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Review

Selective determination of peptides containing specific amino acid residues by high-performance liquid chromatography and capillary electrophoresis

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Abstract

HPLC and CE methods for the selective determination of peptides containing cysteine, arginine, tryptophan, tyrosine and proline are reviewed. Chemical derivatization combined with UV–visible and fluorescent detection is the most often used method. The derivatization conditions, sensitivities, advantages, disadvantages and applications of these methods are discussed. Related methods are also considered.

Contents

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1. Introduction

There is increasing interest in the investigation of bioactive peptides related to most areas of life sciences. For their identification, quantification and purification, a number of methods have been developed. High-performance liquid chromatography (HPLC) is a powerful technique for the separation and determination of peptides owing to the good recovery, high resolution and considerable experimental flexibility [1]. It has been widely used in peptide chemistry. Recently, capillary electrophoresis (CE) [2,3] has made great progress as a complementary method to HPLC. It permits rapid and efficient separations of charged components present in small sample volumes. Increasing numbers of papers have been published about separation and determination of peptides by CE.

A variety of detection techniques in HPLC and CE have been applied to the determination of peptides, including radioimmunoassay, mass spectrometry (MS), ultraviolet (UV)–visible absorption, electrochemical detection and fluorescence measurements. According to Kai et al. [4], radioimmunoassay as an off-line detection method in HPLC generally offers high sensitivity and selectivity for peptides, but it is difficult to obtain specific antibodies, especially for oligopeptides. In addition, the methodology does not provide any structural information about unknown peptides. Mass spectrometric detection coupled with HPLC and CE needs expensive instrumentation and refined operation, although the method shows good structural specificity for peptides. UV detection at wavelengths between 200 and 280 nm is the most frequently used method for the detection of peptides. The detection does not require derivatization of peptide. However, UV detection is not selective for peptides, and UV-absorbing components in crude samples and/or in the mobile phase may interfere with the sensitive determination of peptides. Fluorescence detection is far superior to UV detection in both sensitivity and selectivity, because there are relatively few fluorescent interfering substances. However, introduction of a fluorescent label in the peptide is necessary. Electrochemical detection usually offers better sensitivity and selectivity than UV detection, but is influenced by oxidizing or reducing components in samples and in the mobile phase.

There is a need for the sensitive and selective determination of peptides containing specific amino acid residues in complex samples such as enzymatic digests, degradation products and parent compounds and metabolites of peptide drugs in biological samples. For this purpose, chemical derivatization combined with UV–visible and fluorescent detection in HPLC and CE is the most often used method. A selected number of reagents can react selectively with particular amino acid residues in peptides and proteins under mild conditions and form derivatives with characteristic spectral features that are detectable by UV–visible and/or fluorescent detection. Ideal chemical derivatization should meet the following requirements: (a) the derivatization reagent is selective for a specific amino acid residue; (b) the peptides of interest are rapidly and quantitatively derivatized under mild conditions with a minimum of manipulations, while no degradation of peptides occurs; (c) the label introduces a high degree of sensitivity; (d) the derivatization reagent and its possible degradation products formed during the reaction are either non-detectable or well separable from the derivatized peptides of interest; and (e) for precolumn derivatization, the derivatives should be stable.

The derivatization can take place at the precolumn or postcolumn stage. Both techniques have their own limitations [5]. Precolumn derivatization is the simplest and most commonly used procedure. Because the sample is labelled prior to separation, no modification of the instrument is needed to incorporate the derivatization step. However, the derivative produced has to be stable, otherwise postcolumn derivatization is indicated.

Electrochemical detection without derivatization has been used for the selective determination of tyrosine-containing peptides, but the selectivity of the method is lower than that of the fluorescent derivatization method.

There may be as many as twenty different native amino acids in peptides. It is not yet possible to derivatize selectively amino acid

residues such as asparagine, aspartic acid, glutamic acid, glutamine, glycine, isoleucine, leucine, serine, threonine and valine because of the absence of characteristic side-chains, suitable for selective derivatization. On the other hand, although many chemical reagents for the specific modification of proteins and peptides have been described [6], some of them are not suitable for analytical purposes. Therefore, to date, only methods for the selective determination of peptides containing cysteine, arginine, tryptophan, tyrosine and proline have been reported. We review advances in this area. Some methods for the selective determination of specific amino acid residues in intact proteins are also briefly discussed. These methods are limited for the determination of a single protein without combination with HPLC and CE.

