愛媛大学理学部紀要 第 24 巻 1-8 ページ, 2022 年
Mem. Fac. Sci. Ehime Univ. Vol. 24, p1-8, 2022

Analyses of Free D- and L-Amino Acids in the Perfused Brains of Adult Rats

成体ラットの灌流処理した脳中の遊離 D-および L-アミノ酸分析

Fumi Sato,[1] Masayuki Takahashi,[2] Akihiko Yamagishi [1] and Hisako Sato [3*]

佐藤二美 [1]、高橋正行 [2]、山岸晧彦 [1]、佐藤久子 [3*]

[1] Department of Anatomy, Faculty of Medicine, Toho University, Ota-ku 143-8540, Japan
[1] 東邦大学医学部解剖学講座
[2] Department of Chemistry, Faculty of Science, Hokkaido University, Sapporo 060-0810, Japan
[2] 北海道大学大学院理学研究院化学部門
[3] Graduate School of Science and Engineering, Ehime University, Matsuyama 790-8577, Japan
[3] 愛媛大学大学院理工学研究科環境機能科学専攻
E-mail: sato.hisako.my@ehime-u.ac.jp

Abstract

Free D-amino acids were analyzed in the perfused brains of rats. As samples, left and right cerebrums and a cerebellum were obtained separately from three rats. They were 9 weeks of age. Amino acids were derivatized with 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F). Analyses were performed through two steps. As the first step, a sample was eluted on a reverse phase C_{18}-modified silica gel column. The derivatized amino acids were obtained in a pure form. As the second step, each amino acid was eluted on a chiral column to obtain the D/L ratio. As a result, the L-enantiomers of Ala, Glu, Leu, Lys, Phe and Thr existed at the purity of 99.9 ± 0.1 %, while the D-enantiomer of Ser was present at the level of 11.3 ± 0.5 %. No difference was observed among left and right cerebrums and cerebellums. The results are discussed in comparison with what was reported previously for the cases of mice (5 weeks of age) and mice (3 and 24 months of age).

Keywords: Rat brain, D-Amino acids, Chiral column

1. Introduction

Nowadays it has been established that the D-form of an amino acid exists widely in biological tissues.[1-6] Some are the unnatural products transformed from the L-forms due to aging or disease and others play an essential role in biological processes.[1] In the central nervous systems, for example, D-Ser is present as a co-agonist in neurotransmission with L-Glu for the ion-channel-type N-methyl-D-aspartic acid (NMDA) receptor.[7-11] They are the key regulators of brain plasticity for memory and learning. In another example, the level of D-Ser in a blood represents the normal conditions for kidney function.[12] Its content increases with the lowering of a kidney function. It was also reported that D-Ser existed in a rat brain and

2021 年 11 月 13 日　受付　2022 年 1 月 28 日　受理

that its level varied during growth in connection with the development of the nerves sytems.[13-23]

The field owes its progress substantially to the development of the analytical method of L- and D-amino acids at the extremely low level (~10^{-3} ng).[2] In particular, the multi-step high performance liquid chromatography has been established for this purpose.[5] Until now, however, the reported results have been limited to cases with particular interest. For example, D-Ser is found to exist at the high level in the cerebellum of an infant mammal.[19] It is still unknown, however, whether its level is maintained during growth. Such knowledge might be important to pursue the time course of the production of D-Ser on aging.[21]

In the present study, the analyses of D- and L-amino acids were performed for the brains of rats (9 weeks). It was intended to see how the optical purity of an amino acid in a brain was maintained while a mammal grows up.

2. Experimental

Materials: All chemical reagents (distilled water, methanol, acetonitrile, triethylamine, 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F), a series of DL-amino acids, L-Glu and citric acid) were purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan) and used without preparation.

