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Separation of amino acid and peptide stereoisomers by nonionic micelle-mediated capillary electrophoresis after chiral derivatization

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Abstract

Enantiomers of amino acids and peptides were derivatized with a fluorescent chiral reagent, 4-(3-isothiocyanatopyrrolidin-1-yl)-7-nitro-2,1,3-benzoxadiazole [*R*-(−)- or *S*-(+)-NBD-PyNCS] and the resulting diastereomeric derivatives separated by capillary electrophoresis (CE). The CE running buffer consisted of 25 m*M* acetate buffer (pH 4) and 10 m*M* of the nonionic surfactant Triton X-100. The excitation maximum of NBD-PyNCS at 480 nm matches the major Ar-ion emission line at 488 nm allowing sensitive laser-induced fluorescence detection with limits of detection around 50 n*M*. D-Proline and D-aspartate spiked (at 10^{-4} *M* and 10^{-5} *M* concentrations, respectively) into complex biological matrices (rabbit serum and homogenate of *Aplysia californica* buccal ganglion) are detected without matrix interferences. This method has also been applied to the determination of D- and L-amino acid residues in peptides after acid hydrolysis. Results from the chiral analysis of the naturally-occurring peptide, gramicidin D, are shown. © 1998 Elsevier Science B.V.

Keywords: Enantiomer separation; Derivatization, electrophoresis; Micelles; Amino acids; Peptides

1. Introduction

A number of biomolecules such as amino acids, peptides and proteins possess at least one asymmetric center and therefore, exist as stereoisomers. These stereoisomers are expected to be handled unequally in living organisms during processes of absorption, distribution, metabolism and excretion because asymmetry and optical activity are widely distributed in such biological systems [1]. Researchers have found that enantiomers of some chiral therapeutic drugs such as ibuprofen exhibit different pharmaceutical effects [2]. Advances in methods for chirality analysis over the past decade have enabled scientists to better understand stereochemical phenomena. In the field of neuroscience, for example, Okuma and Abe have found significant amounts of D-amino acids in the nervous tissues and eyes of crustaceans [3]. Also, prevalent amounts of D-serine have been found in rat cortex, hippocampus, striatum, cerebellum, and other brain regions [4]. Very recently, Snyder et al. [5] observed in mammals, discrete neuronal localizations of D-aspartate, especially in the external plexiform layer of the olfactory bulb, hypothalamic supraoptic and paraven-

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tricular nuclei, the medial habenula, and certain brainstem nuclei. Using a high-performance liquid chromatography (HPLC) method they found that the D-aspartate concentration in many pineal glands was similar to L-aspartate concentrations (m*M* levels).

Gas chromatography (GC), HPLC, and thin-layer chromatography (TLC) are analytical techniques widely used for the resolution of stereoisomers [6,7]. Capillary electrophoresis (CE), due to its high separation efficiency, short analysis time, and compatibility with small sample volumes, has been receiving much research interest.

An additional advantage of using CE for enantiomeric separation is the ease of incorporating different chiral selectors. So far, chelate–metal complexes [8], chiral crown ethers [9,10], proteins [11,12], cyclodextrins and their derivatives [13–15], bile salts [16], macrocyclic antibiotics [17], chiral surfactants [18], and other chiral selectors have been used as buffer additives for chiral resolution. However, for many biomedical applications, these direct chiral separation methods are not possible because of insufficient detection sensitivity in CE.

Indirect chiral separation methods involve derivatization of the analytes with an enantiomeric reagent to form corresponding diastereomeric compounds. These diastereomers can be separated by a conventional achiral system and can also have better detectability. This is particularly true when a fluorescent derivatizing reagent is used. Several chiral reagents, e.g. Marfaey's reagents [19], 2,3,4,6-tetra-*O*-acetyl-D-glucopyranosyl isothiocyanate (GITC) [20,21], *O*,*O′*-dibenzoyl-L-tartaric anhydride [22], and 1-(9-fluorenyl)ethyl chloroformate (FLEC) [23] have been used for CE chiral separations. Among them, FLEC derivatization allows the use of laser-induced fluorescence (LIF) detection. The detection limit of (+)-FLEC derivatized D,L-amino acids was obtained at the n*M* level using a KrF (248 nm) laser for excitation [23]. To the best of our knowledge, FLEC is the only fluorescent chiral tagging reagent which has been used to prepare diastereomers of amino acids that have been separated using CE. A disadvantage of FLEC reagent, however, is its deep ultraviolet excitation wavelength (λ_{ex}=260 nm), which hinders the widespread application due to spectral interference from the endogenous compounds in the biological sample matrices and lack of inexpensive or common lasers which operate near 260 nm. Benzofurazan fluorescence tagging reagents are an important group of derivatizing reagents for HPLC analysis. They feature superior spectroscopic properties (λ_{ex} 480 nm, λ_{em}=520 nm) and excellent separation-enhancing capabilities. A comprehensive review of these reagents and their applications to HPLC has recently been published [24]. We describe here the first CE separation of the diastereomers of amino acids and peptides prepared with a benzofurazan reagent, i.e. *R*-(−)- or *S*-(+)-4-(3-isothiocyanatopyrrolidin-1-yl)-7-nitro-2,1,3-benzoxadiazole [*R*-(−)- or *S*-(+)-NBD-PyNCS] (Fig. 1).

