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Determination of D-aspartate *N*-methyltransferase activity in the starfish by direct analysis of *N*-methyl-D-aspartate with high-performance liquid chromatography☆

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ABSTRACT

We describe a method for the detection and quantification of D-aspartate *N*-methyltransferase activity. The enzyme catalyzes the *S*-adenosyl-L-methionine-dependent *N*-methylation of D-aspartate to form *N*-methyl-D-aspartate (NMDA). NMDA is detected directly by high-performance liquid chromatography (HPLC) of their (+)- and/or (−)-1-(9-fluorenyl)ethyl chloroformate fluorescent derivatives. The NMDA production in the assay mixture is linearly proportional to the incubation time and the amount of tissue homogenate. Using a 10 min incubation time, the method allows detection of the enzyme activity below 10 fmol/min. It can be used to analyze kinetic behavior and to quantify the enzyme from a wide variety of organisms.

1. Introduction

N-Methyl-D-aspartate (NMDA) is a potent agonist for a subtype of the L-glutamate receptor in the central nervous system of higher animals, so the receptor has been named the NMDA receptor. NMDA, first known as an artificial compound, was discovered to be a natural product since it was isolated from the bivalve *Scapharca broughtonii* in 1987 [1]. We developed a simple and easy method for the determination of NMDA by high-performance liquid chromatography (HPLC) of their (+)-1-(9-fluorenyl)ethyl chloroformate [(+)-FLEC] fluorescent derivatives, after removal of primary amino acids by treatment with *o*-phthalaldehyde (OPA) [2], and demonstrated occurrence of NMDA in the several bivalves [3]. During these studies, we also found some other compounds that were similar to NMDA in HPLC behavior. One of them is an enantiomer of NMDA, *N*-methyl-L-aspartate (NMLA) that was found only in two bivalve species [3]. Two of them found in *S. broughtonii*, *S. subcrenata* and several other invertebrates are *N*-methyl-D-glutamate (NMDG) and *N*-methyl-L-glutamate (NMLG) [4]. The discovery of the natural occurrence of these *N*-methyl-D,L-amino acids has suggest the possibility that these compounds were widely distributed in many other living organisms with some significant functions.

Two other methods have been developed for the determination of NMA by the HPLC [5,6]. D'Aniello et al. reported an enzymatic HPLC method, which is based on the measurement of the methylamine produced from NMDA by the oxidation with D-aspartate oxidase [5]. Sekine et al. developed a column-switching HPLC system on a reverse-phase column and a chiral column for the separation of NMDA and NMLA after derivatization of NMA with a fluorescent derivatization reagent, 4-fluoro-7-nitro-2,1,3-bezoxadiazole [6]. Recently, using the former method, NMDA was detected in neuroendocrine tissues of rat [5,7] and in other animals [8,9]. Furthermore, it has been reported that NMDA is biosynthesized from D-aspartate as substrate and *S*-adenosyl-L-methionine as a donor of the methyl group, in tissue extracts of these animals, and the enzyme that catalyzes the synthesis is called D-aspartate *N*-methyltransferase (*S*-adenosyl-L-methionine:D-aspartate *N*-methyltransferase or *N*-methyl-D-aspartate synthase) [5,7,9] (Fig. 1). In view of the significance of the discovery, more supporting evidence should be provided for this enzyme system, since the employed assay method leaves some uncertainty: the method detects methylamine and not NMDA itself [5,7–9], and uses D-aspartate oxidase

Abbreviations: NMDA, *N*-methyl-D-aspartate; NMLA, *N*-methyl-L-aspartate; NMA, *N*-methylaspartate; NMDG, *N*-methyl-D-glutamate; NMLG, *N*-methyl-L-glutamate; HPLC, high-performance liquid chromatography; (+)-FLEC, (+)-1-(9-fluorenyl)ethyl chloroformate; (−)-FLEC, (−)-1-(9-fluorenyl)ethyl chloroformate; OPA, *o*-phthalaldehyde.