Preparation of brain samples: The brain samples were taken from three Sprague-Dawley rats (male; 9 weeks old). Our experimental protocol was approved by the Animal Care Committee of Faculty of Medicine, Toho University (No. 20-54-342). After anesthetized with ketamine (7.5 mg/kg)-xylazine (50 mg/kg), the animals were perfused transcardially with 0.9% NaCl and the brains were removed. The right and left cerebrum and the cerebellum were obtained separately from each animal. Thereafter they were homogenized manually in 2 mL of distilled water using a PTFE pestle (YAKUKENSYA Co., Ltd, Chiba, Japan). The mixtures were centrifuged at 16,000 × g for 20 minutes at 4 ºC. The filtrates were stored in the refrigerator at 4 ºC until use. For the analyses of amino acid composition, the brain samples were further treated as follows: The filtrates were centrifuged at 100,000 × g for ten minutes at 4 ºC. For deproteinization, the resulting supernatant were mixed with an equal volume of 10% (v/v) trichloroacetic acid solution and then centrifuged at 22,000 × g for 15 minutes at 4 ºC.

Analytical procedures: The analyses were performed according to the following two steps: (i) the separation of an amino acid as a pure form from the above filtrates; (ii) the determination of the DL ratio of a purified amino acid. As step (i), 0.1 mL of a stored filtrate was added into 0.5 mL of 1:1 (v/v) acetonitrile-water containing 0.1 mg of NBD-F and 5 μL of triethylamine. The solution was placed for 1 hour at room temperature until the derivatization was complete. The progress of derivatization reaction was monitored by the increase of absorbance at 450 nm. After centrifuging the solution, a 50 μL of the supernatant was injected and eluted on a C_{18} column (TSKgel ODS-100Z 5μm, TOSOH Co., Tokyo, Japan). The size of the column was 4.6 mm (i.d.) × 15 cm. The elution was performed under the gradient program using two solutions (A: 16:84 (v/v) CH_3CH/75 mM H_2PO_4; B: 12:39:40 (v/v) CH_3CN/CH_3OH/50mM KH_2PO_4) at a rate of 0.6 $mLmin^{-1}$. The peaks were assigned by comparing the results of eluting an authentic amino acid under the same conditions. Each fraction was collected and

stored below 4 ºC. As a separate experiment, the total abundances of free amino acids were determined by an amino acid analyzer L-8900 (Hitachi High Technologies, Tokyo, Japan) in the Instrumental Analysis Division, Global Facility Center (Hokkaido University). As step (ii), the optical resolution of a collected fraction was performed by use of a chiral column (SUMICHIRAL OA-3200R, Sumika Chemical Analysis Service, Ltd., Osaka, Japan). The size of the column was 4.6 mm (i.d.) × 25 cm. Firstly eluting was performed by methanol containing 50 mM of ammonium acetate as indicated by the manufacturer. In case of glutamic acid, however, the compound was adsorbed too strongly to be eluted from the column. No improvement was achieved when the concentration of ammonium acetate was raised to 0.2 M. For overcoming the problem, it was found that the elution of glutamic acid was possible when a methanol solution containing 30 mM of citric acid was used as an eluant. Based on this, optical resolution was performed with this eluent for all investigated amino acids. The resolution was successful except for proline as tabulated in Table 1.

3. Results and Discussion

Figure 1 shows the total abundances of free amino acids in the left and right cerebrums and cerebellum of a rat. In three tissues, 17 kinds of amino acids were identified to be present. No significant difference was observed among these three tissues. Highest level of Glu reflected the fact that the amino acid acted as an agonist for the NMDA receptor in all of brain tissues.

Figure 2 shows the chromatogram when the sample extracted from the cerebrum was eluted on a C_{18} column. The peaks were assigned by comparing the elution time for the authentic sample of each amino acid. The eluent corresponding to each peak (~ 0.5 mL) was collected for analyzing the DL ratio in the next step.

For checking the resolution capability of the used chiral column (SUMICHIRAL OA-3200R), the authentic sample of a racemic amino acid modified with NBD-F was eluted with methanol containing 30 mM citric acid. Figure 3 shows the example of resolution in case of racemic Glu. Two well separated peaks were obtained. As the separate experiment of using L-Glu alone, the first and second peaks were assigned to the L- and D-enantiomers, respectively.

