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Identification of tryptophan and tyrosine residues in peptides separated by capillary electrophoresis by their second-derivative spectra using diode-array detection

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Abstract

The use of diode-array detection allows the non-destructive identification of tryptophan and tyrosine residues in complex peptide mixtures separated by capillary electrophoresis. Second-order derivative spectra of both amino acids show significant differences while zero-order spectra are overlapping to a great extent. A mixture of peptides containing tryptophan and/or tyrosine residues was used to evaluate this method. Tryptic peptide maps of carbonic anhydrase and of the bacterial chaperonin protein GroEL, and of an autodigest of trypsin were characterized for tryptophan- and tyrosine-containing peptides. Automated spectra library search was performed successfully.

1. Introduction

Diode-array detectors have the major advantage that spectra of eluting or migrating compounds can be stored in a digital form and then manipulated by a variety of algorithms to ascertain, for example, the purity of an eluting peak [1–3]. Such spectral data, in combination with monitoring of the eluate at chosen discrete wavelengths, greatly increases detection specificity over that available from single-channel detectors.

Peptides and proteins exhibit UV-absorption spectra characteristic of their component amino acids [4–6], particularly the aromatic residues tyrosine and tryptophan, and also phenylalanine to a lesser extent. However, analysis of peptides and proteins for the detection of constituent aromatic residues by UV–Vis spectroscopy poses problems because of overlapping absorption bands of tryptophan and tyrosine. Derivative spectroscopy, in particular second-order derivative spectroscopy which transforms peaks and shoulders into minima, enhances resolution of minor spectral features, thus overcoming some of the problems of similar UV spectra. The great use of diode-array detection in combination with reversed-phase HPLC for the specific identification of aromatic amino acids-containing peptides during their elution under gradient conditions followed by protein sequencing has been shown in detail [7,8].

Application of capillary electrophoresis (CE) for peptide separations and analysis is significantly increasing due to the high resolution of this technique, high speed of analysis and small

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amount of sample required. Also fractionation of peptides on CE followed by protein sequencing has been shown already [9]. Hence, the powerful features of two-dimensional UV spectra described above for HPLC are also available for CE and are invaluable for the identification and characterization of peptides.

In this paper we show that the diode-array detector and associated software implemented in the Hewlett-Packard ^{3D}CE system allows to obtain the same valuable information as described for the HPLC system. Automatic evaluation of tyrosine- and tryptophan-containing peptides from peptide maps using spectral library search functions and second-order derivative spectra are demonstrated.

2. Materials and methods

2.1. Peptides and proteins

All peptides and proteins except the bacterial chaperonin protein GroEL were obtained from Sigma (St. Louis, MO, USA). Peptides were dissolved in water at a concentration of 0.5 mg/ml and proteins in water at a concentration of 3 mg/ml. Purified recombinant GroEL at a con-