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from beef kidney, which may oxidize NMDG, as the oxidase from pig kidney does [4,10], and/or other unknown compound(s) present in the tissues to form methylamine. Thus it is not certain, whether all methylamine detected comes from NMDA. This situation prompted us to develop a simple, direct and reliable assay method for D-aspartate *N*-methyltransferase activity in animal tissues. Our method reported in the previous studies using (+)-FLEC is able to separate of NMDA from the other *N*-methylamino acids [3,4]. On the basis of the method [2], we report here the development of a direct and reliable assay method for D-aspartate *N*-methyltransferase activity using a simple HPLC system, and its application in starfish tissues, in which we found a large amount of NMDA together with other acidic *N*-methylamino acids.

2. Experimental

2.1. Chemicals and biological materials

(+)-1-(9-Fluorenyl)ethyl chloroformate [(+)-FLEC], (−)-1-(9-fluorenyl)ethyl chloroformate [(−)-FLEC], NMDA and *N*-methyl-L-aspartate (NMLA) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Solvents for high-performance liquid chromatography (HPLC), such as acetonitrile, tetrahydrofuran and methanol of HPLC grade were obtained from Wako Pure Chemical (Osaka, Japan). The anion-exchange resin, Dowex 1 × 2 (100–200 mesh, chloride form) was obtained from Muromachi Technos (Tokyo, Japan) and converted to acetate form just before use. A reverse-phase HPLC column (150 × 4.6 mm I.D.), packed with 4 μm-diameter J' sphere ODS-M80 was from YMC (Kyoto, Japan). Other chemicals were of analytical purity.

D-Aspartate oxidase was purified as previously reported [10] and had a specific activity of 450 nmol/min per mg protein.

Live specimens of echinoderms Asteroidea: starfish *Asterias amurensis* were collected from a beach in Fukushima Prefecture, Japan. The radial nerve cord of the starfish were removed and washed with ice-cold saline, and were stored at −20 °C until use.

2.2. Analysis of endogenous NMDA together with other acidic N-methylamino acids in the starfish tissues

The frozen tissues (10–300 mg) were homogenized with a Potter–Elvehjem homogenizer equipped with a Teflon pestle in 5 volumes of 16% perchloric acid under cooling in ice water. The acidic homogenates was centrifuged at 12,000 × *g* for 20 min to remove precipitated protein, and the supernatant was neutralized with KOH. After the centrifugation to remove the precipitated $KClO_4$, a portion of the supernatant was chromatographed with Dowex 1 × 2 column, followed by dryness of the eluate, as described in Section 2.4. NMDA together with other acidic *N*-methylamino acids in the starfish tissue was determined as described in Section 2.5.

2.3. Preparation of tissue homogenates from starfish tissues

All the procedures were carried out at 4 °C. The frozen tissues (200–500 mg) were homogenized with a BioMasher (Nippi, Tokyo, Japan). After the addition of about 1 volume of 50 mM potassium phosphate buffer (pH 7.5), the homogenate were dialyzed three times using a EasySep (Tomy Seiko, Tokyo, Japan), for approximately 1.5 h each time, against 1000 volumes of 50 mM potassium phosphate buffer (pH 7.5). The dialyzed tissue homogenates were used immediately for measurement of enzyme activity.

2.4. Assay procedures for D-aspartate N-methyltransferase activity

The assay of enzyme activity was carried out in 1.5-ml Eppendorf tubes. The assay mixture (30 μl) contained 50 mM potassium phosphate buffer (pH 7.4), 33 mM D-aspartate (as precursor for the biosynthesis of NMDA), 20 mM *S*-adenosyl-L-methionine (as methyl group donor) and 20 μl of the dialyzed tissue homogenate prepared as described above. Unless otherwise stated, the assay was carried out for 10 min at 37 °C. The enzyme reactions were stopped by the addition of 500 μl of 8% (v/v) perchloric acid. The solution was centrifuged at 12,000 × *g* for 20 min to remove precipitated protein, and the supernatant was neutralized with KOH. After the centrifugation to remove the precipitated $KClO_4$, the supernatant (5–6 ml) was loaded on a column (2 ml volume) of Dowex 1 × 2 (100–200 mesh, acetate form), and the column was washed with 20 ml of water to remove neutral and basic substances including *S*-adenosyl-L-methionine. Acidic substances including NMDA and unreacted D-aspartate were eluted with 20 ml of 1 M acetic acid. The eluate was concentrated to dryness under reduced pressure with a centrifugal evaporator (SPE-200, Shimadzu Rika, Kyoto, Japan) at 40 °C. The residue was subjected to derivatization for HPLC analysis.

