ELSEVIER

Analytica Chimica Acta 372 (1998) 257–272

ANALYTICA CHIMICA ACTA

Ultraviolet detector response of glycine and alanine homopeptides: Some specific features in capillary electrophoresis

Ilona Hamrníková[a,b], Ivan Mikšík[b], Marie Uhrová[c], Zdeněk Deyl[b,c,*]

[a] *2nd Medical School Charles University, Department of Medicine, Chemistry and Biochemistry, Plzeòská 221/130, CZ 150 00 Prague 5, Czech Republic*

[b] *Institute of Physiology, Academy of Sciences of the Czech Republic, Vídenská 1083, CZ 142 20 Prague 4, Czech Republic*

[c] *Department of Analytical Chemistry, Institute of Chemical Technology, Technická 1905, CZ 166 28 Prague 6, Czech Republic*

Abstract

UV absorbance of a single peptide bond contribution at 200 nm to the overall optical density of the solute was evaluated with two sets of model peptides composed of either 1–14 glycine or alanine residues. It was documented that in capillary electrophoresis using 57 cm (50 cm to the detector) ×75 μm i.d. capillary and 25 mmol l phosphate buffer pH 2.5 containing or devoid of 0.35% hydroxypropyl-methylcellulose (HPMC), the detector's response per peptide bond drops down in peptides containing five glycine or alanine residues. This limiting absorbance value was shown to increase again with the increasing length of the peptide chain reaching either a constant value (glycine and alanine peptides) run in the absence of HPMC at elevated (50°C) temperature or exhibiting a second stepped drop with alanine peptides run at 25°C or in the presence of the polymeric sugar in the background electrolyte. The reasons of this hypochromic effect are discussed.

Keywords: Peptides; UV detection; Hypochromic effects; Capillary electrophoresis

1. Introduction

Recording of UV absorbance in the far UV region appears today the most common detection method for peptides and proteins separated by capillary electromigration or chromatographic methods. While absorbance at 280 nm reflects the presence of aromatic ring possessing peptides and proteins, absorbance at 190 nm reflects the presence of the peptide bond. In practice, however, the detector is usually set to wavelengths anywhere between 200–230 nm. This compromise offers favourable detection limits with simultaneous elimination of interferences caused by the presence of possible contaminants not speaking about the fact that not all commonly used detectors are capable of reliable recording at 190 nm (for review see [1]).

There are several factors to be taken into account when recording peptides in far UV: first the molar absorptivity should be generally higher with longer peptides/proteins. This assumption was confirmed by Becklin and Desiderio [2]. For instance the peptide $(Ala)_3$ exhibits a doubled absorbance when compared to peptide $(Ala)_2$ which is in a good agreement with the fact that the latter peptide contains only one peptide bond while the other contains two. This

*Corresponding author. Tel.: +420-2-4752558; fax: +420-2-4752558

0003-2670/98/$19.00
PII S0003-2670(98)00328-6

observation can be supported by literary data which indicate that the limit of detection for substance P and methionine enkephalin is in the range of 40 fmol [3] while the detection limit for considerably larger polypeptides like collagen type I constituting α-chains (containing about 1000 amino acids) is somewhere in the range of 10–15 amol. Let us emphasise that the limits of detection differ by three orders of magnitude. On the other hand, when comparing the number of peptide bonds present in e.g. methionine enkephalin and collagen α-chain, the difference is much less than the mentioned three orders of magnitude reflecting, perhaps, the high proline and hydroxyproline content in the collagen polypeptide chains. Further support for the relation between absorbance in far UV and the length of the polypeptide chain can be derived from our previous report [4]. Though a general increase in the detector's response can be found with larger peptides, this relation is not simply interpretable [4]. Other factors besides the number of peptide bonds are apparently involved. They can be derived from both the primary and secondary structure of the peptide analysed. The final peak area of a peptide depends not only in its size (number of peptide bonds) but on the nature of amino acids involved as well. Another factor mentioned already in the paper of Simpson and Peterson [5] is based on the exciton theory: a certain hypochromicity can be expected owing to the parallel arrangement of peptide chromophores. This effect could be particularly important in peptides attaining helical conformation. An experimental proof of this assumption comes from the work of Imakori and Tanaka [6], who working with poly-L-glutamic acid were able to demonstrate that at pH values favoring the helical conformation, the extinction coefficient is lower compared to what can be measured at higher pH values favoring the dissociation of the side chain carboxyl and, consequently, random conformation of the polypeptide chain (65% increase of absorbance at 190 nm). Similar conclusions were drawn from the studies on poly-L-lysine by Rosenheck and Doty [7] (random coil information attains 150% absorbance in comparison to the helical arrangement). When increasing the temperature to 52°C, the polypeptide chains of poly-L-lysine are completely converted to β-form as documented by measuring the infrared spectra where a shift of the amide band (I) in a D_2O solution from 1638 to 1610^{-1} cm can be observed.

The far UV absorbance of the β-form shows an additional increase compared to the random coil arrangement and a shift of the maximum from 192 to 194 nm. While the shift of the far UV maximum is hardly discernible when UV absorbance is used for the detection of separated peptides, the changes of absorbance with temperature should be kept in mind in any study regarding detection of peptides in the far UV region. To our knowledge no data are available from the literature about the role of temperature on the detection of peptides separated by e.g. capillary electrophoresis.

In the paper of Becklin and Desiderio [2] it is well documented that glycine polypeptides reveal a lower absorbancy per peptide bond compared to peptides containing alanine only. All di- up to pentapeptides have shown a constant value of absorbance per peptide bond; the peptide $(Ala)_6$ exhibited a distinct hypochromicity which was ascribed to a rudimental α-helix formation (parallel arrangement of the polypeptide chain). A similar hypochromicity was observed with two commercially available peptides, namely PLSRTLSVAAKK and SIGSLAK which are typical in possessing aliphatic side chains. The hypochromic effect was more distinct with the larger peptide compared to the smaller one.

Finally if capillary electrophoresis is used as the separation technique one has to consider also the role of the background electrolyte used. In particular different additives which may be quite helpful in increasing the quality of separation may concomitantly influence the UV detector response by helping the analyte to attain a particular conformation.

In this report we attempted (i) to expand the present knowledge about peptide bond absorbance with the increasing length of the peptide, (ii) to evaluate the role of different aliphatic amino acid side chains (non-ionizable), (iii) to evaluate the role of mobile phase additives upon the detector's response corresponding to a single peptide bond and (iv) to evaluate the role of temperature at which the separation is carried out.

2. Experimental

2.1. Chemicals

Glycine peptides (di- up to hexapeptide) were obtained from Sigma, St. Louis, MO, USA; peptides

containing 7–10 and 13 and 14 glycine residues per molecule were custom prepared in the laboratory using solid phase synthesis, azide and *p*-nitrophenyl ester methods following generally the approach described [8–11]. Alanine polypeptides comprising 1–14 amino acid residues were also custom prepared using solid phase synthesis, N-hydroxy-5-norbornene-2,3-dicarboximide ester, *p*-nitrophenyl ester and azide methods following the procedures described [12–16].