Although sodium dodecyl sulfate (SDS) micelles have been extensively studied and used in CE for many years, few studies report the use of nonionic micelles [25]. Esaka et al. recently reported that by using a mixed micellar system of SDS and Tween-20, improved separation efficiencies were obtained [26]. Moreover, the method allowed the analysis of complex matrix samples such as bovine serum without deproteination. We demonstrate the use of nonionic micelles of Triton X-100 in CE, exploiting the combined effects of separation improvement, analyte solubilization, and luminescence emission enhancement from these micelles.

2. Experimental

2.1. Materials

The chiral fluorescence derivatizing reagents, *S*-(+)- and *R*-(−)-NBD-PyNCS were synthesized as described previously with an optical purity of 99.5% [27]. D-, L- and DL-amino acids, all peptides, Triton X-100 and the SDS were purchased from Sigma (St. Louis, MO, USA). All chemicals and organic solvents were of analytical grade. Milli-Q (Millipore, Bedford, MA, USA) water was used throughout.

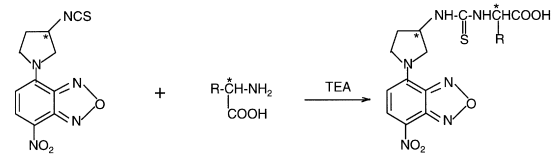

Fig. 1. Structure of the chiral fluorescence tagging reagent, *S*-(+)- or *R*-(−)-NBD-PyNCS and its reaction with amine compounds.

2.2. Chiral derivatization with NBD-PyNCS

In a 0.3-ml reaction vial, 5 μl of amino acid (0.1 mg ml^{-1} in water) or peptide (1 mg ml^{-1} in water) standard solution was mixed with 20 μl of 0.5% triethylamine (TEA) in acetonitrile–water (1:1) and 25 μl of *S*-(+)- or *R*-(−)-NBD-PyNCS acetonitrile solution (2 m*M*). The vial was capped tightly. The mixture was vortexed and then heated with a dry heating block at 60°C for 10 min. After cooling with running water, the vial was opened and 50 μl of 10% (v/v) acetic acid aqueous solution was added. The derivative solution was kept at 5°C before use. It was found to be stable for at least one week in the refrigerator. Before injection the derivative solution was diluted 50 times with the CE running buffer.

2.3. CE

A Beckman P/ACE 2100 (Beckman Instruments, Fullerton, CA, USA) instrument equipped with an argon-ion laser (Omnichrome, Chino, CA, USA) and a LIF detector (Beckman Instruments) was used in all experiments. CE separations were performed in 50 cm×50 μm I.D. fused-silica capillaries (Polymicro Technologies, Phoenix, AZ, USA). Each new capillary was preconditioned prior to its first use by rinsing for 20 min with 0.1 *M* NaOH solution and then 15 min with water. Samples were injected into the capillary by applying pressure ('high pressure') for 3 s. Between runs the capillary was flushed with running buffer for 1 min. The running buffer solution was prepared by mixing 25 ml of 100 m*M* acetate buffer (pH 4.0) with 10 ml of 100 m*M* Triton X-100 and then diluted to 100 ml. This solution was degassed by sonication before use. The separation voltage used was 30 kV, which typically generated a capillary current of about 6.5 μA.