Blanks were run in parallel with each series of assays, and prepared as described above, except that the perchloric acid was added to the assay mixture prior to the addition of the tissue homogenate from the starfish nerves.

2.5. Determination of N-methyl-D-aspartate together with other acidic N-methylamino acids by HPLC analysis

This was performed according to our previous report [2] with minor modifications as follows: the residue, resulting from the procedure described above, was dissolved in 100 μl of 0.1 M sodium borate buffer (pH 9.0). A 10 μl volume of this sample solution was mixed, in a 1.5-ml Eppendorf tube, with 40 μl of OPA solution (50 mg/ml) in acetonitrile and was kept at 50 °C for 15 min to remove D-aspartate unreacted in the enzyme reaction. Then, 5 μl of 18 mM (+)-FLEC in acetone and 5 μl of acetonitrile were added and, after reaction for 15 min at 50 °C, a 10 μl volume of 100 mM aqueous L-cysteic acid in 0.1 M sodium borate buffer (pH 9.0) was added to remove the remaining FLEC. The mixture was allowed to react for another 7 min at the same temperature. After addition of 80 μl of 0.1 M sodium acetate buffer (pH 4.0), the mixture was filtered through a 0.45-μm membrane filter (Millex-LH, Nihon Millipore, Tokyo, Japan).

A portion (10–50 μl) of the filtrate obtained above was injected into a Shimadzu (Kyoto, Japan) HPLC system consisting of two LC-20AD pumps, a SIL-10A auto injector equipped with a sample cooler S (4 °C), a CTO-10AS column oven, a SCL-10AVP system controller, a DUG-20A degasser, an RF-10AXL fluorescence detector and a Chromatopac C-R7A data processor. Unless otherwise stated, the mobile phase consisted of 82% (v/v) 0.1 M sodium acetate buffer (pH 5.60), 10.8% (v/v) methanol and 7.2% (v/v) tetrahydrofuran, and isocratic elution was carried out at a flow-rate of 0.8 ml/min and at a column temperature of 40 °C. For fluorometric detection of eluted FLEC derivatives the excitation and emission wavelengths were set at 260 and 315 nm, respectively.

The identity of detected NMDA was further confirmed as in the same way of our previous studies [2,3], as follows: (a) a procedure using (−)-FLEC as a derivatizing agent, (b) the disappearance of the peak of NMDA derivative by pretreatment of the sample solution with D-aspartate oxidase.