Hydroxypropyl-methylcellulose (HPMC) was a product of Aldrich (Milwaukee, WI, USA). All other chemicals were products of Lachema (Brno, Czech Republic) and were either of p.a. or highest available purity. Milli-Q water (Millipore, Bedford, MA, USA) was used throughout the study; at least 2 mg of each peptide were available for the study.

2.2. Purification of peptides

The crude peptide samples were purified by HPLC using Applied Biosystems Model 140B dual syringe pump (Foster City, CA, USA) equipped with a 150 mm×0.53 mm C18 (5 μm) column. Two solvents were used to elute the peptides: solvent A (0.05% TFA in water) and solvent B (0.05% TFA in acetonitrile-water 80:20). Separations were materialised by running a linear gradient from 5% to 65% B over 30 or 60 min. The mobile phase was delivered at 200 μl min and split 1:10 prior the injector. Loop injections were made with 20 μl sample dissolved in A:B (1:1, v/v).

From each sample purified in this way ESI mass spectra were acquired using the approach described [17]. A Micromass Autospec-Q mass spectrometer with Mark II ESI interface was used for this purpose. The electrospray needle was operated at 7.6 kV and ions were accelerated into the mass spectrometer at 4 kV. Sampling cone voltages in the 75–150 V range appeared optimal. Nitrogen (Very Dry, Liquid Carbonic, Scarborough, Canada) bath was introduced into the interface at a flow rate of 500 l h (80°C); nitrogen nebulizer gas was pumped at a flow rate of 14 l h and the vacuum of 4.10^{-4} and 7.10^{-6} Pa within the source and analyser regions ensured by a rotary and turbomolecular pump. The molecular masses obtained for individual peptides were typically within the 5–30 ppm range which is within the range declared in [10].

The commercially available peptides were of at least 99% purity (except diglycine which was 99.5% pure), the custom synthesized peptides were at least 98% pure. All peptides were stable during half a year storage period in a refrigerator and no discernible peaks were observed at the end of the investigation. The purity of investigated samples at the end of the study was the same as at beginning. Inter-day reproducibility of peak areas during capillary electrophoresis was 99.8±0.03.

2.3. Capillary electrophoresis

Separations of peptide mixtures were done with Beckman P/ACE 5000 (Fullerton, CA, USA) using untreated fused silica capillary 57 cm (50 to the detector) ×75 μm capillary run at 15 kV and 25 mmol l phosphate buffer pH 2.5 containing 0.35% hydroxypropyl-methylcellulose as background electrolyte. Between runs the capillary was washed with Milli-Q water (4 min), 1 mol l NaOH (6 min), Milli-Q water (4 min), 1 mol l HCl (8 min) and finally with Milli-Q water again (4 min). Before each run the capillary was equilibrated with the run electrolyte (2 min). Samples were injected hydrodynamically (3.45 kPa). Statistical evaluation of the peak area differences (extreme values) was done by the Student's *t*-test (n=4, subsequent runs).

Migration areas corrections were done in the following way: measured peak area divided by relative migration to dipeptide; normalization to dipeptide =10 000: corrected area migration recalculated to dipeptide =10 000.

3. Results

With the background electrolyte (25 mmol l sodium phosphate pH 2.5 containing 0.35% hydroxypropyl-methyl cellulose) both the glycine and alanine containing peptides were baseline separated at two different temperatures (25°C and 50°C) as shown in Table 1 and Table 2. The selection of the background electrolyte was based on the fact that this buffer yielded practically the same quality of separation with a large number of proteins differing widely in the aminoacid composition and size and the results were identical with the background electrolyte commer-

Table 1
Separation parameters for series of $(Gly)_{2\text{-}14}$ peptides in 25 mmol l phosphate buffer (containing 0.35% hydroxypropyl-methylcellulose) using 60 cm ×75 μm i.d. capillary

Solute	t_r (min)	$t_r/t_{r,0}$	M_w	$(M_w^{2/3}/z)$	Number of theoretical plates	Resolution
25°C						
$(Gly)_2$	22.12	1			114 232	–
$(Gly)_3$	25.98	1.175	187	31.58	119 993	13.8
$(Gly)_4$	30.53	1.380	243	37.54	114 728	11.1
$(Gly)_5$	34.73	1.570	299	43.04	122 088	9.03
$(Gly)_6$	38.75	1.751	355	48.21	98 191	9.03
$(Gly)_7$	43.94	1.980	411	53.10	90 311	8.70
$(Gly)_8$	48.00	2.170	467	57.77	86 520	8.30
$(Gly)_9$	52.02	2.352	523	62.26	75 380	7.95
$(Gly)_{10}$	55.59	2.513	579	66.58	65 411	6.70
$(Gly)_{13}$	72.77	3.290	747	78.77	59 217	5.40
$(Gly)_{14}$	83.01	3.753	802	82.55	51 720	4.15
50°C						
$(Gly)_2$	14.72	1			114 494	–
$(Gly)_3$	17.31	1.176	187	31.58	125 382	14.1
$(Gly)_4$	20.27	1.377	243	37.54	110 834	10.5
$(Gly)_5$	23.03	1.564	299	43.04	107 603	8.5
$(Gly)_6$	25.65	1.742	355	48.21	92 573	8.5
$(Gly)_7$	28.70	1.950	411	53.10	88 256	8.1
$(Gly)_8$	31.73	2.156	467	57.77	87 315	7.6
$(Gly)_9$	34.90	2.371	523	62.26	80 271	7.3
$(Gly)_{10}$	36.25	2.463	579	66.58	78 315	7.0
$(Gly)_{13}$	47.84	3.250	747	78.77	69 320	6.5
$(Gly)_{14}$	54.05	3.672	802	82.55	61 515	6.2

$t_{r,0}$ – migration time relative to t_r of $(Gly)_2$. Resolution is calculated to the nearest preceeding peak. Individual values represent averages from four subsequent runs; between run differences in t_r were always less than 0.05 min.

cially available from Bio Rad (data not shown). Illustrative examples of separation are demonstrated in Fig. 1 and Fig. 2.

In most cases, both with the glycine and alanine peptides, the number of the theoretical plates was around 1×10^5 (Tables 1 and 2) and usually decreased with the increasing chain length. In most cases, the number of theoretical plates decreased to one half to one third for the 14 amino acids containing peptide as compared to the peptide containing a single peptide bond only.

