Tohru Yoshimura · Toru Nishikawa
Hiroshi Homma *Editors*

D-Amino Acids

Physiology, Metabolism, and Application

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ISBN 978-4-431-56075-3 ISBN 978-4-431-56077-7 (eBook)
DOI 10.1007/978-4-431-56077-7

Library of Congress Control Number: 2016947710

Printed on acid-free paper

This Springer imprint is published by Springer Nature
The registered company is Springer Japan KK

Preface

D-Amino acids were once called unnatural amino acids and were considered to be insignificant for eukaryotes especially in mammals. D-Amino acids had been known to have only a few roles, for example, as components of the peptidoglycan layers of bacterial cell walls and as antibiotics. However, in the 1990s, D-serine was found to serve as a co-agonist of the *N*-methyl-D-aspartate (NMDA) receptor in mammalian brains and to be involved in various brain functions. These findings encouraged further studies on D-amino acids. Currently, they have been revealed to bear important physiological roles. For example, D-serine is implicated in memory formation and learning, and its abnormal concentration in tissues has been reported in various neurological diseases such as schizophrenia and amyotrophic lateral sclerosis. D-Aspartate is found in various mammalian tissues, particularly in the central nervous system and the genitals. D-Aspartate facilitates the endocrine secretion of prolactin, inhibits the secretion of melatonin, and plays a peculiar role in the control of reproductive functions in mammals, including the stimulation of testosterone synthesis.

The objective of this book is to provide an overview of the roles of D-amino acids and to introduce recent progress in studies of them. Part I reviews the indispensable analytical methods by which D-amino acids are studied. Results of studies on D-serine and D-aspartate are shown in Parts II and III, respectively. Remarkable progress in studies on D-amino acids in peptides is described in Part IV. Enzymes producing and degrading D-amino acids are reviewed in Part V, as the knowledge of these enzymes is indispensable for understanding the profound functions of D-amino acids. Part VI introduces their role in foods, especially in fermented foods, which is probably the source of exogenous D-amino acids. It will give me great pleasure if this book facilitates a better understanding of the importance and the fascinating aspects of D-amino acids.

Nagoya, Japan
January 2016

Tohru Yoshimura, on behalf of the editors

Contents

Part I
Detection of D-Amino Acids

Chapter 1
Determination of D-Amino Acids and Their Distribution in Mammals

Yurika Miyoshi, Reiko Koga, and Kenji Hamase

Abstract D-Amino acids are the enantiomers of L-amino acids. Although the L-forms are predominantly present in life systems in the free form and also in the protein-bound form, the optically inversed D-forms were hardly found especially in higher animals. This is due to the very low amounts of these D-amino acids, and the determination is frequently disturbed by large amounts of L-amino acids and uncountable numbers of peptides and amino compounds. However, due to the recent progress in analytical technologies, several D-amino acids have been found in mammals including humans. In this chapter, the analytical methods for determining these D-amino acids and their distribution in mammals are described.

Keywords D-Amino acids • Chiral separation • Analytical method • Distribution

D-Amino acids are the enantiomers of L-amino acids. Although L-amino acids are predominantly present in organisms with distinct physiological functions, the chiral counterparts, D-amino acids, were rarely found, especially in the higher animals. Therefore, these D-amino acids had been long believed that they do not have physiological meanings in mammals. However, along with the progress in analytical techniques, several free D-amino acids have been found in mammals including humans and their distributions, functions, and regulation mechanisms have gradually been clarified. In this chapter, the analytical methods of these free D-amino acids and their distributions in mammals are described. Concerning the D-amino acid residues in proteins, please refer to an excellent review (Fujii et al. 2011).