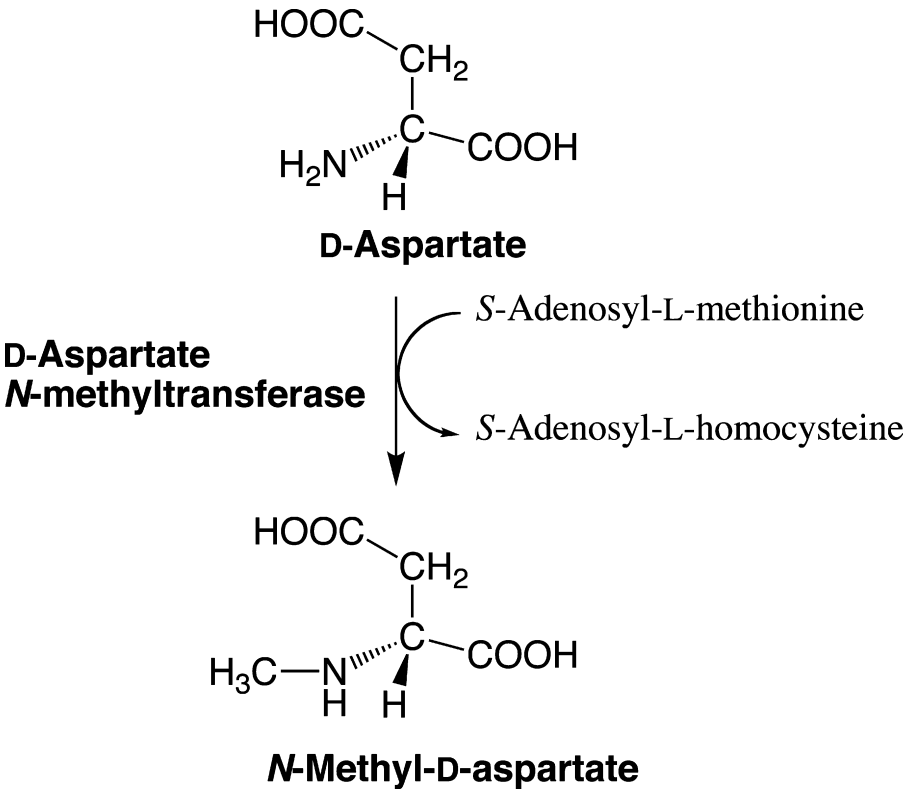

Fig. 1. *S*-Adenosyl-L-methionine-dependent *N*-methylation of D-aspartate to form *N*-methyl-D-aspartate.

3. Results and discussion

3.1. Determination of N-methyl-D-aspartate together with other acidic N-methylamino acids by HPLC analysis

HPLC analysis of NMDA was performed according to our previous report [2] with minor modifications. In this study, when methanol was used in place of acetonitrile in the HPLC solvent system, NMDA and NMLA derivatized with (+)-FLEC were completely resolved as shown in Fig. 2A. Under this condition with authentic compounds of 0.3 pmol each, NMDA was separated from D- and L-aspartate and D- and L-glutamate eluted at the retention time of 47.3 min, 48.2 min, 59.6 min and 61.5 min, respectively (data not shown). However, the OPA treatment before the (+)-FLEC derivatization was indispensable for removal of primary amino acids (Fig. 2C), because NMDA peak was interfered by an overwhelmingly large peak of mainly D- and L-aspartate in the tissue extracts as described in an our previous report [2], or of D-aspartate added to the assay mixture of D-aspartate *N*-methyltransferase as substrate (Fig. 2B).

With the prior treatment with OPA, we found large amounts of NMDA, NMLA and NMLG in the starfish tissues (Table 1). NMDA and NMLA were identified as described below. NMLG was identified by the behaviors of the (+)-FLEC and (−)-FLEC derivatives of NMLG on HPLC chromatograms, as described in our previous study [4] (data not shown). Since radial nerve cord had the highest concentrations of NMDA and NMLA not only among the starfish tissues examined but also in various tissues of invertebrates so far reported [2,3], we decided to measure D-aspartate *N*-methyltransferase activity with radial nerve code as the enzyme source.

Fig. 2D and E shows typical chromatograms of the (+)-FLEC-treated assay mixtures of D-aspartate *N*-methyltransferase of the starfish nerves without and with an incubation for 10 min at 37 °C, respectively. The assay mixture without the incubation was prepared for the blank. Two peaks observed Fig. 2D and E exhibited the same retention times as those of authentic NMDA and NMLA in Fig. 2A. When (−)-FLEC was used in place of (+)-FLEC as derivatizing agent, the retention times of two peaks were exactly switched as well as the previous report [2] (data not shown). In addition, we treated the assay mixtures with pig kidney D-aspartate oxidase, which is known to oxidize NMDA as one of its specific substrate [10]. The treatment with D-aspartate oxidase abolishes the former peak and not the latter peak in the chromatograms of the assay mixtures including the blank. All these data support that the two peaks obtained from the assay mixtures of D-aspartate *N*-methyltransferase of the starfish nerves represent the FLEC derivatives of NMDA and NMLA. To estimate the recovery of NMDA produced through the assay procedure, the assay mixture was received 100 pmol of authentic NMDA and treated in the same manner as described in Section 2.4, followed by NMDA determination. The NMDA recovery was more than 70%.

NMDA and NMLA were detected, even in the assay mixture prepared for the blank (Fig. 2D), indicating that complete removal of endogenous NMDA and NMLA in the homogenates by the dialysis was difficult under the condition employed in the present study. The comparison of the peak areas of NMDA and NMLA derived from the blank mixture containing the homogenate without dialysis with those derived from another blank mixture containing the same homogenate with dialysis showed that approximately 40–50% of endogenous NMDA and NMLA were remained after the dialysis (for example, compare Fig. 2C with D). While the dialysis for much longer time may decrease the level of remained compounds, that might result in a possible loss in the enzyme activity. Further improvement to remove the endogenous compounds may be required.

