Microanalysis of D/L-Amino Acid Residues in Peptides and Proteins

The authors of this article describe the determination of D/L-amino acid residues in peptides and proteins at microgram levels. They fluorescently tagged amino acid residues with naphthalene-2,3-dicarboxaldehyde and performed enantioseparation using β-cyclodextrin-modified micellar electrokinetic chromatography in the presence of methanol as an organic modifier. Their separation was coupled with laser-induced fluorescence detection for sensitive determination. The authors determined configurations of amino acid residues in peptides with 10 μg of hydrolyzed peptide material. They demonstrated the assessment of enantiomeric purity for synthetic peptides by their proposed method. They also applied the method to determining D-aspartic acid, free and bound in proteins in rat brains. Although they detected high levels of free D-aspartic acid, no D-aspartic acid was bound in proteins.

Amino acids are essential components in biological systems; they serve either as individual compounds or as constituents of proteins. D-Amino acids have been considered rare in nature for a long time; however, unexpectedly high levels of D-amino acids recently were found both free and bound in peptides in samples of various origins. L-Amino acid residues in food proteins can be racemized to d-amino acids during food processing. In some cases, the racemization ratios can be greater than 25% (1). Thus, D-amino acids are continuously consumed by animals and humans. In addition, D-amino acids can be synthesized in vivo by certain microorganisms through transformation of L-amino acids. Enzymes such as amino acid oxidase, transaminases, and epimerases catalyze this type of transformation. Several D-amino acid–containing peptides have been discovered in various animals during the past decade (2–4). These peptides exhibit biological functions such as cardio-excitation and muscle activity modulation. Obviously, it is important to assess the factors that influence the formation and biological functions of D-amino acids and their utilization by animals and humans.

Biologically active peptides used as therapeutic drugs should be stereochemically pure because the stereoisomers may produce different effects. In peptide synthesis, the amino acid residues used for synthesis must be optically pure, and analysts must make efforts to reduce racemization in every synthesis step, including residue coupling, deprotection, and cleavage of the resulting peptides from resin (5). Stereochemical purity assessment for synthetic peptide materials always is necessary. However, it has been a challenge to separate peptide stereoisomers, particularly for peptides with more than five amino acid residues. A convenient, reliable way to assess stereochemical purity for synthetic peptides and to determine the configuration of amino acid residues in novel peptides and proteins is to analyze D/L-amino acid residues after hydrolyzing the peptide-protein material (6).

Analytical chemists have achieved enantioseparation of racemic amino acids mainly by chromatographic methods. Recently, chiral capillary electrophoresis (CE) has been drawing considerable research interest (7–10) since its potential in enantiomeric separations was first demonstrated by...
Gassmann and co-workers (11) in 1985. Cyclodextrin-modified micellar electrokinetic capillary chromatography (M EKC) has been particularly useful for separating a wide range of enantiomeric compounds (12,13). One of the most significant advantages of CE-based chiral separations is that they are well suited for analysis of mass- or volume-limited samples.

In this article, we describe an organic modifier–mediated β-cyclodextrin M EKC procedure for the separation of D/L-amino acid enantiomers. The presence of an organic modifier such as methanol in the β-cyclodextrin M EKC running buffer dramatically improved the chiral separation efficiency. Precolumn derivatization with naphthalene-2,3-dicarboxaldehyde enhances detection sensitivity and separation efficiency as well. Coupled with laser-induced fluorescence detection, the described separation is well suited for the microdetermination of D/L-amino acid residues in peptides and proteins. In all analyses, we hydrolyzed 10 μg of peptide material and determined D/L-amino acid residues. These small amounts of peptide material can be obtained easily from semi-preparative or even analytical HPLC purification procedures.

**Experimental**

**Chemicals:** We purchased amino acids, peptides, β-cyclodextrin, sodium dodecyl sulfate, and potassium cyanide from Sigma Chemical Co. (St. Louis, Missouri). The naphthalene-2,3-dicarboxaldehyde was from Molecular Probes (Eugene, Oregon). All other chemicals and organic solvents were of analytical grade.