2.4. Sample analysis

2.4.1. Homogenate of Aplysia buccal ganglion

Specimens of *Aplysia californica* were obtained from the *Aplysia* Research Facility (Miami, FL, USA) and injected with isotonic $MgCl_2$ solution to anaesthetize the animal. The buccal ganglion was dissected under a stereomicroscope and transferred to a small vial. The ganglion was cut into pieces as small as possible by scissors and ground by a manual microhomogenator. Methanol–water (1:3) solution containing 5% trifluoroacetic acid (25 μl) was added. The vial was sonicated for 2 min and centrifuged at 3000 rpm for 5 min. The supernatant was transferred to a second vial. This extraction procedure was repeated once and the extracts were combined. For derivatization, 25 μl of the extract was mixed with 25 μl of 5% TEA in acetonitrile solution and the mixture was dried with a N_2 stream. To the residue, 5 μl of water was added. The derivatization proceeded as described above. The derivative solution was diluted 10 times with the CE buffer before injection.

2.4.2. Rabbit serum

Rabbit serum was obtained from Sigma and diluted with water (1:1). To a 0.3-ml reaction vial, 5 μl of the diluted serum was transferred and the derivatization was performed as described above. The derivative solution was centrifuged at 3000 rpm for 5 min. The supernatant was diluted 10 times with the CE running buffer before injection.

2.4.3. Analysis of gramicidin D

In a glass tube 0.1 mg of gramicidin D was dissolved in 150 μl of constantly-boiling HCl containing 0.1% (w/v) phenol. The tube was sealed under vacuum with a oxygen flame and heated at 110°C for 24 h. After cooling to room temperature, the top of the tube was scored with a diamond pencil and snapped off. The hydrolysate was dried with a N_2 stream. To neutralize the residual acid, 50 μl of 0.5% TEA in acetonitrile–water (1:1) solution was introduced and the content was dried again with an N_2 stream. The residue was dissolved in 50 μl of water and 5 μl was derivatized as described above. The derivative solution was diluted 10 times with CE buffer before injection.

3. Results and discussion

3.1. Fluorescence emission from NBD–PyNCS fluorophor in different media

The effect of organic solvents and micelles on the spectroscopic properties of NBD-PyNCS was studied. In these experiments, a *S*-(+)-NBD-PyNCS-Leu-Gly derivative solution was prepared with ex-

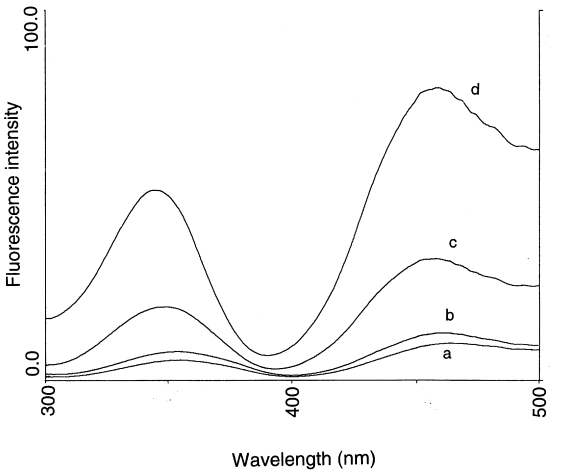

Fig. 2. Excitation spectra (uncorrected) of DL-Leu-Gly-NBD-PyNCS derivatives in: (a) 25 m*M* acetate buffer (pH 4.4); (b) 25 m*M* acetate buffer (pH 4.4) containing 10% (v/v) acetonitrile; (c) 25 m*M* acetate buffer (pH 4.4) containing 50 m*M* SDS; (d) 25 m*M* acetate buffer (pH 4.4) containing 10 m*M* Triton X-100. [NBD-PyNCS] = 10^{-5} *M*. A Hitachi spectrofluorometer (Model F-4010) was used. λ_{em} = 520 nm with both 3-nm ex and em bandpass.

cessive Leu-Gly in the reaction solution. Small portions of the derivative solution (the final concentration of NBD-PyNCS was approximately 10^{-5} *M*) were added to each medium. The organic solvents and micelles tested do not significantly shift the excitation or emission spectra of this fluorophore; however, as shown in Fig. 2, acetonitrile, Triton X-100 micelles, and SDS micelles enhance the observed fluorescence intensity. Micelles interact with the fluorophore through a variety of interactions (i.e, electrostatic interaction, hydrogen bonding, hydrophobic interaction, Van der Waals forces). These interactions solubilize the fluorophore and reduce the probability of nonradioactive decay of the excited state, thus enhancing fluorescence emission. Triton X-100 micelles exhibit the largest increase, and so used to improve the detection sensitivity in CE. Using the P/ACE CE/LIF system, the limits of detection (LODs) for *S*-(+)-NBD-PyNCS-Pro were 10 μ*M* in acetate buffer, and 50 n*M* in buffer containing 10 m*M* Triton X-100, with the LOD of fluorescein being 2 n*M*.