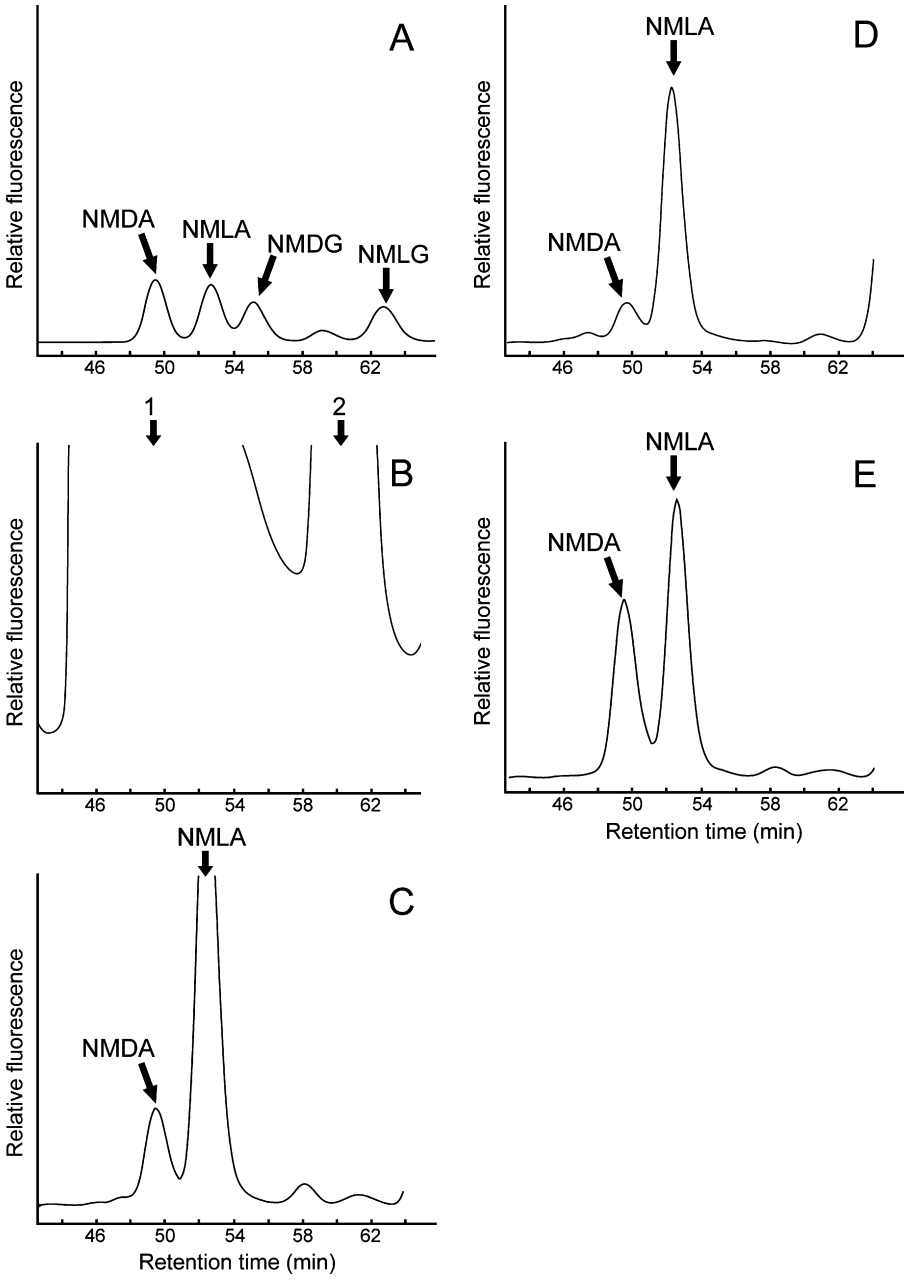

Fig. 2. HPLC chromatograms of (+)-FLEC derivatives of authentic NMDA, NMLA, NMDG and NMLG (A), (+)-FLEC derivatives of the extracts of blank assay mixtures for D-aspartate *N*-methyltransferase in the starfish nerves without (B) and with (C) the prior treatment with OPA and (+)-FLEC derivatives of the extracts of assay mixtures for the enzyme without (D) and with (E) an incubation for 10 min at 37 °C. (A) (+)-FLEC derivatives of authentic NMDA, NMLA, NMDG and NMLG (0.3 pmol each) (B) The assay mixture containing the undialyzed tissue homogenate from the starfish nerves without the incubation was prepared for a blank as described in Section 2.4. The extract of the mixture underwent the (+)-FLEC derivatization as described in Section 2.5, without the prior treatment with OPA. Peaks: 1 = mainly D- and L-aspartate; 2 = mainly D- and L-glutamate. (C) The same extract underwent the (+)-FLEC derivatization with the prior treatment with OPA. (D) The same tissue homogenate used in (B) and (C) was dialyzed. The assay mixture containing the dialyzed tissue homogenate without the incubation was prepared for the blank. The extract of the blank solution underwent the (+)-FLEC derivatization with the prior treatment with OPA. (E) The same dialyzed tissue homogenate used in (D) was incubated with 33 mM D-aspartate and 20 mM *S*-adenosyl-L-methionine for 10 min at 37 °C. The extract of the mixture underwent the (+)-FLEC derivatization with the prior treatment with OPA.

Table 1
Distribution of *N*-methylaspartates and *N*-methylglutamates in the tissues of starfish *Asterias amurensis*.

Tissues	*n*	*N*-Methylaspartate			*N*-Methylglutamate		
		D form (nmol/g tissue)	L form (nmol/g tissue)	D/(D + L) (%)	D form (nmol/g tissue)	L form (nmol/g tissue)	D/(D + L) (%)
Radial nerve cord	3	306 ± 69	17,800 ± 14,300	12.5 ± 15.9	nd	285, nd, nd	–
Testis	3	138 ± 152	1,620 ± 298	6.65 ± 6.23	nd	208 ± 151	–
Ovary	3	42.8 ± 27.4	780 ± 640	8.27 ± 6.34	nd	413 ± 231	–
Pyloric caeca	3	69.2 ± 86.5	224 ± 157	26.5 ± 19.7	nd	110 ± 126	–

The contents and percentages are expressed as means ± S.D., when the number of samples is 3. nd, not detectable.