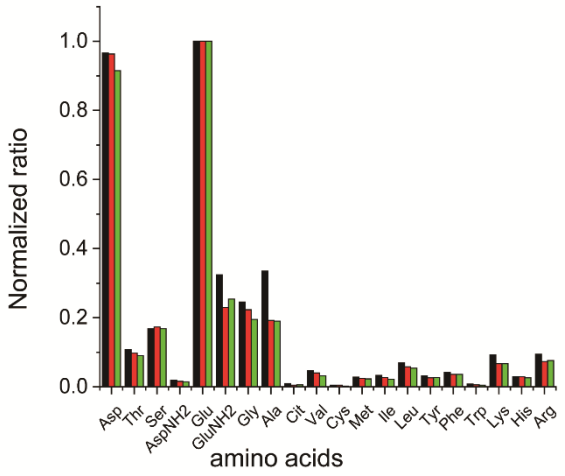


Figure 1. The compositions of free amino acids in brain tissues: (blue) cerebellum, (red) right cerebrum and (green) left cerebrum. The vertical axis was normalized at 1 for Glu. The total amino acids were obtained to be 7.4 μmoles (cerebellum), 14.8 μmoles (left cerebrum), and 12.6 μmoles (right cerebrum), respectively.

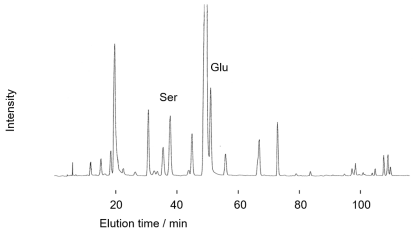


Figure 2. The example of the chromatogram when the sample for a cerebrum (left) was eluted on a C18 column. Amino acids were modified by NBD-F. Graduating conditions are described in the experimental section. Elution was monitored at the absorbance at 450 nm. The flow rate was 0.6 mLmin^{-1}. Temperature was 25 ºC (see the text for details)

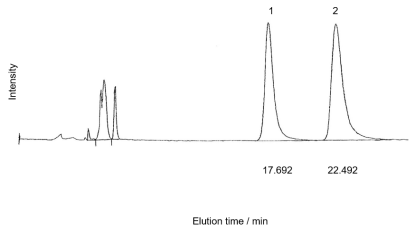


Figure 3. The example of the chromatogram when DL-Glu was eluted on a chiral column. The amino acid was modified by NBD-F. The elution was monitored at the absorbance at 450 nm. The eluant was methanol containing 30 mM of citric acid. The flow rate was 0.7 mLmin^{-1}. Temperature was 25 ºC. Comparing with the elution behavior of the authentic samples, the first and second peaks were assigned to the L- and D-enantiomers, respectively.

The results of chromatographic resolution are summarized in Table 1. As shown in the table, amino acids were separated into each enantiomer nearly on a baseline separation except for Pro. Pro gave only a single peak, indicating that the amino acid was not resolved under the present conditions.

Table 1. The elution time of an amino acid eluted on a chiral column.

Amino acid	L*	D*	SF**
Ala	13.5	18.0	1.37
Glu	17.7	23.9	1.38
Leu	10.1	14.4	1.50
Lys	33.8	48.9	1.46
Phe	15.3	22.9	1.55
Pro	22.5	22.5	1.00
Ser	17.5	22.4	1.30
Thr	14.3	17.6	1.25
Tyr	14.1	18.9	1.38
Val	11.2	13.2	1.22

Column: Sumi Chiral, Elution solvent: methanol (30 mM citric acid), Eluting rate:0.7 ml min^{-1}

(*) Elution time was expressed in terms of min.

(**) Separation factor (the dead time = 1.5 min)

The fraction containing each amino acid in a pure form was obtained from the tissue samples as described above. The sample was injected into a chiral column and eluted under the same conditions. The example is shown in Figure 4 (A) in case of the sample containing Glu. As shown by the figure, the chromatogram gave a single peak corresponding to L-Glu. The results indicated that Glu in the cerebrum of a rat was optically pure. The maximum content of D-Glu was estimated to be 0.1 % of the total Glu.