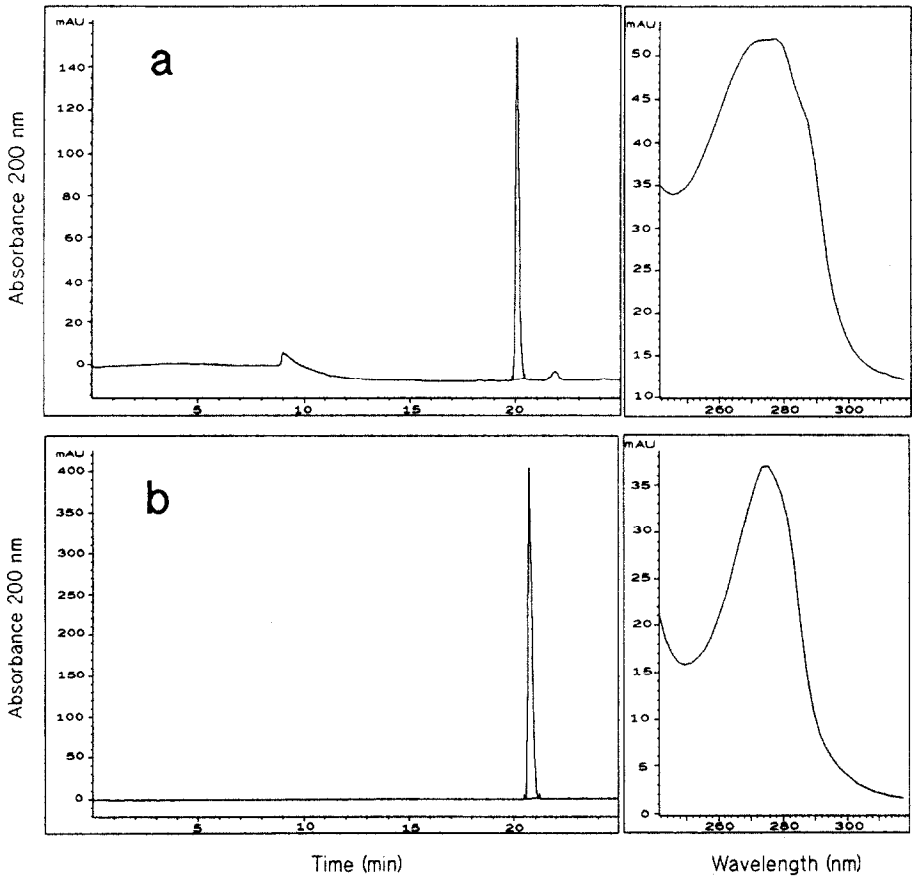

Fig. 1. (a) Electropherogram of tryptophan with recorded zero-order spectrum. (b) Electropherogram of tyrosine with recorded zero-order spectrum.

centration of 4.5 mg/ml was kindly provided by Dr. A.A. Gatenby (DuPont, Wilmington, DE, USA).

2.2. Proteolytic digestion

Carbonic anhydrase and GroEL were incubated with 5% (w/w) trypsin at 37°C for 20 h. An autoproteolytic digestion of trypsin was run at 37°C for 20 h.

2.3. CE system and separation

All experiments were carried out on the Hewlett-Packard ^{3D}CE system with built-in diode-array detector. The system was controlled and all data processing and evaluations were performed using an HP ^{3D}CE ChemStation. Peptide separations were performed on 50 μm and 75 μm bare-fused-silica capillaries with a 3 × extended light path for enhanced sensitive detection. Total length of the capillaries was 64.5 cm, effective