3.2. Parameters influencing the quantification of NMDA production in the enzyme assay

The NMDA production in the assay mixture was investigated as a function of the incubation time, using the tissue homogenates of starfish nerves. The production rate was linearly proportional to the incubation time up to the first 10 min, when the assay mixtures contained the tissue homogenates, corresponding to 25 mg of starfish nerves. After this initial phase, the NMDA production slowed down (data not shown). Routine assays were consequently carried out for 10 min. From these data and the detection limit of <0.1 pmol at a signal to noise ratio of 3 to 1, we can establish that our assay procedure has a low detection limit of approximately 10 fmol/min. The sensitivity of the present assay method is high enough, comparing that reported in the previous studies (e.g., 17.3 nmol NMDA produced/ml assay mixture for 60 min by 200 mg of rat brains) [5,7].

The NMDA production in the assay mixture was also examined as a function of the amount of the tissue homogenates from the starfish nerves. The various amounts of the homogenates, corresponding to 0–42 mg of the starfish nerves in the assay mixture, were used. The amount of NMDA produced was linearly proportional to the amount of tissue homogenates ($r^2 > 0.99$).

Using these conditions, the activity of D-aspartate *N*-methyltransferase found in radial nerve cord of starfish *Asterias amurensis* was 0.231 ± 0.218 nmol/min per g wet tissue (mean ± SD, $n = 4$), which was 0.098 ± 0.086 nmol/min per mg protein in terms of specific activity. Echinoderm, as well as Urochordate and Cephalochordate, situated in a key phylogenic position, as a descent of chordate ancestor. It is interesting that amphioxus nerve cord [9] and ascidian cerebral ganglion [11] displayed the activities of 33.4 nmol/g for 120 min and 10.5 nmol/g for 60 min, respectively, which are similar to 2.31 nmol/g for 10 min, shown by the radial nerve cord of starfish. The present HPLC assay will be useful in measurement of D-aspartate *N*-methyltransferase activity in a wide range of tissues and organisms, owing to elucidating the evolution and biological significant of the enzyme.