The same experiments were performed for other samples collected at the peaks assigned to the known amino acids. The results are summarized in Table 2. According to the table, the investigated amino acids (Ala, Glu, Leu, Lys, Phe and Thr) were present as an optically pure form (99.9 ± 0.1 %). The only exception was Ser. As shown in Figure 4(B), D-Ser was present at the level of 11.3 ± 0.5 % in both left and right cerebrums and cerebellum.

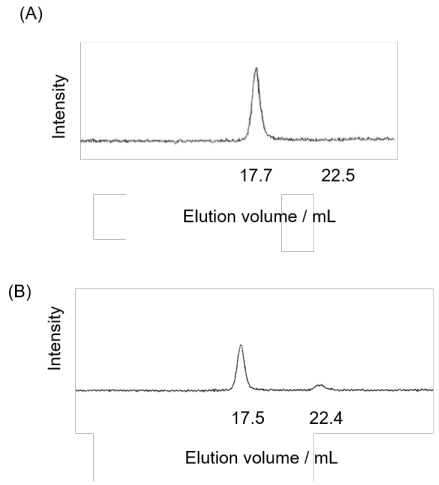


Figure 4. The examples of the chromatogram when the right cerebrum samples collected at the peaks corresponding to (A) Glu and (B) Ser were eluted on a chiral column, respectively (see the text for details). The elution volume at each peak was expressed in terms of mL

When the present results are compared with what was reported previously for the cases of mice (5 weeks) and mice (3 and 24 months of age), the following aspects are noteworthy.[2, 21] Firstly the observed level of D-Ser (11.3 %) was intermediate between that for the brain of mice (5 weeks; ca. 30 %) and those for the cerebellums of mice (3 and 24 months of age; 2.8 % and 1.9 %, respectively).[2,21] One reason might be sought in the difference of nervous systems between mice and rats. Another one, which seemed more probable, was sought in the effects of aging. The tendency of decreasing the content of D-Ser with aging was noted previously.[21] The demand for elasticity of network would decrease with aging.[18] In the pioneering work, the content of D-Ser was reported to be 23 % for the brain of a rat of ca. 7 weeks age.[13] Summarizing these results, the present work suggested that the level of D-Ser continued to decrease on aging for both mice and rats. The aging effect might overcome the differences in species and living circumstances such as stress, disease and health conditions. Regarding as the metabolism of D-Ser, the mechanistic routes are still unclear.[21] The two-base mechanisms by peptide isomerase, for example, are proposed for L-to-D conversion of amino acids.[22,23] If the same mechanism is operative for the case of L-Ser, the efficiency of an isomerase, or the stability of an intermediate state combining with two basic groups, might depend on aging significantly. Moreover the enzymatic activity of peptide isomerase might be dependent on tissues in a body, since it was previously reported that there was heterogeneity in the distribution of D-Ser between the cerebrums and cerebellum of human central nervous system.[19]

Secondly the high optical purity of L-Glu as found in this study was in accord with the case for a mouse (5 weeks). Thus L-Glu might be required to be optically pure all through the lifespan of a rat. This conclusion raises a possibility that D-Glu interferes functions in central nervous systems, acting as an antagonist for NMDA receptor.

Thirdly the other amino acids (Ala, Glu, Thr, Leu, Lys and Phe) were all present as an optically

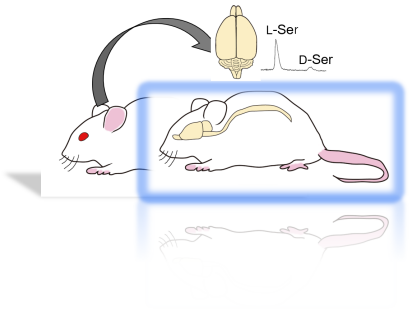
pure form. This was contrasted with the previous results for a mouse that the D-form exited at the level of 10 ~ 30 % in case of Ala, Asp and Phe. Thus the optical purity might approach for 100 % with the growth. One reason might be that the D-form of these amino acids could be present as a source for producing D-Ser at the infant stage. On aging with the decrease of D-Ser, the demand for the D-forms of these amino acids might reduce. It is therefore demanded to pursue the change of D-forms of the free amino acids with the growth of a mammal. Further experiments are demanded to settle this point.

