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A novel capillary electrophoresis method for the determination of D-serine in neural samples

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Abstract

A capillary electrophoresis method has been developed for the determination of D-serine in neural samples. D/L-serine was tagged with naphthalene-2,3-dicarboxaldehyde (CBI-D/L-Ser), and the separation of CBI-D/L-Ser enantiomer was achieved by using a dual chiral selector system consisting of β-cyclodextrin (β-CD) and chiral micelles formed by sodium deoxycholate (SDC). No resolution was observed when either β-CD or SDC was used alone. Moreover, the combined use of β-CD with achiral micelles of sodium dodecylsulfate (SDS) exhibited no resolving effect. With laser induced fluorescence detection, the limit of detection was 3.0×10^{-8} M Ser. Under the separation conditions selected, no other amino acids co-eluted with L-/D-Ser enantiomers. Using the present method, D-Ser level in *Aplysia* ganglia homogenates was found to vary significantly from animal to animal. Interestingly, D-Ser was not detected in single neurons isolated from *Aplysia* ganglia.

Keywords: D-Serine; Capillary electrophoresis; Chiral separation; Neural samples

1. Introduction

Recent studies have shown that D-serine (D-Ser) is an endogenous ligand for the glycine site of N-methyl-D-aspartate (NMDA) receptor. It binds in concert with glutamate to stimulate nerve cells to fire [1–4]. Ser racemase involved in producing D-Ser from L-Ser has been isolated [5–6]. It is believed that inhibition of Ser racemase is a potential therapeutic basis for intervention in diseases associated with NMDA receptor over-excitation [7–9]. Therefore, researches in these areas have promoted the development of novel analytical procedures for the determination of D-serine present in biological samples.

Since the first detection of D-Ser in rat brain tissues in 1992 by using a GC-MS [10] and an HPLC [11] method, several improved analytical methods have been developed for the determination of this neurobiologically important amino acid. Most of these methods involve precolumn derivatization of the amino acid with a chiral reagent and subsequent achiral HPLC separation. O-Phthaldialdehyde (OPA) in conjunction with a chiral thiol compound (e.g. an L-cysteine derivative) was most extensively used [12–13]. However, when chiral derivatization is involved one must ensure the enantiomeric purity of the chiral reagent, identical kinetics towards the two enantiomers, and no racemization during the derivatization process to avoid false analytical signals [14]. In addition to HPLC procedures, capillary electrophoresis (CE)-based separations of Ser enantiomers have also been developed. Amino acid enantiomers including D/L-Ser tagged with fluoresceine-5-isothiocyanate (FITC) [15] or dansyl chloride [16] was resolved by chiral CE. However, derivatization of amino acids with FITC or dansyl chloride tends to produce various by-products, which complicates the subsequent separation [17–19]. D/L-Ser tagged with OPA [20] was also resolved by chiral CE. The method employed a 5 μm i.d. separation capillary and UV laser induced fluorescence detection for sensitive and high through-up analysis, and have been

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applied for the analysis of D-serine in larval tiger salamander retinal homogenates. Recently, another chiral CE method was reported for the separation of D/L-Ser tagged with 3-(4-carboxybenzoyl)-2-quinoline-carboxaldehyde (CBQCA) and the detection of D-serine in the eye with laser-induced fluorescence [21].

Naphthalene-2,3-dicarboxaldehyde (NDA) is a fluorescence tagging reagent developed for primary amines and peptides, which is superior to OPA in terms of derivative's stability and fluorescence optical properties [22], and has been used to tag amino acids in single cell [23] and single secretory vesicles [24]. NDA tagged amino acids exhibit a fluorescence excitation maximum at ∼450 nm, which is very close to the wavelength of the 457.9 nm laser line from a popular argon ion laser. In the present work, capillary electrophoretic resolution of Ser enantiomers fluorescently labeled with naphthalene-2,3-dicarboxaldehyde (NDA) was studied. Application of the separation coupled with laser induced fluorescence detection to quantify Ser enantiomers in neural samples including single *Aplysia* neurons was evaluated.