3.3. Application of the HPLC assay to study kinetics of D-aspartate N-methyltransferase activity

One of the most important uses of the enzyme assay is to determine kinetic parameters and to study enzyme kinetic behavior. In previous studies [5,7–9], there has been no report on kinetic parameters of D-aspartate *N*-methyltransferase. We therefore examined effect of D-aspartate concentration on D-aspartate *N*-methyltransferase activity with the tissue homogenates of starfish nerve, at a fixed concentration of *S*-adenosyl-L-methionine in the assay mixture. Fig. 3 shows the D-aspartate *N*-methyltransferase activity at different D-aspartate concentration. The analysis by Lineweaver–Burk plots suggests the enzyme subjects to Michaelis–Menten kinetics. We tentatively estimated, by the statistical method of Wilkinson [12], that V_{max} and K_m values were respectively 0.55 ± 0.16 nmol/min per g wet tissue and 75 ± 28 mM for D-aspartate, though further detailed analysis is required.

The method used in the present study allows clear separation of NMDA, NMLA, NMDG and NMLG using (+)- and (−)-FLEC, as well as those described in our previous study [2–4]. Therefore, the HPLC assay procedure developed here seems to be compatible with detailed kinetics studies of D-aspartate *N*-methyltransferase in living organisms.

3.4. Activities of L-aspartate or L-glutamate N-methyltransferase

In this study, we also examined separately the activities of L-aspartate or L-glutamate *N*-methyltransferase in radial nerve code with L-aspartate or L-glutamate in place of D-aspartate as substrate, respectively. The tissue had an L-aspartate *N*-methyltransferase activity of 0.657 ± 0.184 nmol/min per g wet tissue (mean ± SD, $n = 3$), consisting with that the tissue had the highest concentration of NMLA among the starfish tissues (Table 1). No activity of L-glutamate *N*-methyltransferase was detected in the same tissue, though a large amount of NMLG was found in a radial nerve code from one of three animals in a separate experiment (Table 1).

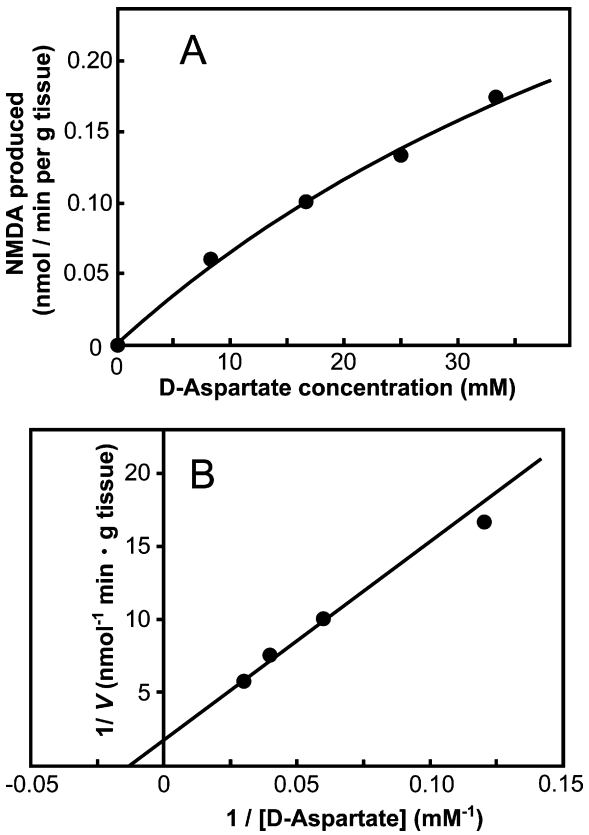

Fig. 3. Effect of D-aspartate concentration on D-aspartate *N*-methyltransferase activity in the tissue homogenate of starfish nerve (A) and Lineweaver–Burk plots (B). The tissue homogenate, corresponding to 25 mg of starfish nerves, was incubated with different concentrations of D-aspartate and 20 mM *S*-adenosyl-L-methionine for 10 min at 37 °C as described in Section 2.4. The amount of NMDA produced in the assay mixture was determined as described in Section 2.5.

The present study suggests that our method could be applied to assay a number of other enzymes producing these acidic *N*-methylamino acids.

4. Conclusion

A direct and reliable assay method for D-aspartate *N*-methyltransferase activity using a simple HPLC system was developed. This assay is based on the detection and quantification of NMDA produced in the enzyme assay. Furthermore, we demonstrate that this method can be used for detailed kinetics studies of D-aspartate *N*-methyltransferase in living organisms.

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