Detector's response was evaluated by peak area. The originally high value of absorbancy generally dropped down with the first three peptides ($P<0.01$, $n=4$) for each peptide involved; $(Gly)_3$ run at 25°C in the presence of 0.35% HPMC and $(Ala)_3$ run in the absence of HPMC, both at 25°C and 50°C showed an insignificant increase in peak area per peptide bond as compared to the respective dipeptide. If the background electrolyte modifier (HPMC) was added, a drop in the peak area area was observed with the tripeptide, but an increased per peptide bond area was observed with $(Ala)_4$ both at 25°C and 50°C. With peptides involving more than five amino acids, an increase of peak area per peptide bond was observed as compared to peptides containing four peptide bonds. This increase was either independent of the increasing chain length of the peptide (for glycine peptides and alanine peptides run in the absence of HPMC at 50°C) or after an initial increase (alanine peptides) that culminated at either $(Ala)_6$ or $(Ala)_8$ (depending on whether the background electrolyte contained HPMC or not), a second stepped drop with the increasing chain length was observed. The differences between the lowest and the highest observed values for a particular set of peptides and separation

Table 2
Separation parameters for series of $(Ala)_{2\text{-}14}$ peptides in 25 mmol l phosphate buffer (containing 0.35% hydroxypropyl-methylcellulose) using 60 cm ×75 μm i.d. capillary

Solute	t_r (min)	$t_r/t_{r,0}$	M_w	$(M_w^{2/3}/z)$	Number of theoretical plates	Resolution
25°C						
$(Ala)_2$	25.58	1			136 295	–
$(Ala)_3$	30.75	1.202	230	36.20	127 360	16.7
$(Ala)_4$	35.98	1.406	301	43.23	121 807	10.9
$(Ala)_5$	40.81	1.595	372	49.72	115 426	9.31
$(Ala)_6$	45.48	1.777	443	55.79	120 165	17.3
$(Ala)_7$	47.32	1.850	514	61.55	115 512	16.4
$(Ala)_8$	50.13	1.960	585	67.03	109 403	15.2
$(Ala)_9$	52.44	2.050	656	72.30	103 216	14.8
$(Ala)_{10}$	53.97	2.110	727	77.37	95 411	13.2
$(Ala)_{11}$	57.04	2.230	798	82.28	87 312	12.1
$(Ala)_{12}$	58.57	2.290	869	87.04	76 593	9.8
$(Ala)_{13}$	59.60	2.330	940	91.67	68 997	7.6
$(Ala)_{14}$	61.14	2.390	1011	96.19	65 312	5.9
50°C						
$(Ala)_2$	16.43	1			118 462	–
$(Ala)_3$	19.77	1.203	230	36.20	124 979	11.5
$(Ala)_4$	23.03	1.402	301	43.23	114 039	10.5
$(Ala)_5$	26.06	1.586	372	49.72	115 503	8.65
$(Ala)_6$	28.93	1.761	443	55.79	104 206	8.65
$(Ala)_7$	30.39	1.850	514	61.55	92 311	7.93
$(Ala)_8$	32.86	2.000	585	67.03	87 516	6.51
$(Ala)_9$	34.99	2.130	656	72.30	74 229	5.47
$(Ala)_{10}$	35.64	2.170	727	77.37	68 915	5.00
$(Ala)_{11}$	38.11	2.320	798	82.28	65 875	4.39
$(Ala)_{12}$	39.58	2.410	869	87.04	59 321	4.15
$(Ala)_{13}$	40.90	2.490	940	91.67	53 211	3.97
$(Ala)_{14}$	42.54	2.590	1011	96.19	49 678	3.03

$t_{r,0}$ – migration time relative to t_r of $(Ala)_2$. Resolution is calculated to the nearest preceeding peak. Individual values represent averages from four subsequent runs; between run differences in t_r were always less than 0.05 min.

conditions used were always highly significant ($P<0.01$, $n=4$ for each peptide involved). The results are shown in Fig. 3. It has to be pointed out that the decrease of a peptide bond absorbance with increasing chain length was observed with the alanine set of peptides only. If the separation was done in the absence of the modifier (HPMC), increasing of the temperature to 50°C equalised the drop observed at 25°C for the longer peptides; however the presence of the polymeric sugar in the background electrolyte prevented this effect (Fig. 3). This character of the peptide bond absorbance upon chain length remained unchanged if the separation was run at elevated temperature (see Figs. 1 and 2). It was interesting to note that the average absorbance per peptide bond was higher with glycine peptides than with peptides consisting of alanine only no matter whether the separation was carried out at 25°C or 50°C (Tables 1 and 2).

Under the specified experimental conditions most of the peptides investigated followed generally the Offord's relation (Figs. 4 and 5, Tables 1 and 2). As expected, the temperature increase did not influence the $(t_r/t_{r,0})$ vs. $(M_w^{2/3}/z)$ dependence. With the alanine peptide set some deviation at longer wavelengths was observed namely at 25°C which may indicate the involvement of another mechanism in the separation process than the charge effect. If the separation was increased (50°C) the Offord's relation had a tendency to approach linearity. However the $(t_r/t_{r,0})$ values

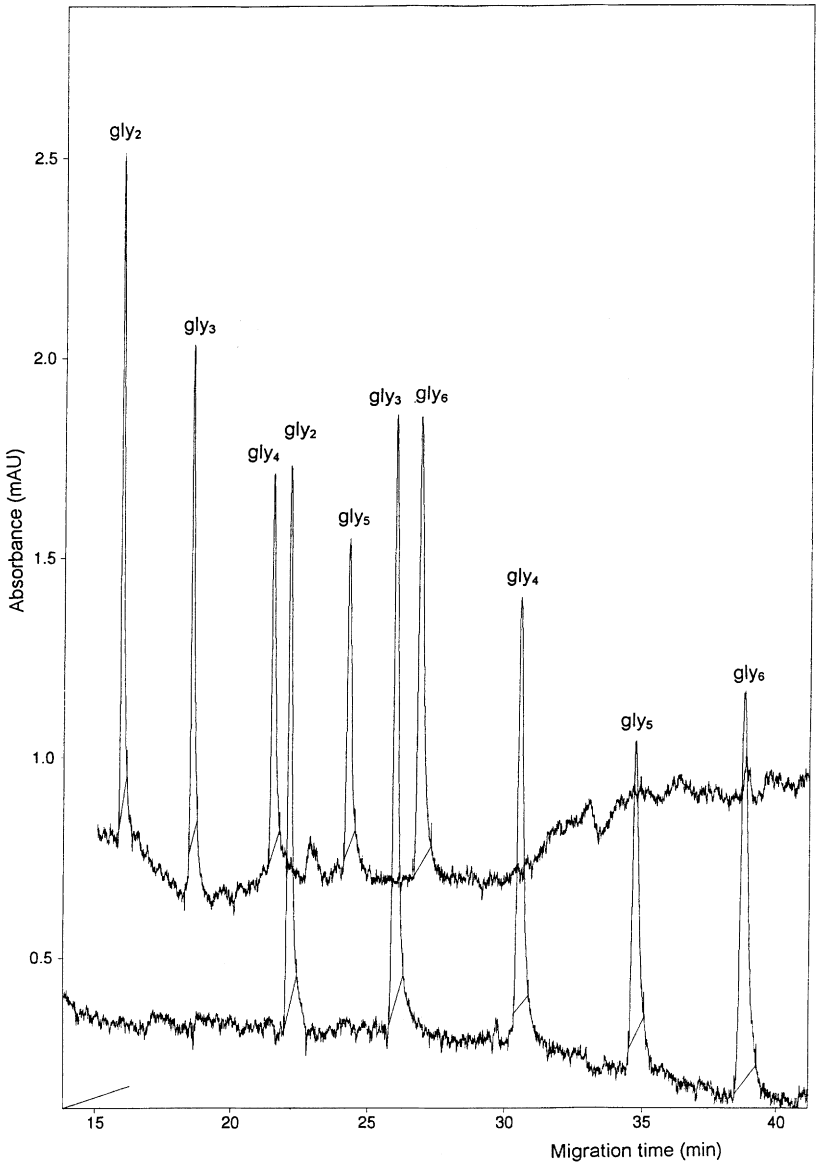

Fig. 1. Comparison of the separation of $(Gly)_{2-6}$ peptides in 25 mmol l phosphate buffer pH 2.5 with 0.35% hydroxypropyl-methylcellulose added at 25°C (bottom recording) and 50°C (upper recording). For additional data see Table 1.

were higher for the temperature with alanine peptides possessing more than eight amino acids per molecule. Glycine peptides possessing 13 and 14 amino acid residues showed a distinctly increased $(t_r/t_{r,0})$ value than expected; this increase was similar no matter whether the run was performed at 25° or 50°C. This further supports our conclusion that for longer peptides other partition mechanisms than electromigration are involved and that the nature of such additional mechanisms depends on the nature of amino acid involved.