2. Selective determination of cysteine-containing peptides

Various thiol-specific reagents has been described for the selective determination of thiol-containing compounds [6–8]. They consist of chromogenic and fluorigenic reagents. Chromogenic reagents, 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) and its analogues, have been reported for the selective determination of biologically important thiols (including small cysteine-containing peptides) through precolumn and postcolumn derivatization [7]. Several types of fluorigenic reagents have been developed for the selective determination of thiol-containing compounds, including dansylaziridine, N-substituted maleimides, bimanes and halogenobenzofurazans. Each reagent has its own merits [8]. Dansylaziridine produces highly fluorescent adducts, but is also fluorescent itself, which results in an interfering peak in the chromatogram. N-substituted maleimides have no blank fluorescence and give highly fluorescent adducts, but form multiple fluorescent products because of hydrolysis of the fluorophores. The reaction of bimanes with thiols is fast, but bimanes produce several reagent peaks in the chromatogram and may also react with non-thiolic functional groups. Halogenobenzofurazans present optimum fluorigenic and physico-chemical features, including lack of native fluorescence, high reactivity towards thiols and excellent solubility and stability of the reagents and their thiol derivatives, but require drastic derivatization conditions. Dansylaziridine, maleimides and bimanes have been applied for the selective detection of cysteine residues in proteins. Halogenobenzofurazans, ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonate (SBD-F) and 4-(aminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole (ABD-F) have been used for the selective determination of cysteine-containing peptides. The selective determination of cysteine-containing peptides focuses mainly on the study of small peptides such as glutathione, cysteinylglycine and γ-glutamylcysteine that occur naturally in human body fluids. DTNB, SBD-F and ABD-F will be discussed in more detail.

2.1. DTNB and its analogues

DTNB (Ellman's reagent) reacts with thiol-containing compounds, resulting in the release of a mixed disulfide and 2-nitro-5-mercaptobenzoic acid (Fig. 1). Precolumn HPLC methods have

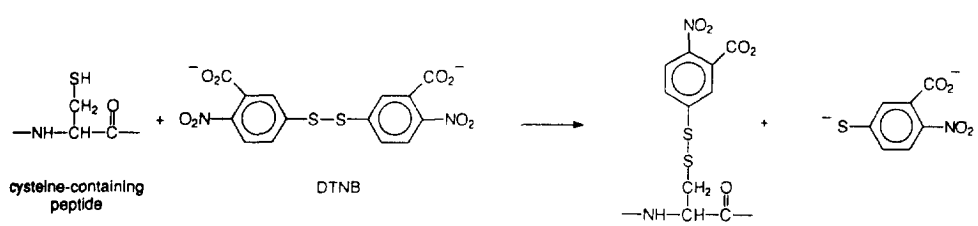

Fig. 1. Reaction of a cysteine-containing peptide with DTNB.

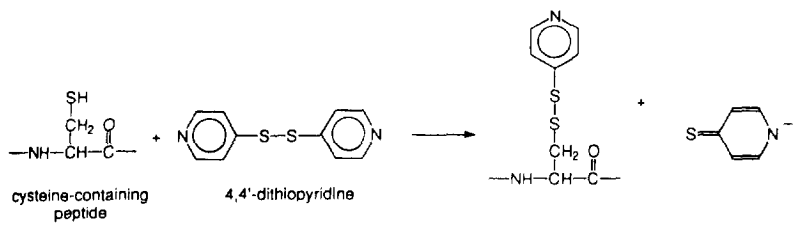

Fig. 2. Reaction of a cysteine-containing peptide with 4,4'-dithiopyridine.

been developed for the selective determination of small cysteine-containing peptides in biological samples [9,10]. The derivatization must be carried out at pH > 8.0. The mixed disulfide is detected at 280 or 330 nm. The methods have been applied for the determination of glutathione in rat liver [9] and of cysteine, γ-glutamylcysteine and glutathione in mouse liver [10]. The weakness of the methods based on DTNB derivatization is that the derivatization reaction requires a high pH.