2. Experimental

2.1. Chemicals and reagents

NDA was purchased from Molecular Probe (Eugene, OR, USA), and its solution was prepared weekly in methanol and kept at 4 °C in dark. Other chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA). The running buffer contained 30 mM β-CD, 60 mM SDC, and 100 mM borate buffer (pH 9.5). Milli-Q water was used throughout.

2.2. Apparatus

Capillary electrophoresis (CE) was performed using a laboratory-built system. A high-voltage supply (0–30 kV, Glassman High Voltage, Whitehouse Station, NJ, USA) was used to drive the electrophoresis. Fused-silica capillaries with an effective length of 50 cm (Polymicro Technologies, Phoenix, AZ, USA) were used for the separation. Samples were injected into the capillary by hydrodynamic flow at a height differential of 20 cm for 10 s. The 457.9 nm line from an argon ion laser (INNOVA 90C FreD, Coherent, Santa Clara, CA, USA) was focused with a 25 mm focal length fused-silica lens onto the detection window of the separation capillary. The detection window was made by removing a 5-mm section of polyimide coating on the capillary. Fluorescence emission was collected from the detection window at an angle of 90° relative to the laser beam via a 40X microscope objective (Melles Griod, Irvine, CA, USA). The image of the collected fluorescence was focused on a spatial filter and passed through a GG495 cut-off filter (Melles Griot) before reaching the photomultiplier tube (R374 equipped with a C1556-50 DA-type socket assembly, Hamamatsu, Shizuoka, Japan). The photomultiplier tube was operated at −950 V provided by a Hamamatsu C1309 regulated high voltage power supply. The output signal was recorded and processed with an IBM compatible computer using home made written software.

2.3. Precolumn derivatization

Sample solution (10 μL) was transferred to a 0.3 mL microcentrifuge vial and dried with a SpeedVac concentrator (ThermoSavant, Holbrook, NY). Borate buffer solution (0.1 M, pH 9.5, 150 μL) was added. The derivatizing reagents, NDA (2 mM in methanol, 50 μL) and KCN (20 mM in water, 50 μL) were added. The mixture was vortexed and let stand for at least 30 min at room temperature. The derivative solution was injected for CE separation without further purification.

2.4. Neural sample preparation

2.4.1. Aplysia ganglion homogenates

Specimens of *Aplysia californica* (120–200 g, obtained from *Aplysia* Research Facility, Miami, FL, USA) were injected with isotonic $MgCl_2$ solution to anaesthetize the animal. The five major ganglia (i.e. buccal, cerebral, pleural, pedal, and abdominal) were dissected out on ice. A ganglion (10–30 mg) was wiped dry, weighed, and then ground in 0.1 M HCl solution (1:3, w/v) with a glass tissue grinder. The homogenate was sonicated for 5 min at 0 °C. Trichloroacetic acid solution (30%, w/v) was added (30 μL per 100 μL homogenate). The mixture was vortexed and let stand for at least 1 h on ice before being centrifuged at 8000 rpm for 10 min. The supernatant was collected and derivatized as described above.

2.4.2. Aplysia single neurons

Aplysia ganglion was dissected from an anesthetized animal, and transferred to a small beaker containing 5 mL of protease solution (1%, w/v). The beakers were kept at 34 °C for about 90 min. The ganglion was transferred to a petri dish containing 4 mL artificial seawater (460 mM NaCl, 10 mM KCl, 55 mM $MgCl_2$, 11 mM $CaCl_2$, 2.5 mM $NaHCO_3$, pH 7.4). Under a microscope, neurons were isolated and moved to another petri dish containing 1 mL artificial seawater. After washing, individual neurons were picked up with a micropipette and placed into 100 μL vials. To a vial containing a single neuron, 5 μL 0.1 M HCl was added. The vial was sonicated for 5 min. The content was dried with a SpeedVac concentrator. The residue was re-dissolved in 2 μL borate buffer (150 mM, pH 9.5), 0.5 μL NDA (4 mM in methanol), and 0.5 μL KCN (40 mM). The mixture was vortexed and kept at room temperature for at least 30 min before injection into the CE system.