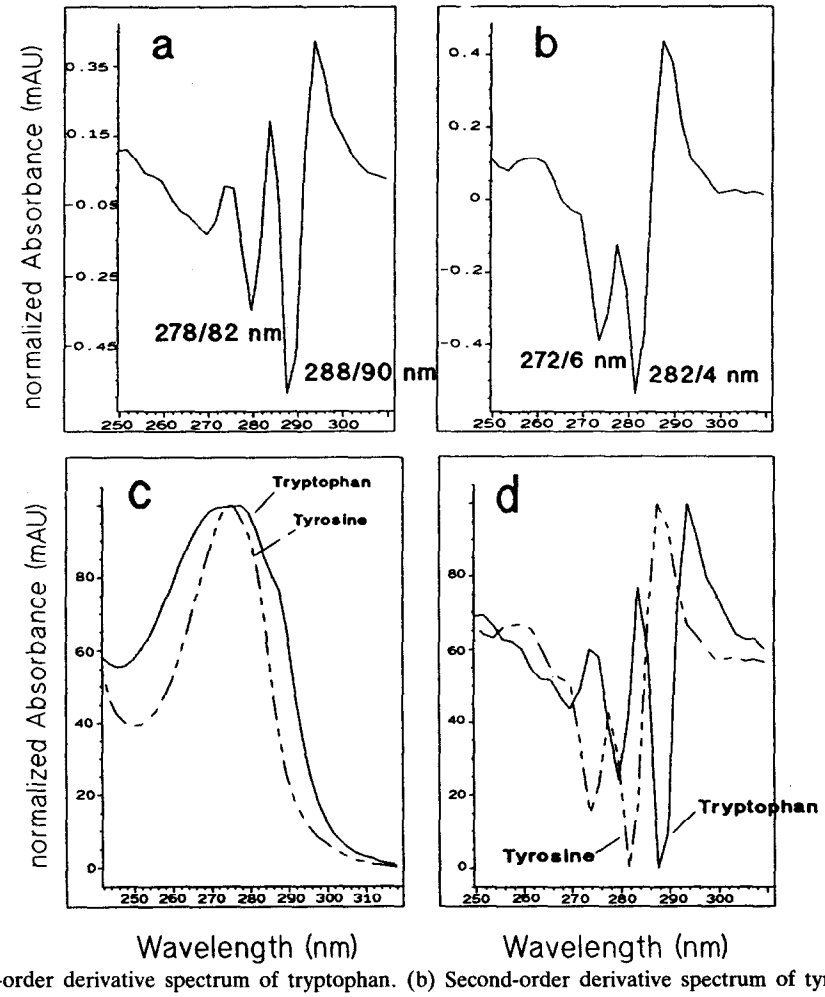

Fig. 2. (a) Second-order derivative spectrum of tryptophan. (b) Second-order derivative spectrum of tyrosine. (c) Overlay of zero-order spectra of tryptophan and tyrosine. (d) Overlay of second-order derivative spectra of tryptophan and tyrosine.

length 56 cm. For the separation of the amino acids and synthetic peptides 20 m*M* phosphate buffer pH 3.0 was used. Protein digests were separated using 50 m*M* phosphate buffer pH 2.5. 30 kV was applied to the capillaries resulting in about 25 μA for the pH 3.0 buffer and about 67 μA for the pH 2.5 buffer. Separations were run at 25°C. Pressure injection for 200 mbar s was used for sample loading into the capillaries. Other conditions were identical.

2.4. Detection system and second-order derivative spectra

Peptides were monitored with the diode-array detector at both 200 and 280 nm. Simultaneously, all spectra were collected during the runs. Second-order derivative spectra for the range 250–320 nm, which is characteristic for the aromatic amino acid residues tyrosine and tryptophan, were automatically calculated using the standard software of the HP ^{3D}CE ChemStation. A spectra library of the second-derivative spectra of tyrosine and tryptophan was created and automated library search was performed using the same software.

3. Results and discussion

In order to evaluate the potential of this method and to create a library with the second-order derivative spectra of tryptophan and tyrosine both amino acids were electrophoresed and zero-order spectra were recorded using diode-array detection as shown in Fig. 1. Using the standard software of the HP ^{3D}CE ChemStation the algorithm calculating the second-order derivatives was carried out. The resulting second-derivative spectra of tryptophan and tyrosine (Fig. 2a and b) were then used for creating the library for automated search of tryptophan- and tyrosine-containing peptides separated by CE. Whilst the zero-order spectra of tryptophan and tyrosine are overlapping with an absorption maximum of about 278 nm for both amino acids (Fig. 2c), the second-order derivative spectra show significant differences.

Table 1
Synthetic peptides used for method evaluation

Peak 1:	VHLTPVEK
Peak 2:	DRV*Y*IHPF
Peak 3:	SISGLAK
Peak 4:	AGCKNFF*W*KTFTSC
Peak 5:	EL*Y*ENKPRRP*W*IL
Peak 6:	EGKRP*W*IL
Peak 7:	*W*MFDamide
Peak 8:	*Y*GGFL

Tyrosine residues (Y) and tryptophan residues (W) are italicized.

The second-order derivative spectrum of tryptophan shows a main minimum at 290 ± 2 nm and a first side minimum at 280 ± 2 nm while that of tyrosine shows the main minimum at 282 ± 2 nm and the first side minimum at 274 ± 2 nm (Fig. 2d).

For evaluation of the automated second-order derivative spectra library search a mixture of short synthetic peptides of known sequence (Table 1) containing either tryptophan, tyrosine, both of them or none were separated. All peptides were almost baseline separated within

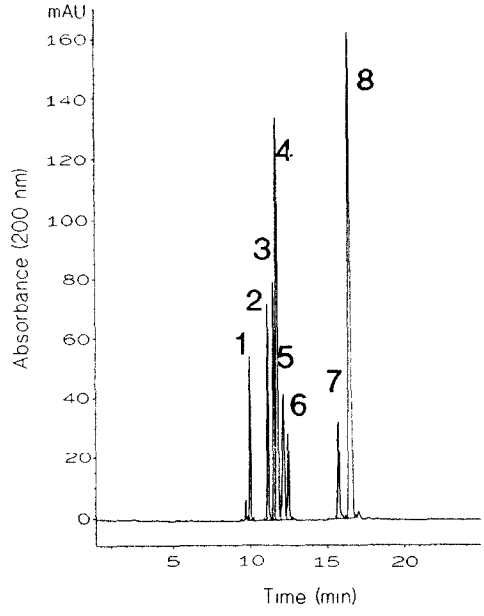

Fig. 3. Electropherogram of 8 synthetic peptides separated by capillary zone electrophoresis (see Table 1 for identification).

