination of various alpha-keto acids in serum and urine. The seven kinds of alpha-keto acids were easily derivatized to their quinoxaline derivatives, and were effectively extracted with ethylacetate from a control serum or urine. The overall sample recovery of the quinoxaline derivatives of alpha-keto acid including de-proteinization, derivatization, and extraction steps, was about 95–100%. Subsequently, they were separated and detected with a reverse-phase HPLC equipped with a fluorescence detector. The useful range of analysis of the seven alpha-keto acids by isocratic HPLC was from 10 to 250 pmol. Pailla et al. also developed the same method for determination of alpha-keto-acid in biological samples [26]. Referring to those methods, separation and detection of the quinoxaline derivatives (PDA-alpha-keto acid), in our method, were carried out using a high-pressure binary-gradient elution on octadecyl silica gel (ODS) column and setting the fluorescence detector at an excitation wavelength of 341 nm, and to detect an emission wavelength of 413 nm. Five kinds of quinoxaline derivatives were determined using HPLC as shown in Fig. 5 after treatment of a 100 μM five-component DAA solution (D-alanine, D-leucine, D-methionine, D-phenylalanine and D-valine;100 μM each in the same manner as described above. No further optimization of the HPLC conditions was required in this experiment.

3.6. Validation of the method

Under the above conditions, validation of the method was examined in terms of reproducibility, linearity, peak response factor and detection limit, and the results are listed in Table 1. The analytical reproducibility was calculated by the peak area and retention time obtained from five identical HPLC runs after pre-column derivatization of a 500 μM five-component DAA solution. The reproducibility of each peak-area and retention time was less than 1.47 and 0.28% of center values (c.v.), respectively. Linearity of peak-area response was also evaluated using the five-component DAA solution at a concentration between 10 and 500 μM and showed correlation factors from 0.994 to 1.000. The peak-area response was normalized to D-alanine at the 500 mM level, and the value of D-leucine at a level 6.65-times greater than that of D-methionine. This difference occurred due to the specificity of DAAO-DAA substrate. The detection limits, obtained from the signal-to-noise ratio (*S*/*N*) of 3, ranged from 0.2 to 1 μM.

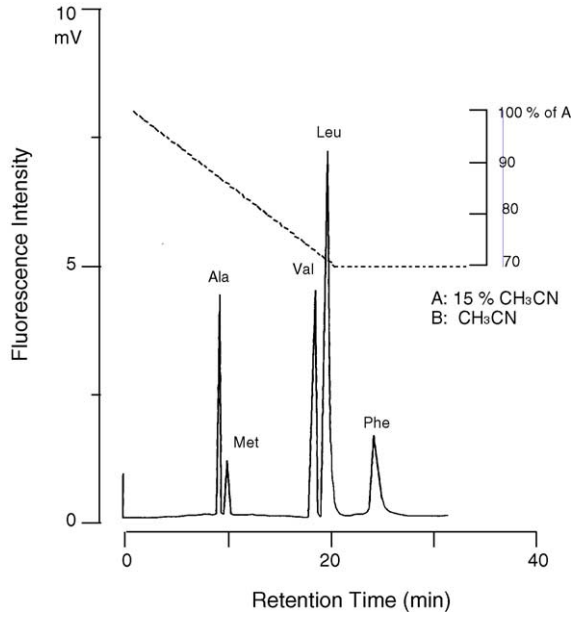

Fig. 5. The typical chromatograms of separation of five kinds of quinoxaline (PDA-alpha-keto acid) derivative driven from DAAs (D-alanine, D-leucine, D-methionine, D-phenylalanine and D-valine; 100 μM each). The high-pressurized gradient profile as eluent was added as indicted in the figure.

It should be noted that the DAAO enzyme used in this study can act only on the neutral DAAs. To do analyses of D-aspartic acid, which is sometimes found in nature, requires that D-AspO be employed instead of the present enzyme. Unfortunately, we did not examine the performance of D-AspO with this method because of the difficulty to obtain it at present. In addition, the high resolution crystal structure of DAAO from the yeast *Rhodotorula gracilis* provided us with the tool to engineer the substrate specificity of this flavo-oxidase. The rational design approach was successful in producing enzymatic activity with a new, broader substrate specificity, and this approach can also be used to develop DAAO variants suitable for use in biotechnological applications [27]. In the near future, it should be possible to obtain DAAO which has special substrate specificity for any kind of DAA.

3.7. Testing of the sample

Although only small amounts of D-alanine or other DAAs are present in mammalian samples, large amounts were found in marine invertebrates at several moles DAA/g of muscle [28–31]. Then, the present method was applied a sample obtained from short-necked clams (in Japanese, *Asari*) for the determination of the presence of DAAs. First, the sample was treated with methanol. Then, the methanolic extract was dried in vacuo. After reconstitution with 0.2 M Tris-HCl buffer (pH 8.3), the solution was submitted to examination using the present method described in the Experimental Section without any further purification. Trace B and A of Fig. 6 show the chromatograms obtained from the sample treated with and without DAAO for the pre-column derivatization, respectively. The large peak (trace B) confirmed the presence of pyruvic acid. By contrast, a very small peak (trace A) was also interpreted as indicating the presence of pyruvic acid, however, this pyruvic acid was believed to be endogenous and to have been converted from DAA and/or LAA with endogenous DAAO or other type of enzymes present in the body.