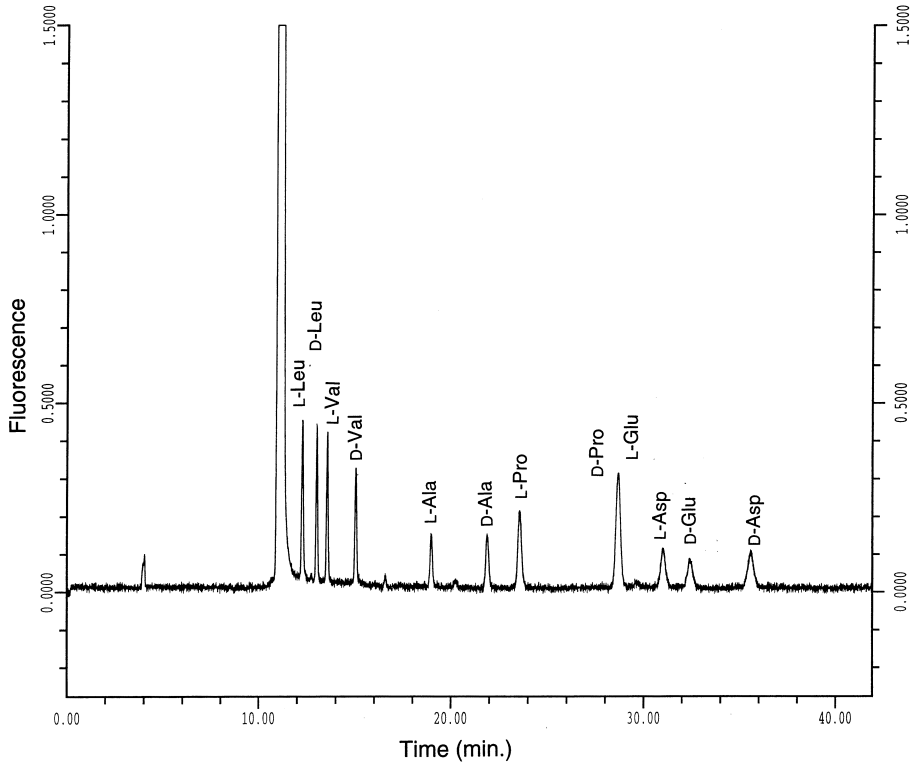

Fig. 3. Separation of a racemic amino acid mixture derivatized with *S*-(+)-NBD-PyNCS. CE running buffer: 25 m*M* acetate (pH 4.0) containing 10 m*M* Triton X-100. Capillary: 50 μm×50 cm; voltage: 30 kV. LIF detection: λ_{ex} = 488 nm, λ_{em} = 520 nm.

3.2. CE separation of NBD-PyNCS tagged amino acids and peptides

The presence of micelles in the running buffer is essential for the chiral separation. CE running buffers which do not contain micelles including 25 m*M* acetate buffer (pH 4), 10 m*M* borate buffer (pH 8.2), and these buffer solutions containing 10% methanol or acetonitrile were tested for separation of *S*-(+)-NBD-PyNCS derivatized D,L-proline and D,L-valine. None of these diastereomeric derivatives were separated. This suggests that the difference in the partitioning coefficients for the diastereomers between the bulk solution and the micelles was important for their separation. Fig. 3 shows a separation of six pairs of amino acid enantiomers using Triton X-100. Amino acids separated under these conditions included neutral and acidic amino acids. For basic amino acids (e.g. arginine), no resolution between enantiomers was obtained under these conditions.

Comparing the resolution obtained here with that which we reported from HPLC [28,29], CE has much higher efficiencies and so CE is better able to resolve NBD-PyNCS-labeled amino acid and peptide diastereomers. For example, using HPLC, *S*-(+)-NBD-PyNCS-D,L-proline diastereomers could not be separated although a variety of mobile phases were tried.

This method can also be used to resolve small peptide stereoisomers. An example of such separations is shown in Fig. 4. The two peptide samples, i.e. Leu-Gly and DL-Leu-Gly, were derivatized with *S*-(+)-NBD-PyNCS. As can be seen, the two isomers D-Leu-Gly and L-Leu-Gly were well separated with the L-Leu-Gly isomer eluting first. The migration order for this small peptide is the same as for those obtained for amino acids (Fig. 3).