Table 2. DL analyses of free amino acids in the rat brain

Amino acid	cerebrum		cerebellum	
	L(%)*	D(%)*	L(%)*	D(%)*
Ala	99.9± 0.1	0.0 ± 0.5	99.9 ± 0.1	0.0 ± 0.5
Glu	99.9± 0.1	0.0 ± 0.5	99.9 ± 0.1	0.0 ± 0.5
Leu	99.9± 0.1	0.0 ± 0.5	99.9± 0.1	0.0 ± 0.5
Lys	99.9± 0.1	0.0 ± 0.5	99.9 ± 0.1	0.0 ± 0.5
Phe	99.9± 0.1	0.0 ± 0.5	99.9 ± 0.1	0.0 ± 0.5
Ser	89.0± 0.1	11.3± 0.5	89 .0± 0.1	11.3± 0.5
Thr	99.9± 0.1	0.0 ± 0.5	99.9 ± 0.1	0.0 ± 0.5

* The errors were the standard deviations for the values obtained of three runs or one for each individual.

Scheme 1 shows the schematic image of a rat. The brain and brainstem are also shown. Among the investigated amino acids, Ser alone existed as a mixture of D- and L-enantiomers at the measurable ratio.



Scheme 1. Schematic image of brain and brainstem of a rat. D-Amino acids were searched in the brain of a rat. Among the investigated amino acids, Ser alone existed as a mixture of D- and L-enantiomers at a ratio of 11:89.

4. Conclusion

The total analyses of amino acids including DL chirality were performed for the brains of adult rats. The optical purity of an amino acid was revealed to be maintained strictly except for Ser.

Acknowledgement

Thanks are due to the Instrumental Analysis Division, Global Facility Center (Hokkaido University) for amino acid analyses. This research was supported by Grants-in-Aid for Scientific Research from the Japanese Ministry of Education & Science (JP 16K15175). The author appreciates Prof. Kei Nomiyama and Prof. Tatsuya Kunisue (Ehime University) for their kind advice.

References

1. N. Fujii, T. Takata, N. Fujii, K. Aki, H. Sakaue, D-Amino acids in protein: The mirror of life as a molecular index of aging, *Biochim. Biophys. Acta: Proteins Proteomics* **2018**, *1866*, 840.
2. C. A. Weatherly, S. Du, C. Parpia, P. T. Santos, A. L. Hartman, D. W. Armstrong, D-amino acid levels in perfused mouse brain tissue and blood: A Comparative Study, *ACS Chem. Neurosci.* **2017**, *8* 1251.
3. I. Livnat, H.-C. Tai, E. T. Jansson, L. Bai, E. V. Romanova, T.-t. Chen, K. Yu, S.-an Chen, Y. Zhang, Z.-yang Wang, D.-dan Liu, K. R. Weiss, J. Jing, J. V. Sweedler, A D-Amino acid-containing neuropeptide discovery funnel, *Anal. Chem.* **2016**, *88*, 11868.
4. M. Qi, M. C. Philip, N. Yang, J. V. Sweedler, Single cell neurometabolomics, *ACS Chem. Neurosci.* **2018**, *9*, 40.
5. A. Furusho, R. Koga, T. Akita, M. Mita, T. Kimura, K. Hamase, Three-dimensional high-performance liquid chromatographic determination of Asn, Ser, Ala, and Pro enantiomers in the plasma of patients with chronic kidney disease, *Anal. Chem.* **2019**, *91*, 11569.
6. A. Yamagishi, K. Takimoto, K. Tamura, F. Sato, H. Sato, Chiral discrimination of dansylated alanine methyl ester on a modified clay surface: Vibrational circular dichroism approach, *Bull. Chem. Soc. Jpn.* **2021**, *94*, 2711.
7. T. Miyamoto, H. Homma, Detection and quantification of D-amino acid residues in peptides and proteins using acid hydrolysis, *Biochim. Biophys. Acta: Proteins Proteomics* **2018**, *1866*, 775.
8. P. Paoletti, C. Bellone, Q. Zhou, NMDA receptor subunit diversity: impact on receptor properties, synaptic plasticity and disease, *Nat. Rev. Neurosci*. **2013**, *14*, 383.
9. H. Wolosker, NMDA receptor regulation by D-serine: new findings and perspectives, *Mol. Neurobiol.* **2007**, *36*, 152.
10. J. De Miranda, R. Panizzutti, V. N. Foltyn, H. Wolosker, Cofactors of serine racemase that physiologically stimulate the synthesis of the *N*-methyl-D-aspartate (NMDA) receptor coagonist D-serine, *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 14542.
11. Y. Tanii, T. Nishikawa, A. Hashimoto, K. Takahashi, Stereoselective antagonism by enantiomers of alanine and serine of phencyclidine-induced hyperactivity, stereotypy and ataxia in the rat., *J. Pharmacol. Exp. Ther.* **1994**, *269*, 1040.
12. J. Sasabe, T. Chiba, M. Yamada, K. Okamoto, I. Nishimoto, M. Matsuoka, S. Aiso, D-Serine is a key determinant of glutamate toxicity in amyotrophic lateral sclerosis, *EMBO J.* **2007**, *26*, 4149.
13. A. Hashimoto, T. Nishikawa, T. Hayashi, N. Fujii, K. Harada, T. Oka, K. Takahashi, The presence of free D-serine in rat brain, *FEBS Lett.* **1992**, *296*, 33.
14. J. M. Seckler, S. J. Lewis, Advances in D-amino acids in neurological research, *Int. J. Mol. Sci.* **2020**, *21*, 7325.
15. M. Suzuki, N. Imanishi, M. Mita, K. Hamase, S. Aiso, J. Sasabe, Heterogeneity of D-serine distribution in the human central nervous system,