4,4'-Dithiopyridine, similarly to DTNB, can derivatize thiol-containing compounds (Fig. 2). Andersson et al. [11] developed a postcolumn derivatization HPLC method for the determination of cysteine, glutathione, cysteinylglycine and γ-glutamylcysteine in plasma. The derivatization was carried out at pH 7.3 and the thione anion was detected at 324 nm. The detection limit is 50 nmol/l in plasma. 4,4'-Dithiopyridine was compared with DTNB. DTNB may be preferable to dithiopyridine for precolumn derivatization, whereas postcolumn derivatization with 4,4'-dithiopyridine gives better sensitivity owing to the higher absorbance coefficient of the thione anion.

Other aromatic disulfides such as 2,2'-dithiodipyridine and 6,6'-dithiodinicotinic acid have lower absorptivities, making them less attractive sulfhydryl reagents than 4,4'-dithiopyridine and DTNB.

2.2. SBD-F

SBD-F was described in 1983 as a fluorigenic reagent for the determination of thiol-containing compounds [12]. Small cysteine-containing peptides such as glutathione and other biological thiols in blood and plasma are typically derivatized at pH 9.5 and 60°C for 1 h and determined by HPLC [13–16] and CE [17] with fluorescence detection at 515 nm with excitation at 385 nm. The derivatization scheme is shown in Fig. 3. Sueyoshi et al. [18] used SBD-F as a precolumn derivatization reagent for the selective detection of cysteine-containing peptides in RP-HPLC. A peptide containing a disulfide bond was reduced and cleaved with tributylphosphine, and then reacted with SBD-F at pH 8.5 and 60°C for 1 h. The range of quantification was 100 pmol to 10 nmol. The method has been applied to the selective detection of cysteine-containing fragments in an enzymatic digest of bovine high-molecular-mass kininogen.

Ling and Baeyens [19] carried out a comparative study of micro-LC and capillary zone electrophoresis for the analysis of thiols (including small peptides containing cysteine) using SBD-F as a precolumn derivatization reagent. Both systems have been applied to the specific detection of reduced glutathione in human whole blood.

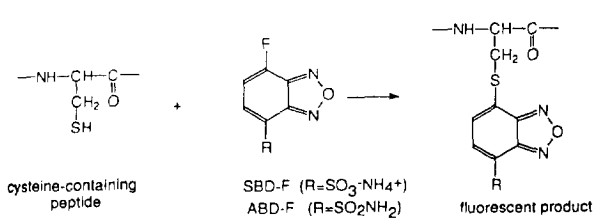

Fig. 3. Reaction of a cysteine-containing peptide with SBD-F and ABD-F.

In conclusion, the methods based on SBD-F are simple, sensitive and reliable and there are no interfering reagent peaks in the chromatogram. Disadvantages include the low reactivity of SBD-F, the long reaction time and alkaline pH.

2.3. ABD-F

ABD-F is similar to SBD-F. It was designed and synthesized in order to obtain milder derivatization conditions than with SBD-F and to overcome possible thiol degradation [20]. The derivatization reaction is typically carried out at pH 8.0 and 50°C for 5 min (Fig. 3). The fluorescence is monitored at 510 nm with excitation at 380 nm. ABD-labelled small peptides such as glutathione and other biological thiols were separated by HPLC and detected fluorimetrically with a detection limit of 0.4 pmol. ABD-F is also used for the differentiation of cysteine residues in proteins of large molecular mass. Toyo'oka and Imai [21] isolated and characterized cysteine-containing regions of proteins using ABD-F and HPLC. Egg albumin as a model for thiol-containing proteins was treated with ABD-F. In the presence of 0.5% sodium dodecyl sulfate (SDS) at pH 8.0 and 60°C for 1 h, all four cysteine residues (Nos. 11, 30, 367 and 382) were specifically labelled. In the absence of SDS at pH 8.0 and 40°C for 1 h, however, selective labelling of only one cysteine residue (No. 367) occurred.