In order to evaluate the role of hydroxypropyl-methylcellulose a similar set of experiments as described was run in a background electrolyte which

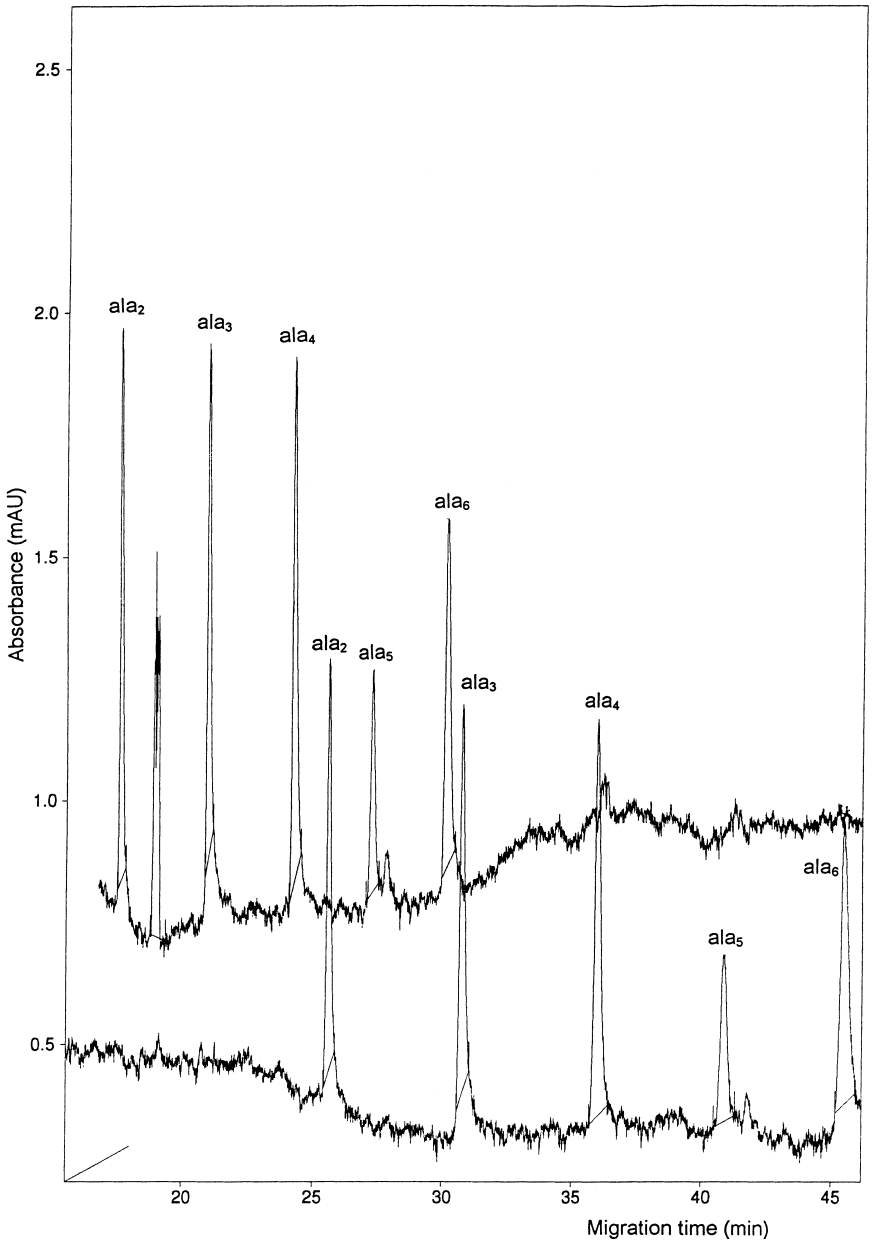

Fig. 2. Comparison of the separation of $(Ala)_{2-6}$ peptides in 25 mmol l phosphate buffer pH 2.5 with 0.35% hydroxypropyl-methylcellulose added at 25°C (bottom recording) and 50°C (upper recording).For additional data see Table 2.

was devoid of this modifier. It was demonstrated that hydroxypropyl-methylcellulose addition has, as expected, a slowing down effect upon the separation and with alanine peptides (as mentioned already) leads to a drop of peptide bond absorbance with increasing peptide length (Fig. 6, Fig. 7). Typical separation of the whole series of glycine peptides is shown in Fig. 8.

Offord's plots are shown in Figs. 4 and 5. They demonstrate a deviation from linearity with long