**Instrumentation:** We performed CE with a laboratory-built system and fused-silica capillaries (Supelco, Pennsylvania) with an effective length of 50 cm for separation. The samples were injected into the capillary by hydrodynamic flow with a height differential of 20 cm for the two ends of the capillary. The capillary was rinsed sequentially with 0.1 M hydrochloric acid, distilled water, and buffer for approximately 1 min each before successive electrophoretic runs. The 457.9-nm line from an argon ion laser (Innova 90C FreD, Coherent Inc., Laser Group, Santa Clara, California) was focused with a 25-cm-focal-length fused-silica lens onto the detection window of the separation capillary. Fluorescence emission was collected at an angle of 90° relative to the laser beam by a 40× microscope objective (Melles Griot, Irvine, California). The image of the collected fluorescence was focused on a spatial filter and passed through a GG 495 cutoff filter (Melles Griot) before reaching the photomultiplier tube, which was operated at −950 V. The output signal was recorded and processed with an Agilent model 3395 integrator (Agilent Technolo
gies, Wilmington, Delaware) or an IBM-compatible computer using software written in-house.

**Procedures:** Precolumn derivatization: We mixed 100 μL of the sample solution with 700 μL of 0.1 M borate buffer solution (pH 9.5) in a 1-mL vial. We also added derivatizing reagents—100 μL of naphthalene-2,3-dicarboxaldehyde (2 mM in methanol) and 100 μL of potassium cyanide (20 mM in water). The mixture was vortexed and left to stand for 30 min at room temperature. The derivative solution was injected for separation without additional purification.

Acid hydrolysis of peptides: A portion of the peptide sample solution containing approximately 10 μg of peptide was transferred into a 1-mL vacuum hydrolysis tube (Pierce Chemical Co., Rockford, Illinois) and dried with a nitrogen stream. Then we added constant boiling hydrochloric acid that contained 100 μL of 0.1% (w/v) phenol. The tube was sealed under vacuum and heated to 110°C for 24 h. After cooling to room temperature, we transferred the hydrolysate into a 0.3-mL vial and maintained it at 0°C or lower until use. For derivatization, we transferred 20 μL of the hydrolysate to a 0.3-mL vial and dried it with a nitrogen stream. To neutralize the residual acid, we introduced 20 μL of 0.5% (v/v) triethylamine in 1:1 (v/v) methanol–water solution and dried the content again. The residue was dissolved in 10 μL water and derivatized as described above, except the amounts of all reagents were reduced proportionally.

Hydrolysates of soluble proteins in rat brains: We used an approximately 30-mg sample taken from the brains of two-day-old rats. The rat brain sample was homogenized with a micro tissue grinder and transferred to a 0.3-mL centrifuge tube. We then added 200 μL of 0.1 M hydrochloric acid solution. The mixture was vortexed and then sonicated for 15 min. After centrifugation at 11,000 rpm for 10 min, a 100-μL portion of the supernatant was transferred to another 0.3-mL vial and 15 μL of 30% trichloroacetic acid solution (w/v) was added. We vortexed and centrifuged the mixture at 13,000 rpm for 15 min. The supernatant was used to determine free D-aspartic acid. Protein precipitated out was transferred into a vacuum hydrolysis tube. We performed acid hydrolysis and derivatized the hydrolysate as described above.

**Results and Discussion**

Naphthalene-2,3-dicarboxaldehyde reacts with primary amines in the presence of cyanide to form 1-cyano-2-substituted benz[f]isoindole derivatives. Separations of 1-cyano-2-substituted benz[f]isoindole amino acid enantiomers by β-cyclodextrin M EKC have been described elsewhere (13,14). From the data published, however, enantiomers of certain amino acids such as arginine (Arg), aspartic acid (Asp), and leucine (Leu) (14), and phenylalanine (Phe) (15) cannot be resolved adequately by using β-cyclodextrin as the chiral selector. Fortunately, adding methanol to the β-cyclodextrin M EKC running buffer dramatically improves the chiral separation efficiency, as shown in Figure 1.