It is concluded that ABD-F is a useful reagent for the sensitive and selective determination of cysteine-containing peptides by HPLC because of its excellent reactivity, selectivity to the thiol group, low fluorescence background, and good fluorophore stability. Moreover, its solubility in water may be also advantageous for reaction with native proteins.

3. Selective determination of arginine-containing peptides

The specific determination of arginine-containing peptides is relatively difficult to achieve. The high pK_a of the guanidine functional group ($pK_a = 12–13$) necessitates fairly drastic reaction conditions (pH 12) to generate an effective nucleophile. Most proteins and peptides are not stable to extremely alkaline pH. However, benzoin as a fluorescent derivatization reagent has been reported for the selective determination of arginine-containing peptides by HPLC and CE. Although 9,10-phenanthrenequinone [22,23] and ninhydrin [24] have been used as fluorescent derivatization reagents for the determination of monosubstituted guanidino compounds by HPLC, they have not been applied to the selective determination of arginine-containing peptides by HPLC. In principle, they are applicable to the determination of arginine-containing peptides, although the derivatization conditions may need to be modified. However, the sensitivity of methods for monosubstituted guanidino compounds using 9,10-phenanthrenequinone and ninhydrin is lower than with benzoin. In addition, 9,10-phenanthrenequinone is insoluble in water and must be dissolved in dimethylformamide, which often causes incompatibility with the mobile phase in HPLC.

3.1. Benzoin

Benzoin was found to react selectively with guanidine and monosubstituted guanidino compounds (including arginine containing peptides) in alkaline solution (Fig. 4). The adduct 2-substituted amino-4,5-diphenylimidazole shows strong fluorescence at 435 nm with excitation at 325 nm [25–27]. Several HPLC methods based on this reaction have been developed for the selective determination of arginine-containing peptides.

Ohno et al. [28] proposed a reversed-phase HPLC method coupled with on-line postcolumn derivatization with benzoin and fluorescence detection for the selective determination of arginine-containing peptides. The arginine-containing peptides kyotorphin, kallidin, bradykinin, angiotensin (ANG) I, II and III, substance P and β-melanocyte stimulating hormone (β-MSH) could be determined with detection limits of 5–15 pmol. The facile detection of arginine-con-

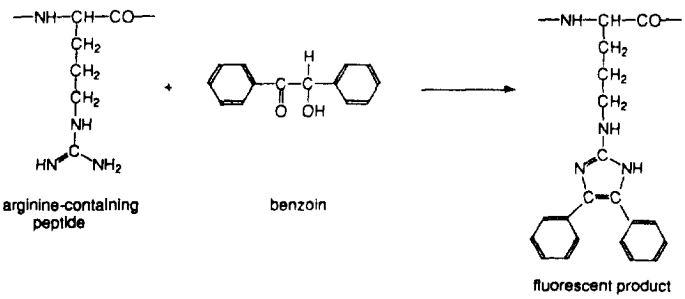

Fig. 4. Reaction of an arginine-containing peptide with benzoin.

taining fragments in the tryptic digest of β-melanocyte stimulating hormone as a model compound showed satisfactory result.

Precolumn fluorescence derivatization is more sensitive than postcolumn derivatization, which allows detection levels in the femtomole range for arginine-containing peptides [29]. The derivatization reaction is typically carried out at 100°C for 90 s in 0.2 *M* potassium hydroxide containing 3 m*M* benzoin. The HPLC method allows the determination of ANG I, II and III. The benzoin derivatives of ANGs and their analogues can be successfully resolved on a reversed-phase column with isocratic elution. This HPLC method can be applied not only to enzyme assays in the renin–angiotensin system but also to the investigation of the enzymatic degradation of various ANGs in biological samples [30]. The disadvantage of this method is that the column slowly deteriorates because of the alkaline mobile phase (pH 8–9).