It should be pointed out, however, the migration order can be reversed if the other enantiomer of the reagent, i.e. *R*-(−)-NBD-PyNCS, is used as shown in

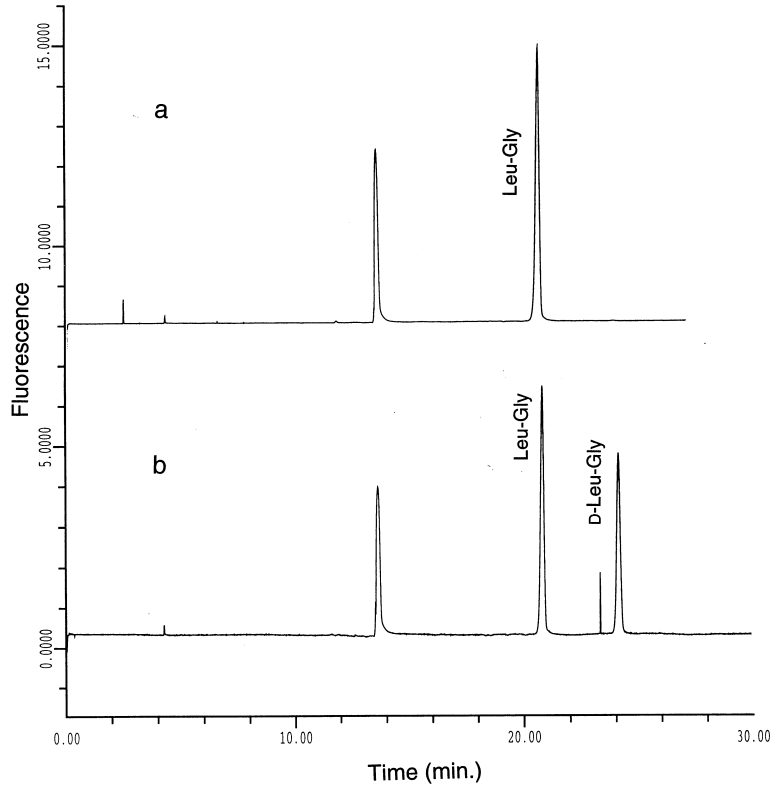

Fig. 4. Separation of peptide stereoisomers: (a) Leu-Gly derivatized with *S*-(+)-NBD-PyNCS; (b) DL-Leu-Gly derivatized with *S*-(+)-NBD-PyNCS. Separation and detection conditions as in Fig. 3.

Fig. 5. In this experiment D- and L-proline were derivatized with *S*-(+)-NBD-PyNCS and separately with *R*-(−)-NBD-PyNCS. With *S*-(+)-NBD-PyNCS, L-proline migrated faster than D-proline and with *R*-(−)-NBD-PyNCS, the migration order was reversed. These migration orders are consistent for all the amino acid enantiomers tested including leucine, valine, aspartate, glutamate, etc. Thus, we can control the elution order of enantiomers by choosing the right enantiomeric form of the reagent. This is an advantage for optical-purity analysis where the content of the minor component can be less than 0.05% of its antipode. In these cases, the minor peak can be made to elute before the major peak for better quantitation.

The CE migration order of amino acid enantiomers is the same as the HPLC elution order [28]. Considering the similarities of these two separation methods, i.e. the pseudostationary phase in this micellar CE versus the C_{18} solid-phase in the HPLC, these results appear reasonable. In addition, the same migration orders of the D- and L-amino acid isomers were found with FLEC as with the chiral derivatization reagent in SDS-based MEKC separations [23].

3.3. Matrix effects

LIF detection is the most sensitive detection scheme available today for HPLC and CE. A concern about using this technique is the potential interference from the endogenous components in complex sample matrices. The possibility of having such interference is dependent upon the excitation wavelength used for the LIF detection. The fluorescence tagging reagent used in this work, NBD-PyNCS, has an excitation maximum around 480 nm (Fig. 1), which offers several advantages compared to FLEC. First, at such a long excitation wavelength, few endogenous components fluoresce. Secondly,