ASN Neuro. **2017**, 1.

16. C. Madeira, M. V. Lourence, C. Vargas-Lopes, C. K. Suemoto, C. O. Brandão, T. Reis, R. E. P. Leite, J. Laks, W. Jacob-Filho, C. A. Pasqualucci, L. T. Grinberg, S. T. Ferreira, R. Panizzutti, D-serine levels in Alzheimer's disease: implications for novel biomarker development, *Transl. Psychiatry* **2015**, *5*, 561.
17. Y. Miyoshi, K. Hamase, T. Okamura, R. Konno, N. Kasai, Y. Tojo, K. Zaitsu, Simultaneous two-dimensional HPLC determination of free D-serine and D-alanine in the brain and periphery of mutant rats lacking D-amino-acid oxidase, *J. Chromatogr. B* **2011**, *879*, 3184.
18. A. Hashimoto, T. Nishikawa, T. Oka, K. Takahashi, Endogenous D-serine in rat brain: *N*-methyl-D-aspartate receptor-related distribution and aging, *J. Neurochem.* **1993**, *60*, 783.
19. Y. Nagata, K. Horiike, T. Maeda, Distribution of freeD-serine in vertebrate brains, *Brain Res.* **1994,** *634,* 291.
20. Z. Li, Y. Xing, X. Guo, Y. Cui, Development of an UPLC-MS/MS method for simultaneous quantitation of 11 d-amino acids in different regions of rat brains: Application to a study on the associations of D-amino acid concentration changes and Alzheimer's disease, *J. Chromatogr. B* **2017**, *1058*, 40.
21. T. Yamamoto, K. Yaku, T. Nakagawa, Simultaneous Measurement of amino acid enantiomers in aged mouse brain samples by LC/MS/MS combined with derivatization using N-(5-Fluoro-2,4-dinitrophenyl)-L-leucinamide (L-FDLA), *Metabolites* **2021**, *11*, 57.
22. P. S. Basal, A. M. Torres, B. Crossett, K. K. Y. Wong, J. M. S. Koh, D. P. Geraghty, J. I. Vandenberg, P. W. Kuchel, Substrate specificity of platypus venom L-to-D-peptide isomerase, *J. Biol. Chem.* **2008**, *283*, 8969.
23. M. Katane, *The D-amino acid Research Society* **2015**, *4*, 1