17 min (Fig. 3). Peptides containing tryptophan (peaks 4–7) clearly can be identified by their second-order derivative spectrum with the characteristic minimum at 290 ± 2 nm (Fig. 4). The presence of a tyrosine residue besides tryptophan in peptide peak 5 did not affect the identification of tryptophan. Automated library search of those peptides resulted in match factors of about 750–990 for tryptophan and of about 0.02–1.0 for tyrosine. A match factor of 1000 would be the ideal match factor, while matches > 750 indicate undoubtful presence and matches < 100 absence of the search species, respectively.

Similarly, tyrosine-containing peptides can be clearly identified by their characteristic second-order derivative spectral minima around 282 ± 2 nm as shown for peptides in peak 2 and 8 (Fig. 5). Match factors for tyrosine and tryptophan are in the same range as shown for tryptophan-containing peptides.

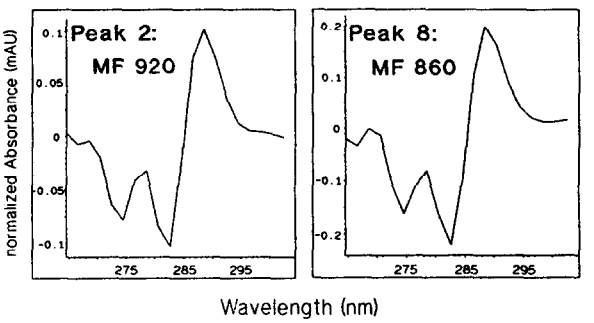

Fig. 5. Second-order derivative spectra of peptides 2 and 8 containing a tyrosine residue.

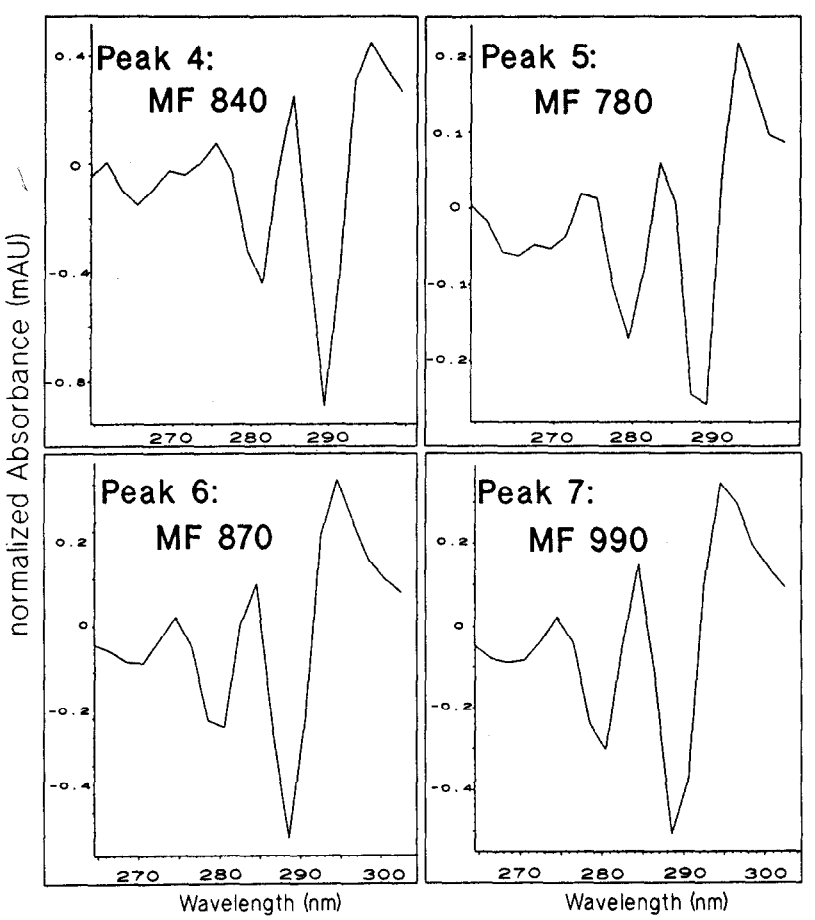

Fig. 4. Second-order derivative spectra of peptides 4–7 containing a tryptophan residue. MF = Match factor.