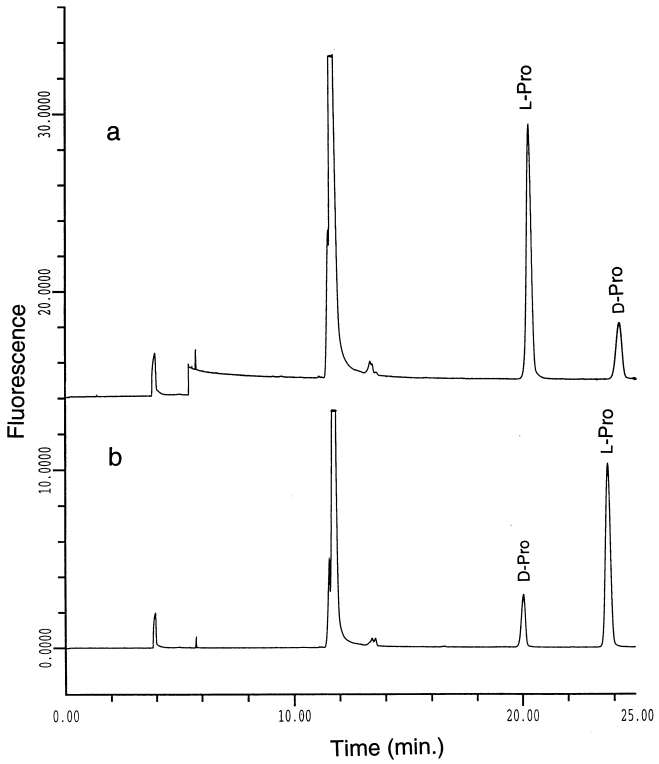

Fig. 5. Migration order is reversed by changing the enantiomeric form of the tagging reagent: (a) *S*-(+)-NBD-PyNCS; (b) *R*-(−)-NBD-PyNCS. The concentration of L-proline was four times that of D-proline. Separation and detection conditions as in Fig. 3.