Cobb and Novotny [31] proposed a CE method with laser-induced fluorescence detection for the selective determination of arginine-containing peptides using the reaction with benzoin. ANG I, II and III and their analogues were used as model arginine-containing peptides. Derivatization was performed prior to separation under the conditions used in precolumn derivatization HPLC. A detection limit of 270 amol was achieved for model arginine-containing peptide. The selective detection of arginine-containing fragments in the tryptic digest of chicken egg white lysozyme also demonstrates the applicability of the method for high-sensitivity peptide mapping. The use of CE permits rapid and highly efficient separations with minimal sample volumes, while the laser-based fluorescence detection system yields three orders of magnitude lower detection limits than HPLC with conventional fluorescence detection [27].

Tomori [32] investigated the fluorescence derivatization of DLL-MePhe-Pro-Arg-H (LY-DLL, LY-294468) with benzoin. It was found that the guanidino group of the arginine residue was not converted into a fluorescent derivative by reaction with benzoin. However, if the LY-DLL was first converted into an LY-DLL–Tris adduct with Tris–HCl buffer (pH 8.5) for 3–24 h at room temperature, the LY-DLL–Tris adduct could be derivatized by benzoin at 65°C for 5 min in 0.8 *M* sodium hydroxide. The HPLC method based on the modification has been proposed for the determination of LY-DLL in biological fluids.

Recently, we studied the precolumn fluorescence derivatization of antagonist [Arg^6, D-$Trp^{7,9}$, $MePhe^8$]–substance P {6–11} (antagonist G) with benzoin by HPLC (unpublished results). It was found that under the derivatization conditions used in the literature [29] antagonist G was partly degraded so that several peaks were observed in the chromatogram. Therefore, milder conditions for antagonist G were chosen. Under the conditions used (0.067 *M* NaOH, heating at 100°C for 10 s), good results were obtained and degradation was negligible. The detection limit was 0.21 nmol/ml. The method has been successfully applied to the selective detection of arginine-containing fragments in

chemical degradation products of antagonist G. One and three fragments were found in acidic and alkaline solution, respectively.

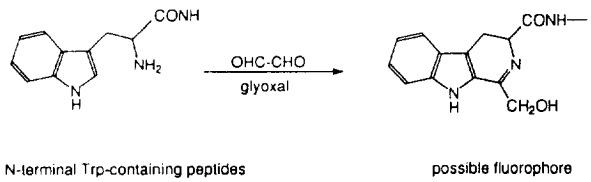

Fig. 5. Reaction of an N-terminal trypophan peptide with glyoxal.

4. Selective determination of tryptophan-containing peptides

There are few reports on the selective determination of tryptophan-containing peptides. However, some papers have been published on the determination of tryptophan in intact proteins, by a direct spectrophotometric determination or a spectrophotometric determination based on colour reactions of intact protein with a variety of reagents, such as sulfenyl halides, 2-hydroxy-5-nitrobenzyl bromide and acid ninhydrin. Direct spectrophotometric determination is based on the absorption of the aromatic group of tryptophan, tyrosine and phenylalanine in the UV region between 250 and 300 nm. Each amino acid has its own characteristic absorption pattern. Derivative spectrophotometric techniques have been introduced to measure tryptophan in the presence of tyrosine and phenylalanine [33–38]. The methods need a considerably large amount of sample proteins and are affected by other absorbing components. Spectrophotometric determinations based on colour reactions with sulfonyl halides [39–41], 2-hydroxy-5-nitrobenzyl bromide [42,43] and acid ninhydrin [44] are not strictly selective for tryptophan and the reagents also react with cysteine. In principle, these methods are applicable as a basis for the selective determination of tryptophan-containing peptides. However, the methods have not so far been combined with HPLC and CE and measure only one peptide or protein in a single analysis. Only the fluorescence derivatization reagent glyoxal has been reported for the selective determination of tryptophan-containing peptides.