3. Results and discussion

3.1. Separation of NDA tagged Ser enantiomers

NDA reacts with Ser in the presence of cyanide, forming highly fluorescent 1-cyano-2-substituted benz[f]isoindole (CBI)-Ser derivatives. The reaction proceeds readily in aqueous solutions. CBI-Ser derivatives can be sensitively detected using the 457.9 nm laser line from an argon ion laser for fluorescence excitation. However, the separation of CBI-L/D-Ser enantiomers had been a challenge. Although a separation was reported by using a MEKC running buffer containing 10 mM γ-CD, 50 mM SDS, and 100 mM borate (pH 9.0) [25], but the baseline separation of CBI-L/D-Ser enantiomers cannot be achieved. Other CD chiral selectors including α-CD, β-CD, sulfated β-CD, carboxymehtyl β-CD (CM-β-CD), heptakis (2,3,5-tri-O-benzoyl)-β-CD, HP-γ-CD, carboxymehtyl-γ-CD(CM-γ-CD), octakis (2,3,6-tri-O-acetyl)-γ-CD, and sodium taurocholate were also tested with little success. Fortunately, it was found that CBI-Ser enantiomers could be base line resolved by using a mixed chiral selector system consisting of β-CD and chiral micelles formed by SDC as shown in Fig. 1a. It was noted that neither β-CD nor SDC used alone exhibited such resolving effects (Fig. 1b and c, respectively). Further, It was found that no resolution was achieved by the combined use of β-CD with achiral micelles such as those formed by SDS. Other mixed chiral selector systems tested included α-CD/SDC, γ-CD/SDC, HP-β-CD/SDC, as well as CM-β-CD/SDC. The most effective medium for CBI-Ser enantiomers was β-CD/SDC. After a careful study on the effects of chiral selector concentrations, the concentration and pH value of the borate buffer, as well as the applied voltage, the separation conditions were selected as following: 50 μm i.d. × 50 cm long capillary, 15 kV voltage, and a running buffer containing 30 mM β-CD, 60 mM SDC, and 100 mM borate at pH 9.5.

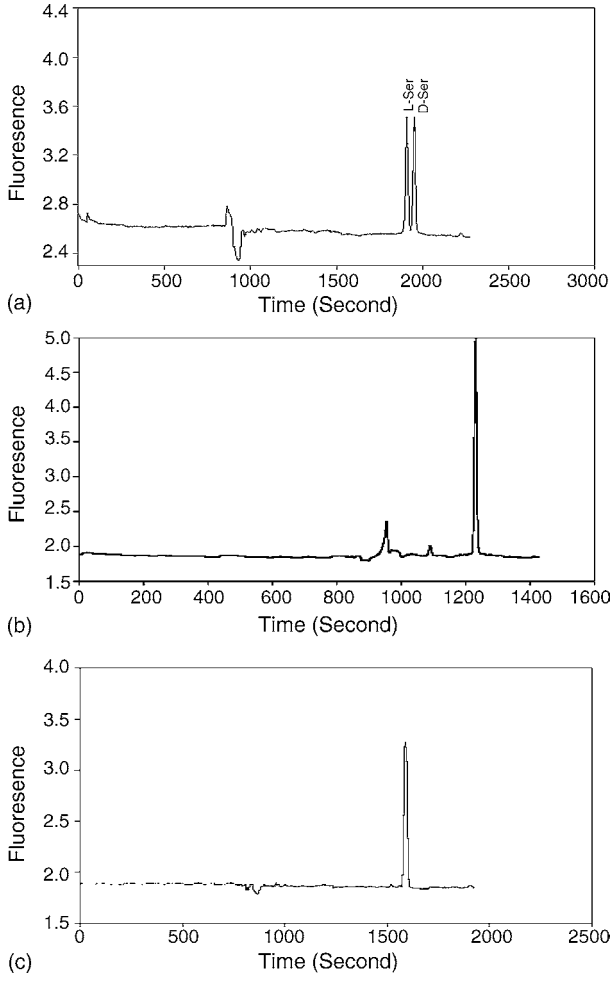

Fig. 1. Separation of CBI-Ser enantiomers using different running buffers: (a) 100 mM borate (pH 9.5), 30 mM β-CD, and 60 mM SDC; (b) 100 mM borate (pH 9.5), and 30 mM β-CD; (c) 100 mM borate (pH 10.0), 60 mM SDC. Capillary was 50 μm i.d. × 50 cm effective length. Voltage applied was 15 kV. Ser enantiomer concentration was 2.0 μM.