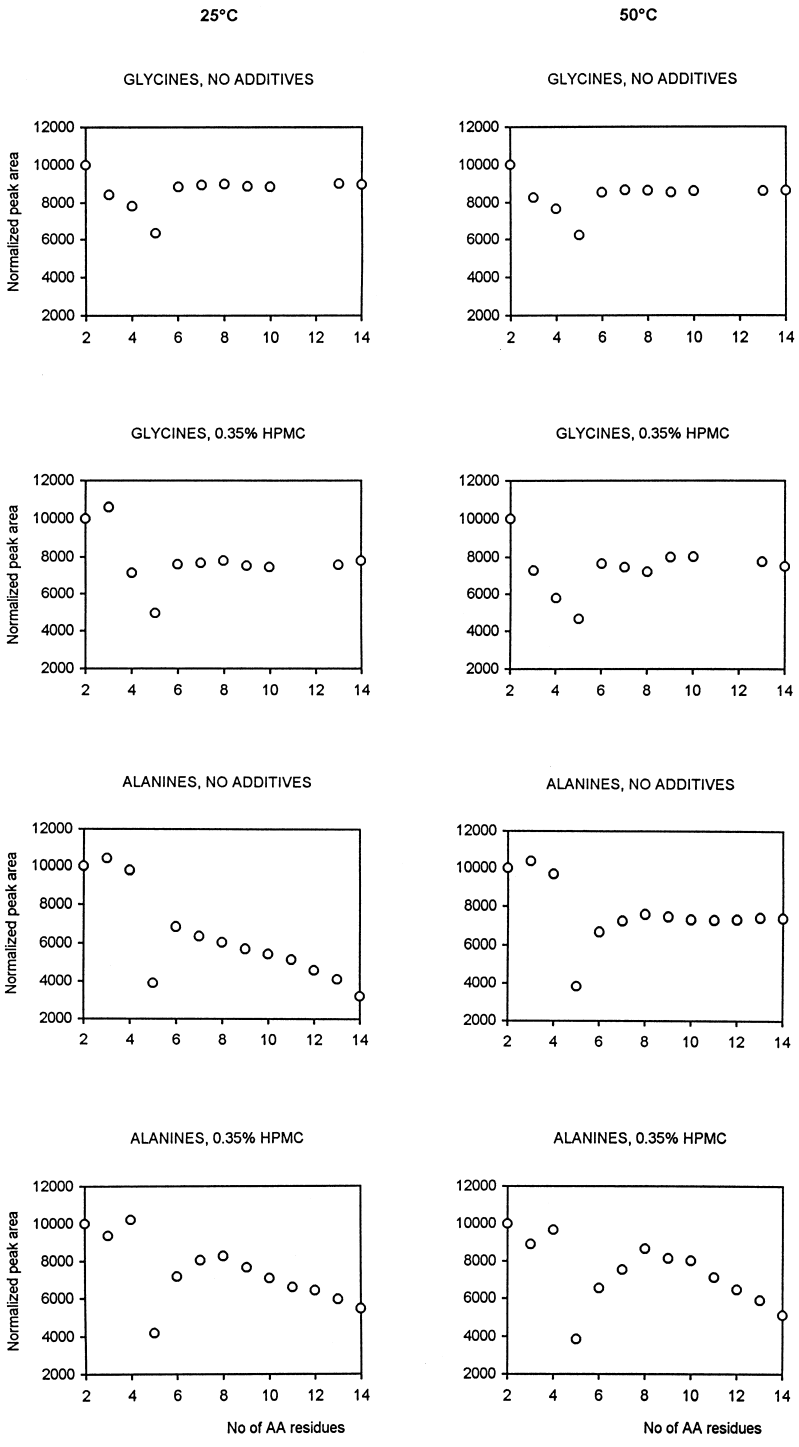

Fig. 3. Relation between the normalized peak area corresponding to a single peptide bond and the number of the amino acid residues in the particular peptide

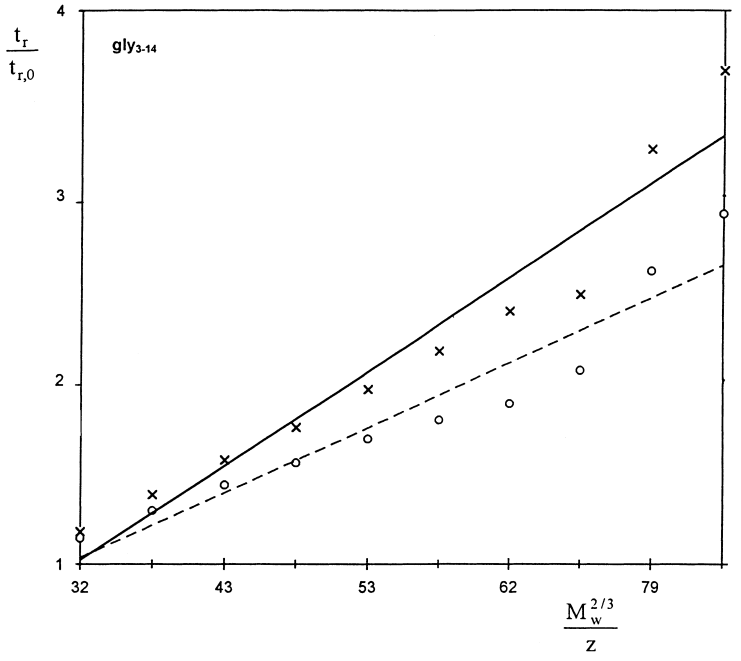

Fig. 4. Offord's plot for $(Gly)_{2–14}$ peptides in the presence (×, ————) and absence (○, – – –) of hydroxypropyl-methylcellulose in the background electrolyte. The data represent the results obtained at 50°C.

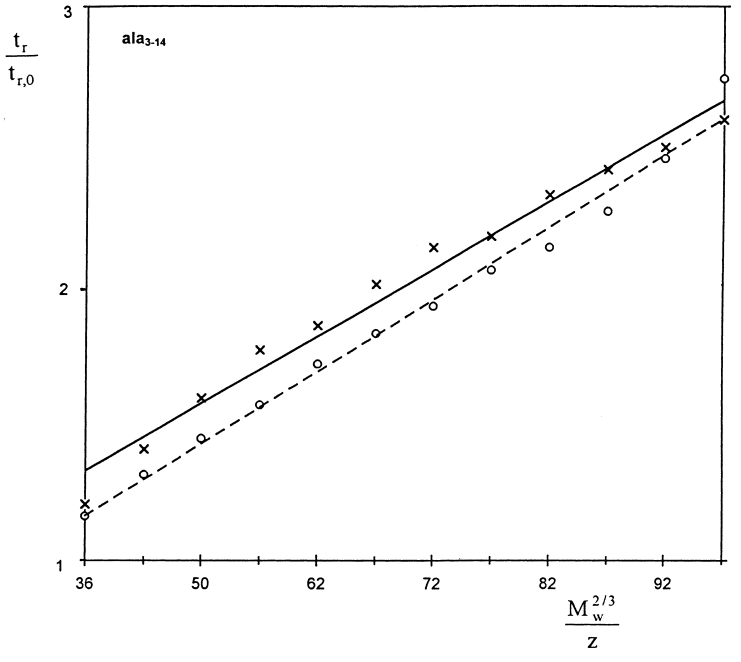

Fig. 5. Offord's plot for $(Ala)_{2–14}$ peptides in the presence (×, ————) and absence (○, – – –) of hydroxypropyl-methylcellulose in the background electrolyte. The data represent the results obtained at 50°C.