4.1. Glyoxal

It was found that phenylglyoxal and glyoxal react selectively with N-terminal tryptophan-containing peptides and form highly fluorescence derivatives (Fig. 5). However, the reaction of phenylglyoxal with the N-terminal tryptophan peptide results in several fluorescent products, whereas the reaction of glyoxal gives a single fluorescence product [45]. Kai et al. [4] proposed an HPLC method for the sensitive and selective determination of N-terminal tryptophan peptides using glyoxal as a precolumn fluorescence derivatization reagent. The derivatization takes place in acidic medium (pH 4.5) at 100°C for 30 min. The fluorescence was detected at 465 nm with excitation at 275 nm. The detection limits for N-terminal tryptophan-containing peptides Trp-Gly-Gly, Trp-Gly, Trp-Ala, Trp-Leu and Trp-Met-Asp-Phe-NH_2 were 55–382 fmol per 100-μl injection volume. The method also allowed the facile detection of an N-terminal tryptophan fragment in the enzyme reaction mixture of dynophin A with trypsin.

5. Selective determination of tyrosine-containing peptides

Derivative spectrophotometry can also be used for the determination of tyrosine in intact proteins [33–38]. Several good methods have been reported for the selective determination of tyrosine-containing peptides by HPLC and CE by use of the fluorescence derivatization reagents 1,2-diamino-4,5-dimethoxybenzene and borate–hydroxylamine–cobalt(II). In addition, HPLC combined with electrochemical detection has been used for this purpose.

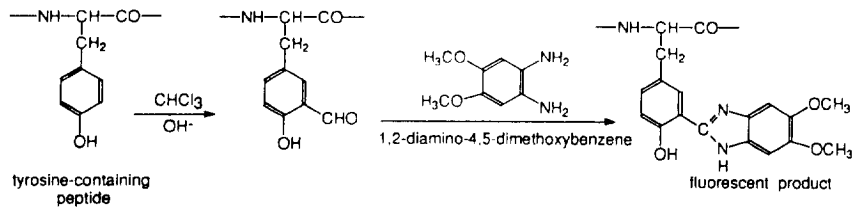

Fig. 6. Reaction of a tyrosine-containing peptide with 1,2-diamino-4,5-dimethoxybenzene.

5.1. 1,2-Diamino-4,5-dimethoxybenzene

A precolumn fluorescence derivatization method has been described for the HPLC determination of tyrosine-containing peptides [46]. The tyrosine residue in the peptide is first formylated in 0.6 *M* potassium hydroxide at 60°C for 10 min in the presence of 50% (v/v) chloroform, and the resulting formylaldehyde is then converted into a fluorescent derivative by reaction with 1,2-diamino-4,5-dimethoxybenzene at 60°C for 18 min in a weakly acidic solution (pH 3–4). The derivatization scheme is shown in Fig. 6. The peptide derivatives fluoresce most intensely at pH 1.3–3.5. The derivatives were separated on a reversed-phase column by isocratic elution and detected at 425 nm with excitation at 350 nm. The detection limits for the tyrosine-containing peptides tested (Tyr-Gly, Gly-Tyr, Tyr-Arg, Tyr-Phe, Tyr-Gly-Gly, Tyr-Gly-Gly-Phe, methionine enkephalin, leucine enkephalin and ANG (I, II and III)) are in the range 3.4–26.2 pmol.

1,2-Diamino-4-methoxybenzene, resembling 1,2-diamino-4,5-dimethoxybenzene, has also been utilized for the selective determination of tyrosine-containing peptides in a combination with CE and laser-induced fluorescence detection [31]. The model tyrosine-containing peptides Gly-Leu-Tyr, Tyr-Arg, ANG II, methionine enkephalin and leucine enkephalin were detected at 438 nm with excitation at 330 nm. The detection limit for leucine enkephalin was 390 amol, which is four orders of magnitude lower than with HPLC and conventional fluorescence detection. This method has been applied to the detection of tyrosine-containing peptides in the chymotryptic digest of horse heart cytochrome *c* for peptide mapping purposes.