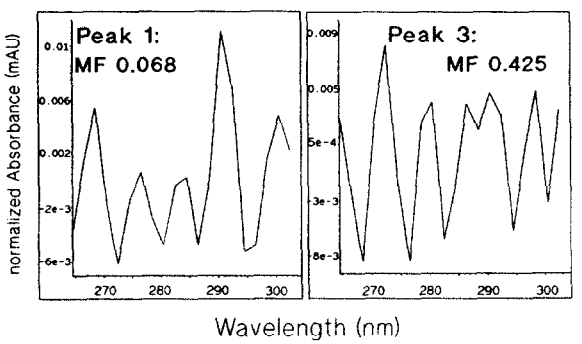

Fig. 6. Second-order derivative spectra of peptides 1 and 3 containing no tyrosine and no tryptophan residue.

As negative control Fig. 6 shows the characteristic second-order derivative spectra of two peptides (peaks 1 and 3) which contain neither tyrosine nor tryptophan residues. No clear minimum can be observed in the wavelength range between 250 and 320 nm and no matches were found. In this case typically spectra look like "baseline noise".

The peptide containing one tryptophan and one tyrosine residue (peak 5) resulted only in the clear identification of tryptophan with a match factor of 780, but not of tyrosine which resulted only in a match factor of 80 due to the overlap of the major minimum of tyrosine with the side minimum of tryptophan (Fig. 4). This is coincident with the finding of Grego et al. [7] who stated that tyrosine can only be determined in the presence of one tryptophan residue, if three tyrosine residues are present. In this case, the major minimum of tyrosine at around 282 nm will be larger then that of tryptophan at around 290 nm [7].

The method was then applied to tryptic digests of the enzyme carbonic anhydrase, GroEL and the protease itself. Carbonic anhydrase was used due to its high content of tryptophan residues