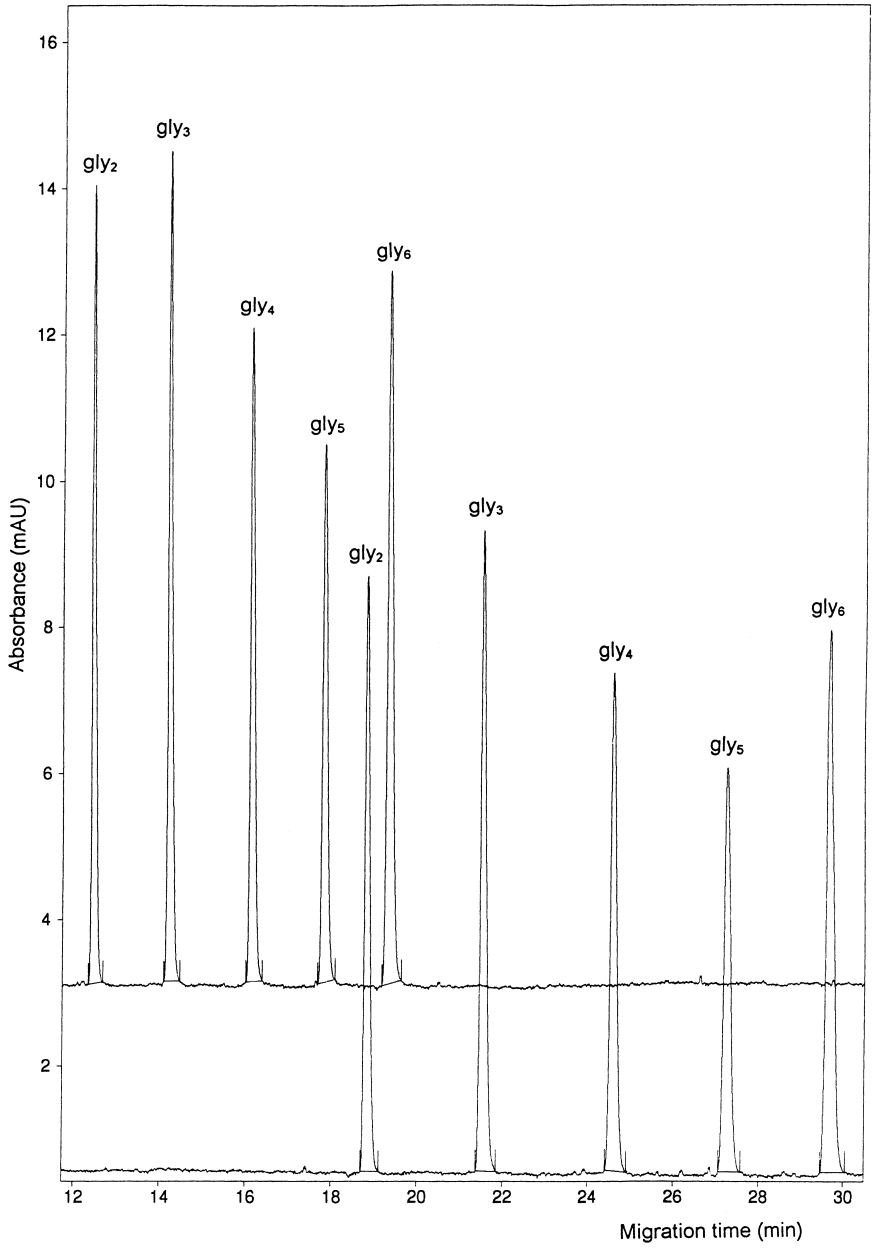

Fig. 6. Comparison of the separation of $(Gly)_{2-6}$ peptides in 25 mmol l phosphate buffer pH 2.5 at 25°C (bottom recording) and 50°C (upper recording).

polypeptide chains (13 and 14 amino acids) in favour of longer retention. The effect of temperature on this increased retention is positive (further retention increase with increasing temperature) for glycine containing peptides and negative for polyalanines. Changes in the peak area representing a single peptide bond in the solute are summarised in Table 3 and Table 4. It can be stated that the value of peptide bond absorbance is higher in the absence of hydroxypropylmethylcellulose than in its presence. No systematic

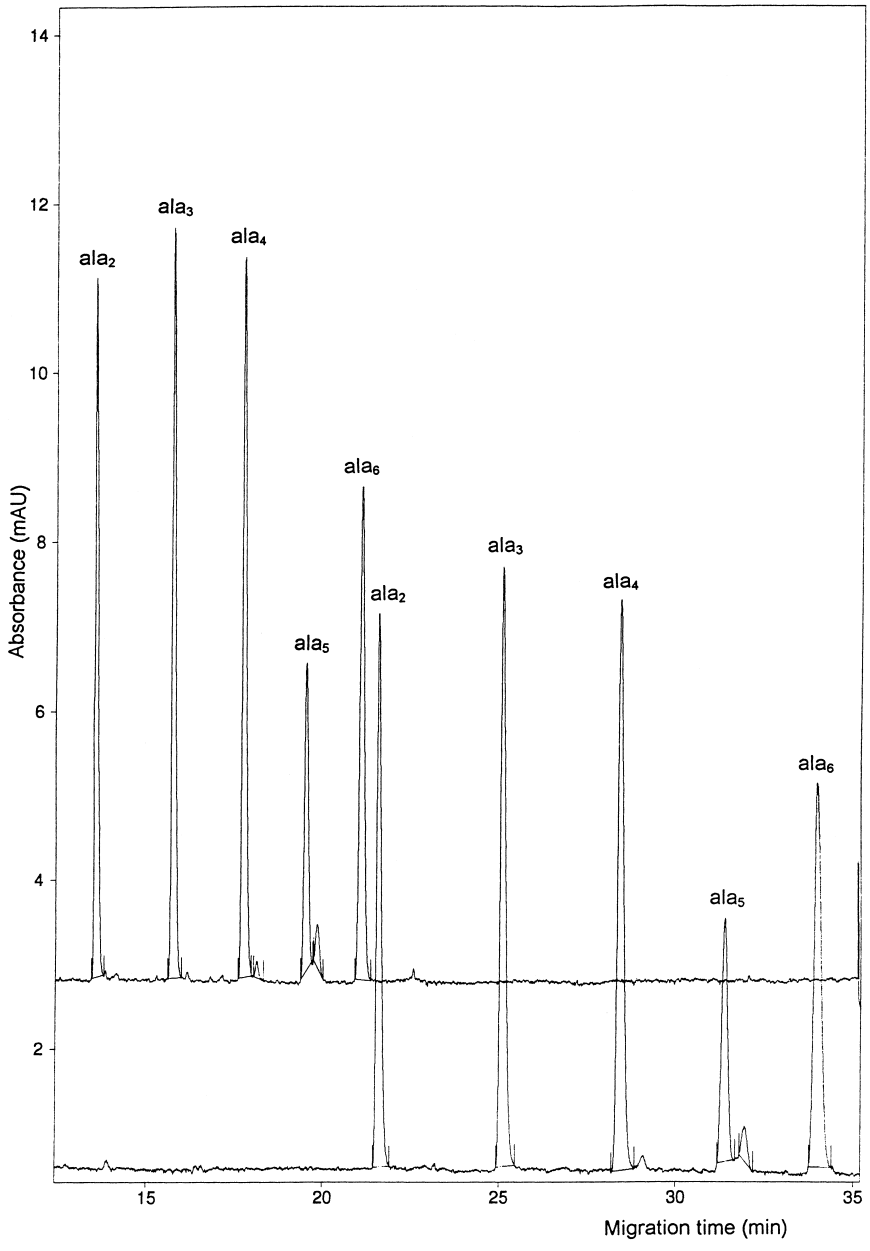

Fig. 7. Comparison of the separation of $(Ala)_{2–6}$ peptides in 25 mmol l phosphate buffer pH 2.5 at 25°C (bottom recording) and 50°C (upper recording).

difference was revealed when comparing poly(Gly) and poly(Ala) peptides.

It has to be emphasised that under the experimental conditions the endoosmotic flow was very slow (migration time of the thiourea or benzylalcohol exceeded 180 min), and, in accordance with what would have been expected, the migration of individual peptides is predominantly influenced by their charge.