One of the challenges in the future with regard to the determination of real free DAAs in a sample using the present method, will be to remove the endogenous pyruvic acid in the sample matrix before pre-column derivatization. Subsequently, the concentration of pyruvic acid was calculated at 1.75 mg D-alanine/g of muscle by subtracting the difference in peak area of the trace A from that of the trace B. To check whether pre-column derivatization reaction progressed smoothly in the seafood sample extract solution, the sample was spiked with 2.0 mg/g D-valine and subsequently treated in the same manner as described above. The reason why D-valine was chosen was that the sample did not contain either D-valine or its oxidation product alpha-ketoisovaleric acid, as shown in Fig. 6A and B. The result is shown by trace C of Fig. 6. The content of D-valine in the sample was calculated from the peak (retention time:19.2 min) corresponding to alpha-ketoisovaleric acid using the standard calibration curve, and was measured at 2.1 mg (yield: 105%). From this result we infer that D-alanine is derivatized as along with D-valine in the seafood sample. In addition, a large

Table 1
Method of validation

D-Amino acid	Reproduciblity (CV%)[a]		Correlation factor[b]	Peak responce factor[c]	Detection limit (μM, $S/N = 3$)
	Peak-Area	Retention time			
D-Alanine	1.12	0.26	0.9998	1.00	0.5
D-Methionine	1.09	0.24	0.9940	0.40	1.0
D-Valine	1.18	0.26	0.9999	1.06	0.5
D-Leucine	1.25	0.28	0.9963	2.66	0.2
D-Phenylalanine	1.47	0.23	0.9995	0.80	0.5

[a] Each reproducibility value was from five identical HPLC runs of 500 mM five-component D-amino acid solution.
[b] Each linearity of peak area as correlation factor corresponding to each amino acid between 10 and 500 mM was calculated by the least-squares regression method.
[c] Each peak responce factor was calculated by normalization to D-alanine at a concentration of 500 μM.

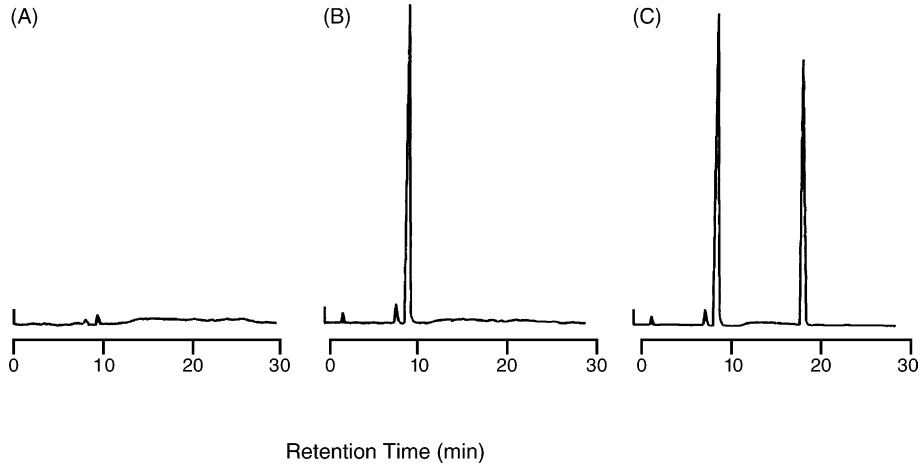

Fig. 6. Typical chromatograms obtained from a shellfish, (Japanese *Asari* clam) after pre-column derivatization in the presence of DAAO (B) or absence of DAAO (A). Chromatogram C was obtained from a sample spiked with D-valine (2.0 mg/g) prior to being given the same treatment as given to sample B. The other HPLC conditions were as described above in the Experimental Section.

amount of DAAO present in the reaction mixture did not disturbed the reaction between alpha-keto acid and PDA in this method.

During 1999 and 2001, Nagahisa and his group conducted a large-scale survey of the presence of free D-alanine in forty-one or more kinds of invertebrates inhabiting the Sanriku Coast of Japan sponsored jointly by the United Nations University, the Ocean Research Institute of the University of Tokyo and the Iwate Prefectural Government, in order to ascertain data on variations in metabolic products due to differences in type of organism, habitat, season, etc. According to their report [32], regarding DAA in *Asari*, a large amount of D-alanine was present and showed seasonal changes in concentration. The maximum concentration of D-alanine in summer reached a level of about 1.5 mg/g of muscle. Although we did not verify our results using a previously proven method, the result of 1.75 mg D-alanine/g *Asari* muscle obtained here is almost the same level as that of 1.5 mg D-alanine/g of *Asari* muscle reported from Nagahisa's survey.

4. Concluding remarks

Until now, bioanalytical chemistry has developed along separate lines, based primarily either on biological or chemical principles. While both approaches have merits and demerits, their combinations hold out the promise of more powerful and productive analytic techniques. With this concept in mind, in this paper we presented a method for pre-column derivatization using enzymatic modification of DAAO prior to PDA chemical modifications. Applying the pre-column derivatization method in HPLC, a selective determination of DAAs was demonstrated for the first time without any use of traditional enantiomeric separation techniques. Although the present method still has some problems as addressed above, we believe that the technique demonstrated here will help to solve important problems in the field of bio-analysis.

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