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T. Yoshimura et al. (eds.), *D-Amino Acids*, DOI 10.1007/978-4-431-56077-7_1

1.1 Determination of D-Amino Acids in Biological Samples

In most cases, the amounts of D-amino acids are at trace levels in the tissues and physiological fluids of higher animals, and the determination is interfered by many of the intrinsic substances (large amounts of L-amino acids, peptides, and amines). Therefore, sensitive and selective analytical methods consisting of chromatographic or electro-driven separation techniques, or using highly selective enzymes/antibodies, have been established. In this section, colorimetric methods using enzymes/antibodies and separation analyses including GC, HPCE, and HPLC are described.

1.1.1 Colorimetric Analysis Using Enzymes and Antibodies

Colorimetric analyses are useful as simple and rapid methods. Concerning the D-amino acid analysis, several methods have already been established. Historically, simple methods using D-amino acid oxidase (DAO, EC 1.4.3.3) and D-aspartic acid oxidase (DDO, EC 1.4.3.1) have frequently been used. These enzymes react with the D-amino acids to form α-keto acids, and the keto acids further react with hydrazine to form hydrazones. Therefore, D-amino acids could be determined by a simple colorimetric analysis (Nagata et al. 1985). However, the substrate specificity of these enzymes is broad, and the D-amino acids are determined as their sum in the target matrices. The combination of DAO and HPLC is also used to separately determine various D-amino acids (Kato et al. 2011). For the specific determination of D-Ser, D-Ser dehydratase (DSD, EC 4.3.1.18) could also be used (Suzuki et al. 2011). Concerning the method using an antibody, an enzyme immunoassay using a specific monoclonal antibody against D-Asp has been established (Ohgusu et al. 2006). These methods are rapid and simple, and the amounts of the D-amino acids could therefore be easily determined. However, a variety of substances is present in the tissues and physiological fluids; thus, a thorough confirmation of the determined values would be necessary.

1.1.2 GC Methods for the Determination of D-Amino Acids

For the chiral amino acid analysis, enantioselective separations should be carried out. For the chromatographic and electrophoretic separations, the use of chiral derivatizing reagents, chiral stationary phases, or chiral mobile phases is essential. Concerning the GC methods, chiral derivatization reagents and chiral stationary phases have been widely used. As the chiral derivatization reagents, historically, a chiral alcohol ((+)-2-butanol) and a chiral amino acid derivative (*N*-trifluoroacetyl-L-prolyl chloride (TPC)) were used (Hamase et al. 2002). A chiral derivatization

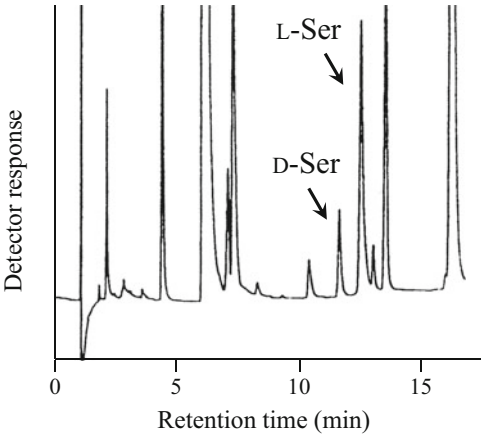

Fig. 1.1 Chiral GC determination of D-Ser in rat brain. Ser enantiomers were determined as their *N*,*O*-PFP-isopropyl derivatives using Chirasil-L-Val as a chiral stationary phase (Reproduced from a reference (Hashimoto et al. 1992a) with permission)

reagent, (*S*)-α-methoxy-α-trifluoromethylphenylacetyl chloride (MPTA-Cl, Gal and Ames 1977), was also used as a promising reagent without racemization during the derivatization procedure. As the chiral stationary phase, Chirasil-L-Val and Chirasil-γ-Dex are reported as useful columns, and most of the proteinogenic amino acids could be separated within 60 min (Schurig 2011). Using Chirasil-L-Val, a large amount of D-Ser in mammalian brain was discovered for the first time (Hashimoto et al. 1992a, Fig. 1.1.). For the enantioselective analysis of amino acids using GC, please refer to a previous review (Schurig 2011). In order to obtain a higher selectivity, two-dimensional GC methods have also been established (Junge et al. 2007; Waldhier et al. 2011). For the two-dimensional analysis, a long enantioselective column was used as the first dimension, and a short non-enantioselective column was used as the rapid second dimension. Although complicated analytical instruments are needed, the 2D-GC methods provide a selective and comprehensive determination of the D-amino acids in biological samples.