Regarding peptide bond absorbance with the increasing polypeptide chain length, a decrease

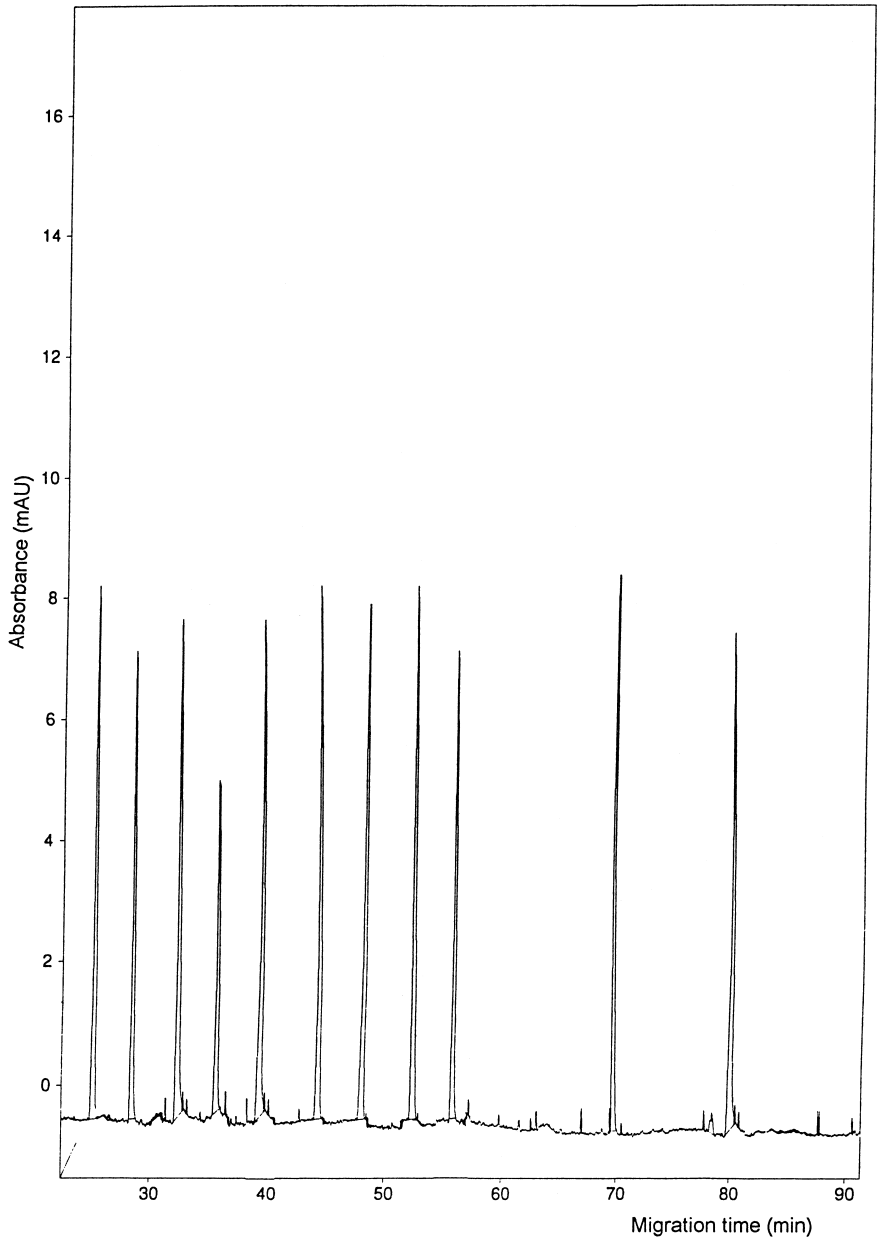

Fig. 8. Typical separation of $(Gly)_{1-14}$ peptides in 25 mmol l phosphate buffer, pH 2.5, in the presence of 0.35% hydroxypropylmethylcellulose. Peak identification from the left to the right: $(Gly)_2$, $(Gly)_3$, $(Gly)_4$, $(Gly)_5$, $(Gly)_6$, $(Gly)_7$, $(Gly)_8$, $(Gly)_9$, $(Gly)_{10}$, $(Gly)_{13}$, $(Gly)_{14}$.

between di- and tetrapeptides was observed reaching a minimum value with five amino acids containing peptide; this drop was not abolishible by increasing the temperature of the separation run. Hexapeptides attained a certain level of absorbance which with glycine polypeptides remained constant up to 14 amino acids per molecule; with alanine containing peptides a drop in absorbance was observed with

Table 3
Peak areas for $(Gly)_{2-14}$ peptides after migration correction and $(Gly)_2$ peak normalization. Background electrolyte without any additive

Solute	25°C			50°C		
	Area measured	Area migration corrected	Area normalized to $(Gly)_2$ =10 000	Area measured	Area migration corrected	Area normalized to $(Gly)_2$ =10 000
$(Gly)_2$	56 137	56 137	10 000	62 139	62 139	10 000
$(Gly)_3$	66 790	47 368	8 431	72 827	51 650	8 264
$(Gly)_4$	57 018	43 962	7 825	61 966	47 776	7 644
$(Gly)_5$	51 148	35 717	6 357	55 836	38 991	6 238
$(Gly)_6$	77 170	49 690	8 841	82 809	53 321	8 531
$(Gly)_7$	83 632	50 230	8 940	89 910	54 000	8 640
$(Gly)_8$	89 432	50 470	8 983	95 457	53 870	8 619
$(Gly)_9$	93 230	49 750	8 855	100 016	53 200	8 512
$(Gly)_{10}$	98 803	49 600	8 828	106 970	53 700	8 592
$(Gly)_{13}$	126 375	50 550	8 997	134 300	53 680	8 588
$(Gly)_{14}$	136 518	50 320	8 956	146 312	53 930	8 628

Individual data represent averages from four subsequent runs.
Differences between the extreme values of peak areas are significant at the $P<0.01$ level.
Migration correction: measured peak area divided by relative migration to $(Gly)_2$; normalization to $(Gly)_2$ =10 000: corrected area migration recalculated to $(Gly)_2$ =10 000. Significance by the Student's *t*-test.

increasing chain length. This, however, is temperature dependent and can be eliminated by increasing the temperature. On the contrary, addition of hydroxypropyl-methylcellulose to the background electrolyte stabilises this effect.

4. Discussion

Though detection of peptides even in complex mixtures represents the most widely used approach in e.g. peptide mapping, in capillary electrophoresis

Table 4
Peak areas for $(Ala)_{2-14}$ peptides after migration correction and $(Ala)_2$ peak normalization. Background electrolyte without any additive.