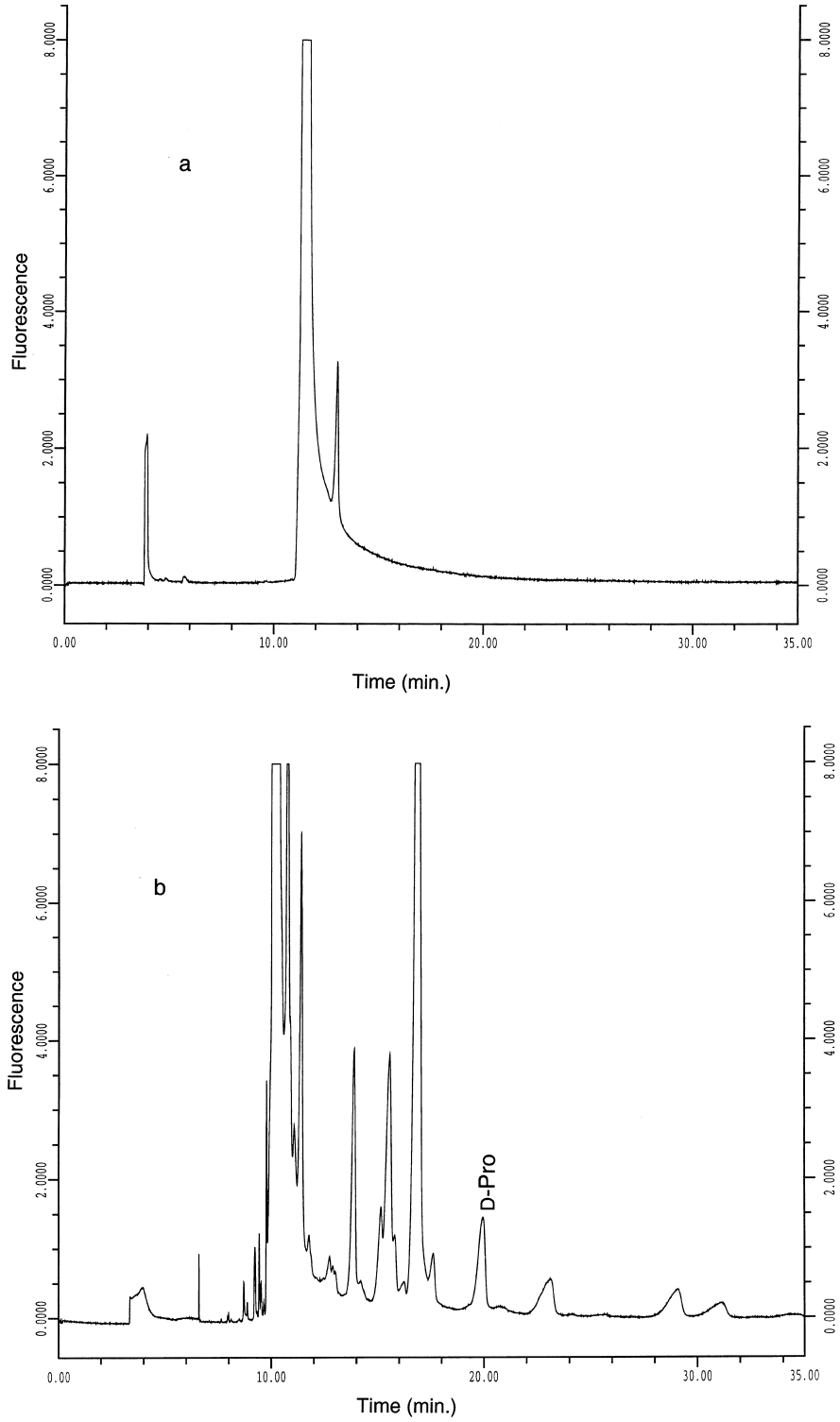

Fig. 6. D-Amino acid determination in biological matrices: (a) water blank; (b) D-proline spiked (10^{-4} *M*) rabbit serum; (c) D-aspartate spiked (10^{-5} *M*) homogenate of *Aplysia* buccal ganglion. *R*-(−)-NBD-PyNCS was used for derivatization in all cases and the separation and detection conditions were as in Fig. 3.

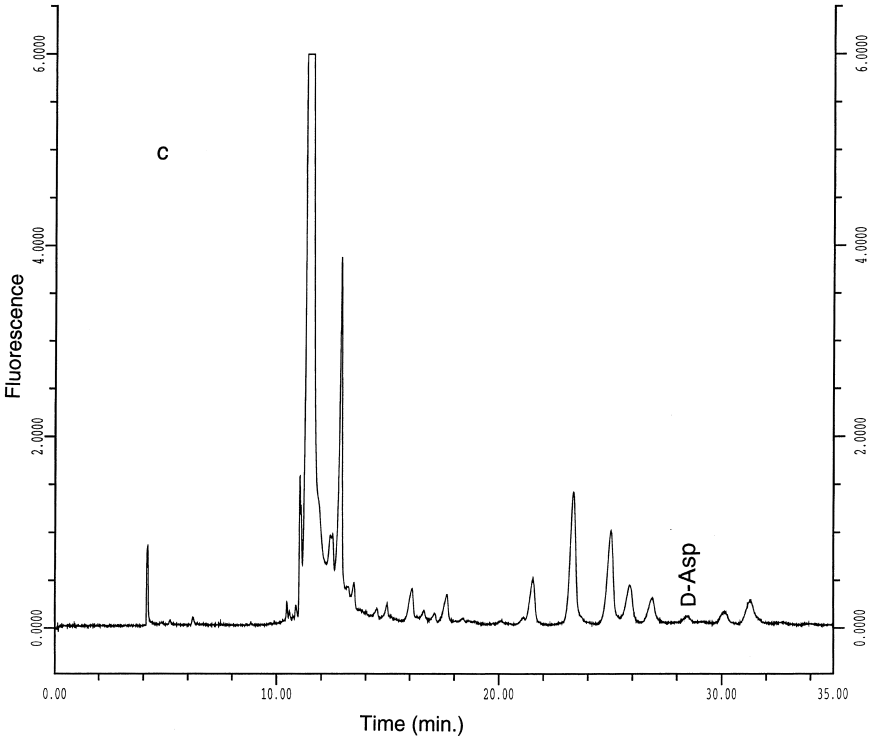

Fig. 6. (*continued*)

this wavelength matches the major argon-ion laser line at 488 nm, thus permitting the effective use of this laser source which is stable, cheap, and available to many scientific laboratories.

Fig. 6 shows electropherograms from the analysis of D-proline and D-aspartate in biological samples (a homogenate of *Aplysia* buccal ganglion and rabbit serum). Since no D-proline and D-aspartate were found in these samples (data not shown), the two D-amino acid enantiomers were added to the sample (10^{-4} *M* D-proline to the rabbit serum and 10^{-5} *M* D-aspartate to the *Aplysia* ganglion sample). In both cases, these amino acid enantiomers could be detected adequately without interference from the sample matrices. Although D-Pro comigrates with L-Glu when *S*-(+)-NBD-PyNCS is used (see Fig. 3), using the *R*-(−)-NBD-PyNCS reagent avoids this potential problem. The availability and use of both reagent forms allows verification of results when assaying complex matrices.