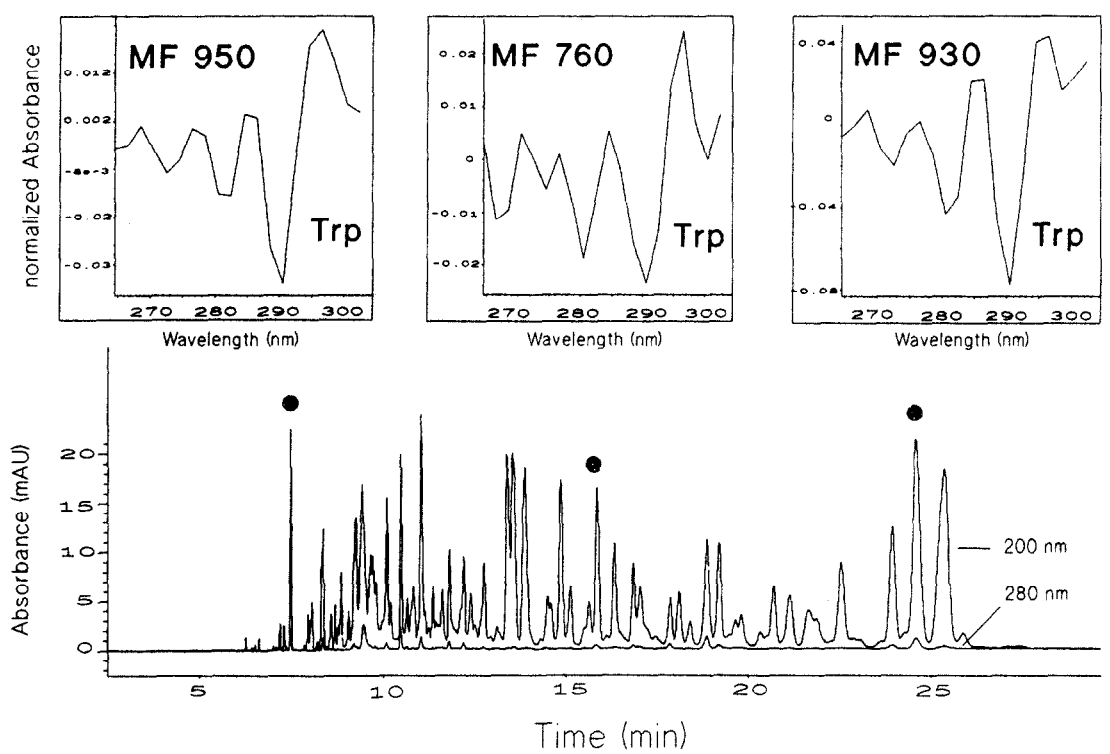

Fig. 7. Electropherogram of the tryptic digest of carbonic anhydrase with three representative second-order derivative spectra of peptides. Electropherogram was recorded at 200 and 280 nm.

while GroEL contains only tyrosine residues and no tryptophan residue. As shown in Figs. 7–9, in the various digests tryptophan- and tyrosine-containing peptides can be easily discriminated and identified by their characteristic second-order derivative spectra. In the electropherograms of Figs. 7 and 9 peptide detection was recorded at 200 nm and in addition at 280 nm to generally identify the aromatic amino acids-containing peptides. In the electropherogram of the tryptic digest of GroEL detection was recorded only at 200 nm. Detection at 280 nm was neglected to show that with diode-array detection and associated software second-order derivative spectra can automatically be calculated and automatic search can be performed allowing unambiguous identification of tyrosine residues in this particular protein digest.

In this paper it was shown that second-order derivative spectroscopy is a very valuable tool for the unambiguous identification of tryptophan- and tyrosine-containing peptides separated by CE using diode-array detection. In peptides containing both amino acids with the same number only the presence of tryptophan can be clearly identified. The method described will be especially useful for micropreparative isolation of these peptides for further analysis by protein sequencing, similar as previously described for the separations and fraction collection of tryptophan-containing peptides by HPLC [7].

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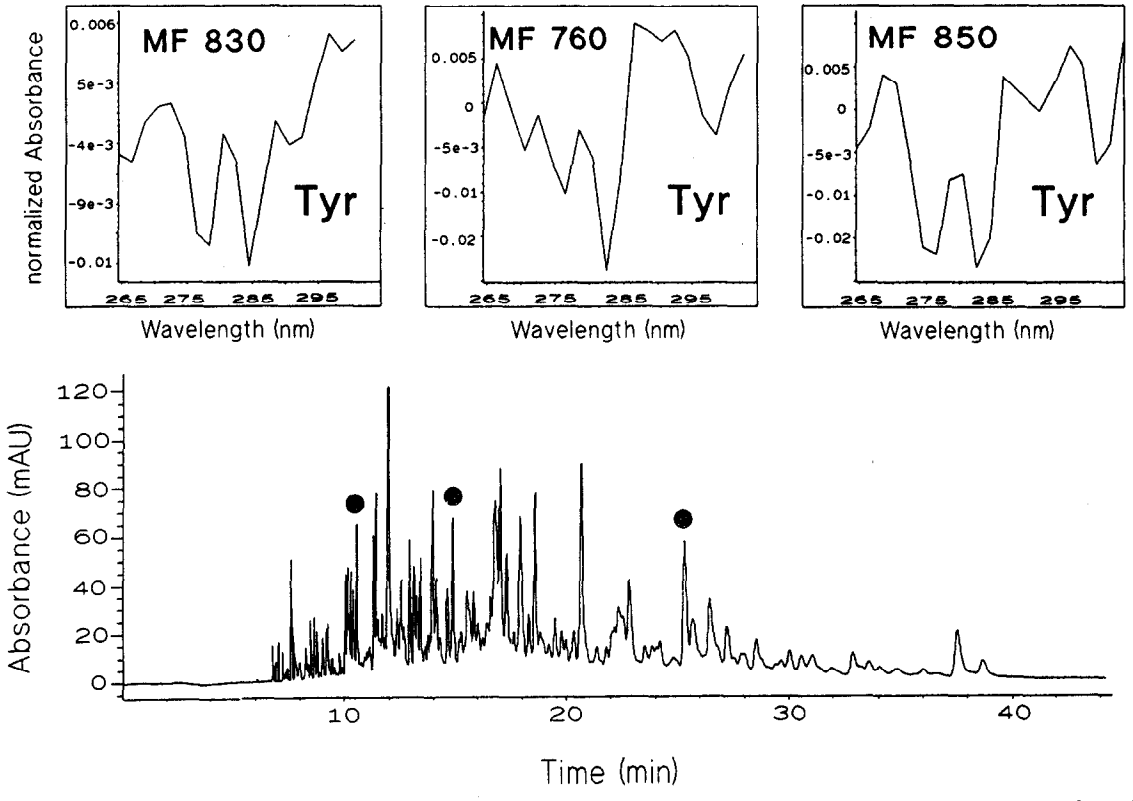