1-Cyano-2-substituted benz[f]isoindole amino acid enantiomers, including aspartic acid, phenylalanine, and iso-leucine, were well resolved in the presence of methanol. The fluorescence excitation maximum for 1-cyano-2-substituted benz[f]isoindole amino acids is 445 nm, which is close to the wavelength of a laser line from a widely used argon ion laser (457 nm). Another significant advantage of using naphthalene-2,3-dicarboxaldehyde as the tagging reagent is that the excess naphthalene-2,3-dicarboxaldehyde from the derivatization has little influence on the fluorescence detection, because naphthalene-2,3-dicarboxaldehyde fluoresces little at the excitation wavelength. With laser-induced fluorescence detection, the limits of detection for amino acid enantiomers were approximately 1 × 10⁻⁸ M (signal-to-noise ratio of 3).

We also have investigated the racemization of amino acid residues during the acid hydrolysis process. We subjected amino acid enantiomers to the acid hydrolysis process and then determined the optical antipodes present in the hydrolysate. The racemization ratios were less than 0.2% in the hydrolysates prepared from tyrosine, aspartic acid, and phenylalanine enantiomers. These results are consistent with those reported by Toyooka and Liu (6) using an HPLC method.

Optical purity of synthetic peptides can be assessed conveniently using the present method. Importantly, only small amounts of peptide samples are needed for analysis. In all cases described in this article, we hydrolyzed approximately 10 μg of the pep-
Chiral separations of 1-cyano-2-substituted benz[f]isoindole amino acid mixture by β-cyclodextrin MEKC (a) without or (b) with methanol (15% v/v) added into the running buffer. Electolyte: 20 mM β-cyclodextrin, 50 mM sodium dodecyl sulfate, 50 mM borate buffer (pH 9.0); applied voltage: 15 kV. A 50-μm i.d. capillary was used. Peaks: 1 = L-tyrosine, 2 = D-tyrosine, 3 = D-isoleucine, 4 = L-isoleucine, 5 = D-aspartic acid, 6 = L-aspartic acid, 7 = D-methionine, 8 = L-methionine, 9 = L-tryptophan, 10 = D-tryptophan, 11 = L-phenylalanine, 12 = D-phenylalanine.

Figure 1: Chiral separations of a 1-cyano-2-substituted benz[f]isoindole amino acid mixture by β-cyclodextrin MEKC (a) without or (b) with methanol (15% v/v) added into the running buffer. Electolyte: 20 mM β-cyclodextrin, 50 mM sodium dodecyl sulfate, 50 mM borate buffer (pH 9.0); applied voltage: 15 kV. A 50-μm i.d. capillary was used. Peaks: 1 = L-tyrosine, 2 = D-tyrosine, 3 = D-isoleucine, 4 = L-isoleucine, 5 = D-aspartic acid, 6 = L-aspartic acid, 7 = D-methionine, 8 = L-methionine, 9 = L-tryptophan, 10 = D-tryptophan, 11 = L-phenylalanine, 12 = D-phenylalanine.

Figure 2: Optical purity assessment through analysis of D/L-amino acid residues for dipeptides (a) glycine-L-phenylalanine and (b) glycine-D-phenylalanine. Electrolyte: 20 mM β-cyclodextrin, 50 mM sodium dodecyl sulfate, 50 mM borate buffer (pH 9.0); 15% (v/v) methanol; applied voltage: 17 kV. A 50-μm i.d. capillary was used. A 10-μg peptide sample was hydrolyzed and then analyzed in both cases. Peaks: 1 = glycine, 2 = L-phenylalanine, 3 = D-phenylalanine.
Our proposed method offers a convenient way to examine synthetic peptides for their enantiomeric purity and to acquire the first-shot information about the absolute configuration of amino acid residues in novel peptides and proteins.

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References