3.2. Interference studies

Many endogenous amino acids and primary amines are present in nerve tissue samples and react with NDA forming fluorescent derivatives, which may interfere with the determination of L/D-Ser. Therefore, effects from L-, D-amino acids and amines including L-Arg, Gly, L-Gln, L-His, L-Val, L-Leu, L-Met, L-Tyr, L-Thr, L-Trp, L-Phe, L-Ile, L-Asn, L/D-Ala, L/D-Asp, L/D-Glu, taurine, and γ-amino-*n*-butyric were investigated. These compounds were tested because they would most likely interfere with the determination. As can be seen from Fig. 2, all of them were separated from L/D-Ser enantiomers under the separation conditions selected, i.e. none of them would interfere with the determination of L/D-Ser.

3.3. Analysis of neural samples

A five-point calibration curve was prepared using D-Ser standard solutions. The concentration of D-Ser in the derivative solutions ranged from 0.2 to 2.0 μM. Fluorescence intensities were used for the quantification. The following regression equation was obtained:

$$F = 0.4990X + 0.0025 \quad (r^2 = 0.9987)$$

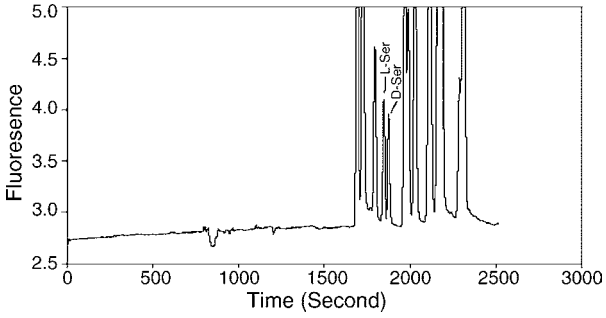

Fig. 2. Electropherogram obtained from separating D/L-Ser enantiomers in the presence of 19 other amino acids. CE conditions were as in Fig. 1a.

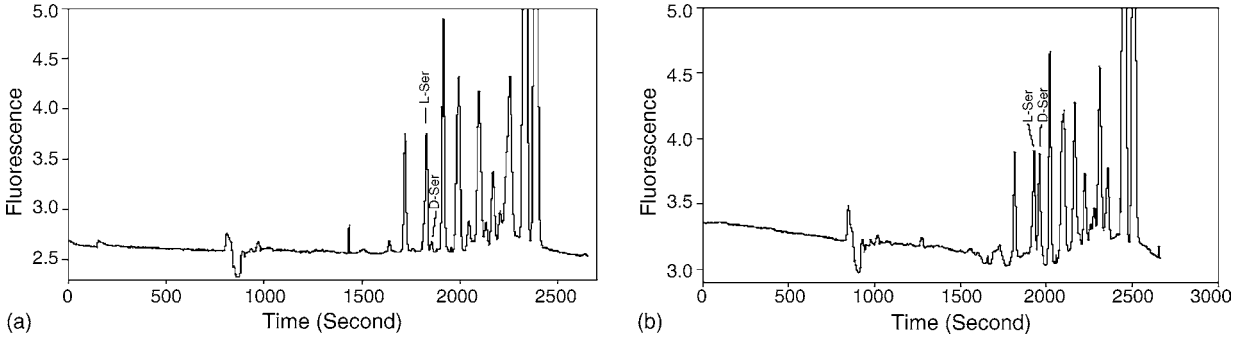

Fig. 3. Electropherograms obtained from analyzing an *Aplysia* pedal ganglion homogenate (a) and the homogenate sample spiked with D-Ser at 1.1 μM. CE conditions were as in Fig. 1a.

where F is the fluorescence intensity, and X is the concentration of D-Ser in the derivative solution (μM). The amounts of D-Sere in the samples were calculated from the regression equation. From this calibration curve, the limit of detection (signal/noise = 3) was estimated to be 3.0×10^{-8} M D-Ser.