3.4. Peptide assays

An important application for this chiral separation method is the analysis of D- and L-amino acid residues in peptides. This technique offers a simple and reliable way to examine synthetic peptides for chemical purity and to evaluate the configuration of amino acid residues in newly-discovered peptides and proteins. Fig. 7 shows the results from one such analysis using Gramicidin D. Its sequence is:

O = CH − L-Val − Gly − L-Ala − D-Leu −
L-Ala − D-Val − L-Val − D-Val −
$[$L-Trp − D-Leu$]_3$ − L-Trp − $NHCH_2CH_2OH$

As can be seen from Fig. 7, the amino acid residue information obtained from the electropherogram matches well with the known chemical structure. For example, the D-Leu is the most intense peak (4 in the peptide), followed by a peak with unresolved Gly

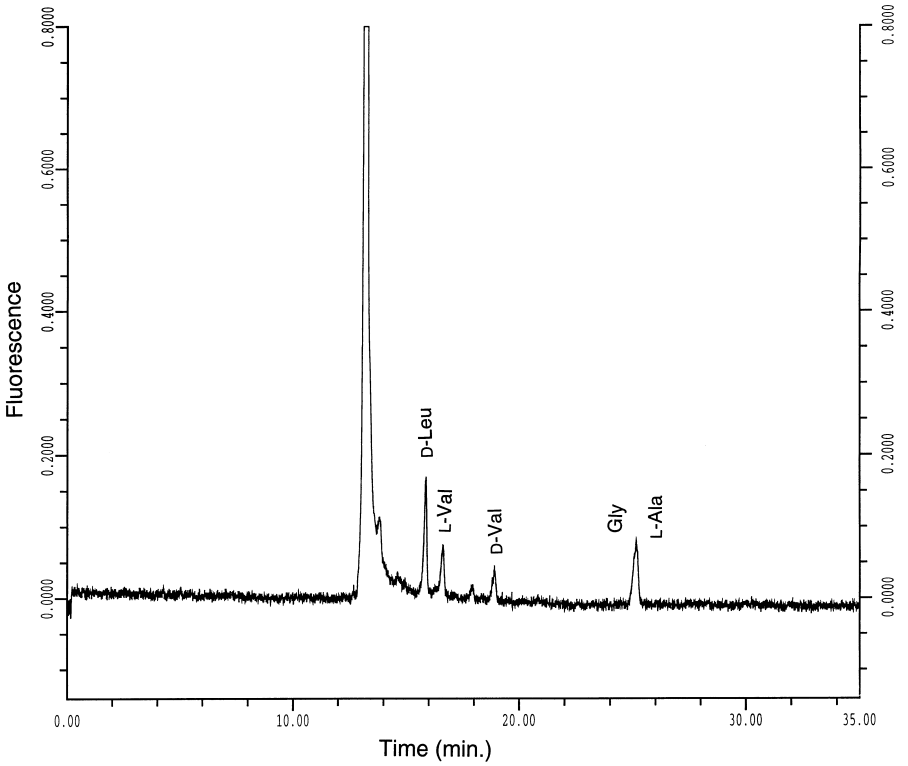

Fig. 7. Analysis of D- and L-amino acid residues in the peptide gramicidin D. Separation and detection conditions as in Fig. 3.

(not optically active) and L-Ala (3 total amino acids in the peak), and well-resolved L-val and D-Val peaks (2 each in the peptide). The only amino acid not detected is L-Trp which coelutes with the large reagent peak at about 13 min. Thus, this method successfully determines all unusual D-amino acid residues in this peptide.

4. Conclusions

The chiral fluorescence tagging reagent, *S*-(+)- or *R*-(−)-NBD-PyNCS, reacts with amino acids and peptides without observable racemization. The resulting diastereomers can be separated by Triton X-100 micelle-mediated CE. The presence of micelles in the CE running buffer is essential for the chiral separation, and it also improves the CE detection sensitivity by enhancing the fluorescence emission from the tagged analytes. An argon-ion laser at 488 nm is suitable for the LIF detection. This method has been applied to detect D-proline and D-aspartate amino acid enantiomers in biological matrices and has been used for analysis of D- and L-amino acid residues in the peptide Gramicidin D. Future studies involve using this method to screen the neuroactive peptides and hormones in *Aplysia californica* for D-amino acids.

Acknowledgements

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