Fig. 8. Electropherogram of the tryptic digest of GroEL with three representative second-order derivative spectra of peptides. Electropherogram was recorded only at 200 nm.

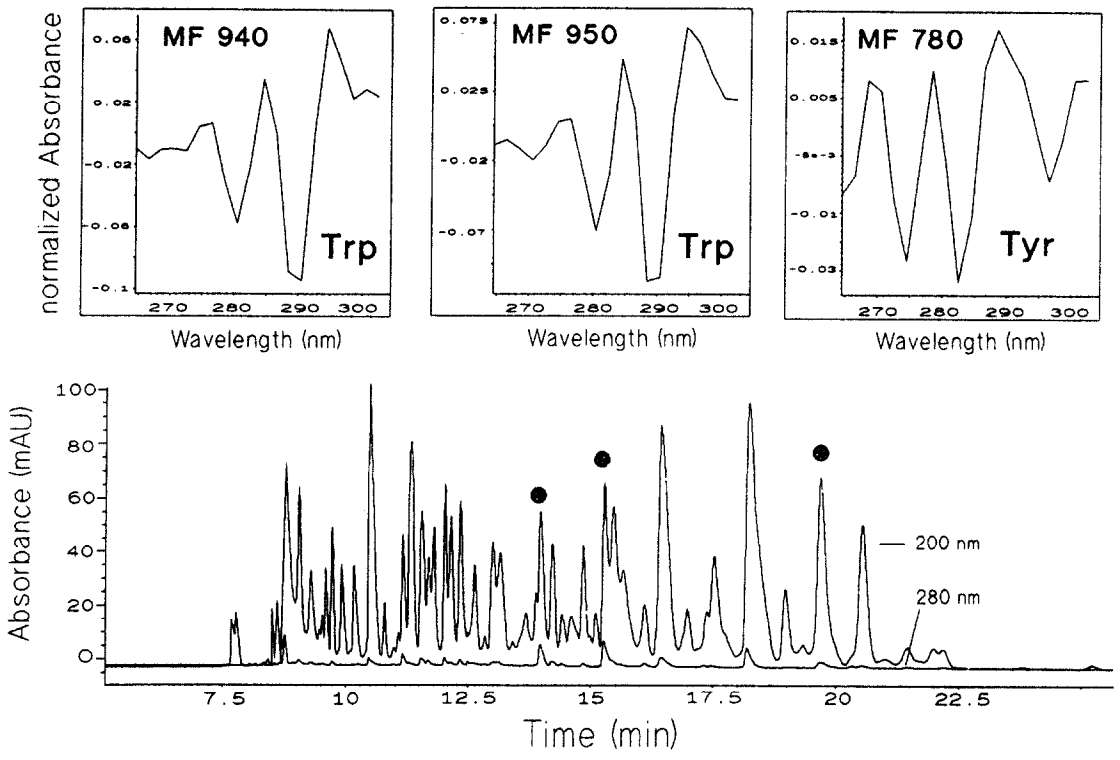

Fig. 9. Electropherogram of the autodigest of trypsin with three representative second-order derivative spectra of peptides. Electropherogram was recorded at 200 and 280 nm.

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