3.3.1. Analysis of Aplysia ganglia

Aplysia californica, a sea mollusk, is a widely used neuronal model. Although D-Ser has been detected in higher vertebrates such as rats, there is so far no report on the occurrence of D-Ser in invertebrates including *Aplysia*. In the present work, ganglia dissected from five specimens of *Aplysia californica* were analyzed. Fig. 3a shows an electropherogram obtained from an analysis of a pedal ganglion homogenate. The peaks corresponding to L-Ser and D-Ser can be well identified. To verify the peak identification, authentic D-Ser was added to this sample, and then the spiked sample was again derivatized and separated. The electropherogram obtained is shown in Fig. 3b. As can be seen by comparing Fig. 3a with b, the height of D-Ser peak increased markedly in the electropherogram. It was noticed that while the levels of L-Ser were similar, D-Ser levels varied substantially from animal to animal. For example, D-Ser contents in pedal ganglion homogenates ranged from 0 to 32 nmol/g wet tissue. The determination results are summarized in Table 1. Recovery of D-serine from a pedal ganglion sample was studied. D-Serine was spiked to three portions of a pedal ganglion sample at 1.1 μM. The recovery was found to be 105.2 ± 3% (mean of the three measurements). Similar results were obtained for the other four major *Aplysia* ganglia, i.e. cerebral, buccal, abdominal, and pleural. More studies are needed before any conclusion can be drawn on why D-Ser levels in *Aplysia* ganglia varied so much from animal to animal.

Table 1
Results of D-serine determination in *Aplysia* pedal ganglion

Sample number	Content of D-serine (nmol/g)	R.S.D., $n = 4$ (%)
1	32.2	3.8
2	27.6	4.6
3	17.5	4.2
4	0	

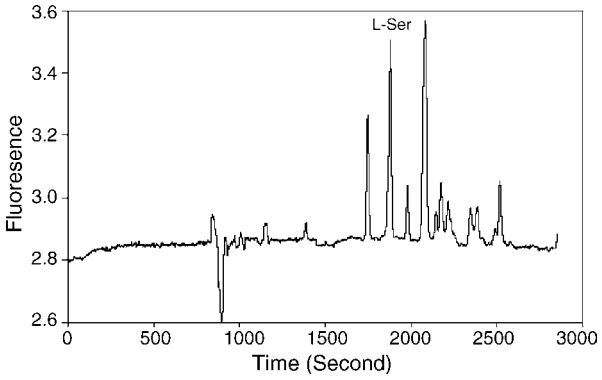

Fig. 4. Analysis of a single neuron dissected from an *Aplysia* pedal ganglion. CE conditions were as in Fig. 1a. No D-Ser was detected in this neuron.

3.3.2. Analysis of single Aplysia neurons

With the use of laser induced fluorescence detection, the present method may be used to analyze single *Aplysia* neurons for determine D-Ser contents. Fig. 4 shows a typical electropherogram from the analyses of single neurons dissected from an *Aplysia* pedal ganglion. As can be seen, no D-Ser was detected. Interestingly, D-Ser was not detected in all of the neurons analyzed. D-Ser might perhaps not occur in neurons of *Aplysia* or the contents in single neurons too low which can not be detected by the present method. Therefore, these results need to be further confirmed.

4. Conclusion

The highly fluorescent L/D-Ser enantiomers tagged with naphthalene-2,3-dicarboxaldehyde was base line resolved by employing a dual chiral selector system composed of β-CD and SDC. Coupled with laser induced fluorescence detection, the separation was well suited for sensitive determination of L/D-Ser in neural samples. *Aplysia* ganglia homogenates, and single neurons dissected from *Aplysia* ganglia were analyzed. For the first time, the occurrence of D-Ser in the central nervous system of *Aplysia californica* (sea mollusk, an extensively studied neuronal model) was investigated. D-Serine contents in *Aplysia* ganglion homogenates were found to vary

significantly from animal to animal. Analyses of *Aplysia* single neurons revealed that D-Ser might not occur in neurons.