1.1.3 HPCE Methods for the Determination of D-Amino Acids

The separation of chiral amino acids using HPCE is mainly performed by chiral mobile phase methods along with adding some chiral selectors to the electrophoresis buffer system. For the sensitive determination, amino acids are normally derivatized with a fluorescence reagent and determined by a laser-induced fluorescence detector. As the derivatization reagent, naphthalene-2,3-dicarboxaldehyde (NDA) was often used, and cyclodextrin derivatives are added to the buffer system as the chiral selector. Using this method, D-Ser, D-Asp, D-Glu and D-Ala could be determined (Miao et al. 2005; Ota et al. 2014). 4-Fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F)

and fluorescein isothiocyanate (FITC) are also used as the derivatization reagent and successfully applied to the cyclodextrin-based separation system. For the HPCE analysis of D-amino acids, please refer to a previous review (Kitagawa and Otsuka 2011).

1.1.4 HPLC Methods for the Determination of D-Amino Acids

Concerning HPLC, chiral derivatization reagents and chiral stationary phases are frequently used for the determination of D-amino acids in real biological samples. Historically, a chiral derivatizing reagent, 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (FDAA, Marfey's reagent), was reported in 1984 (Marfey 1984). This reagent reacts with amino acids to form peptide-type diastereomers and could be separated by a widely used reversed-phase column. The FDAA-derivatized amino acids could be monitored by the absorbance at 340 nm, and various FDAA analogues are currently used as simple analytical tools for a user-friendly HPLC system equipped with a UV detector (Bhushan and Brückner 2011). *o*-Phthalaldehyde (OPA) plus chiral thiol is also historically used for the determination of chiral amino acids. OPA was originally developed for the highly sensitive fluorescence derivatization of amino acids as a post-column derivatization reagent and also a pre-column derivatization reagent with the a chiral thiol, 2-mercaptoethanol. In 1984, Aswad established a method using OPA and a chiral thiol compound, *N*-acetyl-L-cysteine (Aswad 1984). Subsequently, various chiral thiols were designed and used for the D-amino acid analysis (Buck and Krummen 1984; Nimura and Kinoshita 1986; Brückner et al. 1994). The OPA method enables the highly sensitive fluorescence determination of chiral amino acids in combination with a reversed-phase HPLC system and widely used for biological samples. Brain D-Ser is easily determined by OPA plus a chiral thiol, *N-tert*-butyloxycarbonyl-L-cysteine (Boc-L-Cys, Hashimoto et al. 1992b). An analogue of 9-fluorenylmethyl chloroformate (Fmoc-Cl), (+)-1-(9-fluorenyl)ethyl chloroformate (FLEC), was designed (Einarsson et al. 1987). FLEC has a chiral carbon, and most of the proteinogenic amino acids were nicely separated using a reversed-phase column. For the OPA and FLEC methods, an HPLC system with a fluorescence detector was used, and the highly sensitive determination of chiral amino acids could be carried out. These days, several chiral derivatization reagents, such as *R*(−)-4-(3-isothiocyanatopyrrolidin-1-yl)-7-(*N*,*N*-dimethylaminosulfonyl)-2,1,3-benzoxadiazole (*R*(−)-DBD-PyNCS (Min et al. 2011) and (*S*)-*N*-(4-nitrophenoxycarbonyl)-L-phenylalanine-2-methoxyethyl ester (*S*)-NIFE (Visser et al. 2011), have also been developed for the HPLC-MS/MS technique, and the highly sensitive and selective determination of D-amino acids in biological matrices was successfully performed. Although the chiral derivatization methods have several fundamental problems, such as the optical purity of the reagents and a