5.2. Borate–hydroxylamine–cobalt(II)

Kai and Ohkura [47] described a fluorescence derivatization method for N-terminal tyrosine-containing peptides. The derivatives of the peptide are formed by heating at 100°C for 1–5 min in a weakly alkaline (pH 8–9) solution containing 3.3 m*M* hydroxylamine, 17 μM Co^{2+} and 0.1 *M* borate. The fluorescence is most intense at 440 nm with excitation at 330 nm in a weakly alkaline solution. The structure of the fluorescent product is unknown. The method can be applied to both precolumn and postcolumn derivatization systems for HPLC. Precolumn HPLC has been applied to endogenous enkephalins in a tissue extract at the 300-fmol level [48].

5.3. HPLC–electrochemical detection

Some amino acids have electroactive substituents. The most important of these are the phenol, indole and thiol substituents of tyrosine, tryptophan and cysteine, respectively. Peptides containing these amino acids should be detectable by electrochemical detection (ED). White [49] developed an HPLC method for the determination of tyrosine-containing peptides (oxytocin, Leu-enkephalin, ANG II and Lys-vasopressin) with ED (electrode potential +0.9 V). Although most peptides are probably electroactive by virtue of the terminal amino group, selectivity is reached by selection of a suitable electrode potential. ED is more selective than UV detection, but oxidizing and/or reducing compounds in biological samples should be removed before subjecting the samples to HPLC. Mousa and Couri [50] applied an HPLC–ED

method for the determination of enkephalins, β-endorphins, tyrosine, tyrosylglycine and tyrosylglycylglycines (electrode potential +1.25 V). The detection level was in the 10–100-pg range. This method can be used to determine tissue levels and to study pharmacodynamics of enkephalins and β-endorphin. A highly specific measurement of the different enzymes involved in the metabolism of enkephalin has been achieved.

6. Selective determination of proline-containing peptides

Only one paper has been published on the selective determination of proline-containing peptides [51]. Short-chain peptides with an N-terminal proline were determined by HPLC with laser-induced fluorescence (LIF) detection. The peptides were derivatized with 4-(N,N-dimethylaminosulfonyl) - 7 - fluoro - 2,1,3 - benzoxadiazole (DBD-F) at 50°C for 1 h in 0.1 *M* borax (pH 9.3)–acetonitrile (Fig. 7). The rate of reaction decreases with increase in the molecular mass of the peptide. The proline peptides, including bioactive peptides such as Pro-Leu-Gly-NH_2, Pro-Thr-Pro-Ser-NH_2 and Pro-Asp-Val-Asp-His-Val-Phe-Leu-Arg-Phe-NH_2, were well separated by reversed-phase HPLC. The detection limits with a 15-mW argon ion laser at 488 nm were in the 6–28-fmol range. The detection limits were improved to 2–5 fmol with a microbore column, which was two orders of magnitude higher than with a conventional fluorescence detector using a xenon arc lamp.

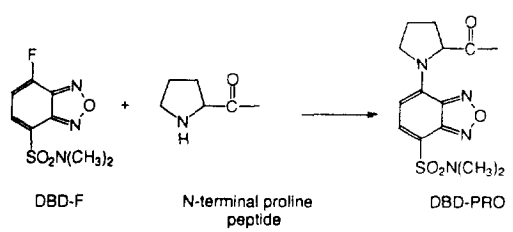

Fig. 7. Reaction of an N-terminal proline peptide with DBD-F.

7. Conclusions and future trends

Only a limited number of amino acids in peptides can be derivatized selectively. Extension of this number will be difficult and may be feasible only for the functional groups thiol–ether (methionine), primary aliphatic amino (lysine) and imidazole (histidine).

Development of more sensitive and selective detection methods can improve the future determination of peptides. Especially HPLC and CE combined with MS detection or LIF detection together with selective derivatization are expected to be fruitful.

The selective derivatization of amino acids in proteins is even more complicated compared with peptides because of steric hindrance by the three-dimensional structure of proteins. On the other hand, sometimes it is possible to differentiate between "outer" and "inner" amino acids in proteins.

The methods discussed can be applied to the analysis of complex mixtures such as enzyme digests, degradation products and biological fluids. Determination of peptides and proteins in biological fluids is of paramount interest for pharmacological studies of these compounds. Extensive application studies will be needed for different purposes.

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