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References

[1] S.H. Snyder, P.N. Kim, Neurochem. Res. 25 (2000) 553–560.
[2] J.-P. Mothet, A.T. Parent, H. Wolosker, R.O. Brady Jr., D.J. Linden, C.D. Ferris, M.A. Rogawski, S.H. Snyder, Proc. Natl. Acad. Sci. U.S.A. 97 (2000) 4926–4931.
[3] Y. Yang, W. Ge, Y. Chen, Z. Zhang, W. Shen, C. Wu, M. Poo, S. Duan, Proc. Natl. Acad. Sci. U.S.A. 100 (2003) 15194–15199.
[4] D. Boehning, S.H. Snyder, Annu. Rev. Neurosci. 26 (2003) 105–131.
[5] H. Wolosker, S. Blackshaw, S.H. Snyder, Proc. Natl. Acad. Sci. U.S.A. 96 (1999) 13409–13414.
[6] H. Wolosker, K.N. Sheth, M. Takahashi, J.P. Mothet, R.O. Brady Jr., C.D. Ferris, S.H. Snyder, Proc. Natl. Acad. Sci. U.S.A. 96 (1999) 721–725.
[7] S.H. Snyder, C.D. Ferris, Am. J. Psychiatry 157 (2000) 1738–1751.
[8] H. Wolosker, PanizzuttiR. De Miranda, J. Neurochem. Int. 41 (2002) 327–332.
[9] A. Sawa, S.H. Snyder, Mol. Med. 9 (2003) 3–9.
[10] A. Hashimoto, T. Nishikawa, T. Hayashi, N. Fuji, K. Harada, T. Oka, K. Takahashi, FEBS Lett. 296 (1992) 33–36.
[11] A. Hashimoto, T. Nishikawa, T. Oka, K. Takahashi, H. Hayashi, J. Chromatogr. 582 (1992) 41–48.
[12] A. Hashimoto, T. Oka, Prog. Neurobiol. 52 (1997) 325–353.
[13] K. Hamase, A. Morikawa, K. Zaitsu, J. Chromatogr. B: Biomed. Appl. 781 (2002) 73–91.
[14] H. Wan, L.G. Blomberg, J. Chromatogr. A 875 (2000) 43–88.
[15] X. Lu, Y. Chen, J. Chromatogr. A 955 (2002) 133–140.
[16] L. Yang, Z. Yuan, Fresenius J. Anal. Chem. 365 (1999) 541–544.
[17] E. Bayer, E. Grom, B. Kaltenegger, R. Uhmann, Anal. Chem. 48 (1976) 1106–1109.
[18] Y. Tapuhi, D.E. Schmidt, W. Lindner, B.L. Karger, Anal. Biochem. 115 (1981) 123–129.
[19] C. DeJong, G.J. Hughes, E.V. Wieringen, K.J. Wilson, J. Chromatogr. 241 (1982) 345–359.
[20] K.B. O'Brien, M. Esguerra, C.T. Klug, R.F. Miller, M.T. Bowser, Electrophoresis 24 (2003) 1227–1235.
[21] K. Thongkhao-On, S. Kottegoda, J.S. Pulido, S.A. Shippy, Electrophoresis 25 (2004) 2978–2984.
[22] R.G. Carson, K. Srinvasachar, R.S. Givens, B.K. Matuszewski, J. Org. Chem. 51 (1986) 3978–3983.
[23] S.D. Gilman, A.G. Ewing, Anal. Chem. 67 (1995) 58–64.
[24] D.T. Chiu, S.J. Lillard, R.H. Scheller, R.N. Zare, S.E. Rodriguez-Cruz, E.R. Williams, O. Orwar, M. Sandberg, J.A. Lundqvist, Science 279 (1998) 1190–1193.
[25] T. Ueda, R. Mitchell, F. Kitamura, T. Metcalf, J. Chromatogr. 593 (1992) 265–274.