Solute	25°C			50°C		
	Area measured	Area migration corrected	Area normalized to $(Ala)_2$=10 000	Area measured	Area migration corrected	Area normalized to $(Ala)_2$=10 000
$(Ala)_2$	59 682	59 682	10 000	50 667	50 667	10 000
$(Ala)_3$	72 333	62 356	10 413	61 058	52 636	10 369
$(Ala)_4$	76 734	58 575	9 782	64 381	49 145	9 681
$(Ala)_5$	34 010	23 455	3 916	28 100	19 513	3 844
$(Ala)_6$	64 122	41 103	6 864	52 629	33 954	6 688
$(Ala)_7$	65 139	38 092	6 361	62 791	36 720	7 233
$(Ala)_8$	66 033	36 281	6 058	70 095	38 514	7 587
$(Ala)_9$	66 045	34 220	5 714	72 806	37 920	7 470
$(Ala)_{10}$	65 930	32 638	5 450	76 085	37 115	7 311
$(Ala)_{11}$	65 870	30 924	5 164	78 692	36 945	7 278
$(Ala)_{12}$	64 936	27 632	4 614	83 807	37 083	7 305
$(Ala)_{13}$	65 086	24 747	4 132	92 144	37 610	7 410
$(Ala)_{14}$	64 926	19 153	3 198	102 791	37 515	7 390

Individual data represent averages from four subsequent runs; differences between the extreme values of peak areas are significant at the $P<0.01$ level.
Migration correction: measured peak area divided by relative migration to $(Ala)_2$; normalization to $(Ala)_2$ =10 000: corrected area migration recalculated to $(Ala)_2$ =10 000. Significance by the Student's *t*-test.

surprisingly the number of systematic studies on this subject is very limited [2]. There are obviously two reasons for this situation. First for being able to exploit the absorbancy contribution of different peptide bonds to the overall absorbance of a particular peptide would need the evaluation of a large number of combinations considering the number of sequences that can be formed from the naturally occurring 20 amino acids which makes any study on this subject very complex. The second reason is that a study of this type would need a number of sets of custom made peptides which is a tedious business to materialise. In a recent paper Becklin and Desiderio [2] have demonstrated that the contribution of a single peptide bond to the overall absorbance in a set of glycine and alanine (2–6 amino acids) peptides is constant. In their paper, the only difference observed was with the $(Ala)_6$ peptide which exhibited a hypochromic effect. This hypochromicity was tentatively ascribed to the parallel alignment of this peptide. The background electrolyte used was 20 mmol/l ammonium formate the pH of which was brought to 2.5 by trifluoroacetic acid.

At an earlier stage of our experiments we have used the same buffer; however, later we have abandoned it for two reasons. First, we obtained a rather large spread of experimental data (similar to that reported in the quoted paper [2]) and, second, we used a buffer system which gave identical results (regarding quality of separation) as the commercially available Bio Rad buffer. This buffer can be successively used for separating the most diverse protein and peptide mixtures, even for those which are otherwise very difficult to separate – like collagen CNBr peptides [18].

In contrast to the quoted results of Becklin and Desiderio [2] we have observed the decrease of absorbance per peptide bond at both glycine and alanine pentapeptides. Admittedly we have used another background electrolyte differing namely in the presence of 0.3% hydroxypropyl-methylcellulose, but similar results were obtained with a buffer devoid of this modifier. It appears feasible to conclude that the absorbance drop (the length of the polypeptide at which it occurs) depends on the background electrolyte composition.

In our experiment the contribution of a peptide bond dropped sharply at the beginning of the dependence of absorbance contribution vs. peptide length and reached a minimum for $(Gly)_5$ or $(Ala)_5$ followed by a general increase in longer peptides. With glycine polypeptides a constant value (within the experimental error) was achieved while with the alanine polypeptides the absorbance contribution per peptide bond dropped again between $(Ala)_6$ and $(Ala)_{14}$; this drop could be eliminated by temperature increase or stabilised by adding hydroxypropyl-methylcellulose to the background electrolyte. The value of a peptide bond absorbance was significantly lower for alanine peptides in comparison to the glycine containing ones.

One of the ways how to explain the drop of the peptide band absorbance with increasing length of the peptide observed both with the alanine and glycine series should probably reflect an intrinsic property of the peptides studied. It is interesting to note two facts. The limiting value which the contribution finally reaches is different for alanine and glycine polypeptides, respectively (lower for alanine peptides) and is higher in the absence of hydroxypropyl-methylcellulose than in the presence of this modifier. The fact that this difference cannot be eliminated by elevated temperature speaks against considerations about secondary structure effect or must be caused by such a structural alteration that could not be eliminated by a simple temperature increase. Also a decreased recovery owing to irreversible sticking of particularly the large peptides to the capillary wall is unlikely to explain this drop as no detectable amounts of any of the analyzed peptides could be detected in the washes after 10 repeated runs (with no-washes inbetween the individual runs, data not shown).

As documented most of the peptides follow in their behaviour Offord's theory (for more detailed discussion see Refs. [19–22]); distinct exceptions were seen with 13 and 14 membered peptides which exhibited higher $(t_r/t_{r.0})$ ratio than predicted by the theory. This means that their retention in the capillary is higher than expected and a plausible explanation is that peptides possessing more than 13 amino acids tend to stick to the bare capillary wall. However, with alanine polypeptides addition of hydroxypropyl-methylcellulose brings about additional effects which we were unable to interpret, namely an increase in retention with increasing temperature. The effect was small but well reproducible. The size of the peptide which starts to stick to the wall would probably be affected by the nature of the side chain, however, data

in this respect are not available to us so far. Our own experience revealed that e.g. peptides possessing C-terminal proline exhibit very strong interactions with the capillary wall which makes them difficult to elute and to obtain acceptable peak shapes.

In order to avoid large scatter of data we have used internal standard (Gly_2 or Ala_2). It is evident that the main source of the scatter of data is caused by sample injection by pressure. Electrokinetic injection was avoided because of the extremely slow endo-osmotic flow.

It is also noticeable that there is a difference in peptide bond absorbance contribution with different amino acid side chains. We have evaluated systematically only the difference between $(Gly)_n$ and $(Ala)_n$; comparing these two sets of peptides, the alanine containing solutes revealed a lower absorbance contribution per peptide bond as compared to polypeptides containing glycine.

The last phenomenon to be discussed is the observation that for peptides containing six and more glycine residues the peptide bond absorbance contribution attains a constant value, while with alanine polypeptides there is an increase up to the octapeptide followed by a decrease with further chain length prolongation. This effect can be eliminated (on the contrary to the pentapeptide drop) by increasing the temperature from 25°C to 50°C, or can be stabilised by adding hydroxypropyl-methylcellulose to the background electrolyte. In this case, behaviour of the polypeptides seems to reflect a structural change and/or solute-modifier (hydroxypropyl-methylcellulose) interaction.

It may be argued to what extent 50°C is adequate for disrupting the rudimental secondary structure of the peptides investigated. Application of a higher temperature was, however, precluded by the possibilities offered by the equipment used. It should be noted that a brief heating to 100°C before the electrophoretic run yielded practically the same results as with running the sample simply at 50°C (data not shown).

5. Conclusions

By investigating the UV absorbance of a set of glycine and alanine homopeptides (containing 2–14 amino acids) and using a pH 2.5 background electrolyte both containing and devoid of 0.35% hydroxypropyl-methyl cellulose we were able to demonstrate that a tendency towards decreasing peptide bond absorbance occurs with short peptides reaching a minimum for glycine and alanine pentapeptides (with some irregularities seen with tri- and tetrapeptides). With a further increase of the polypeptide chain a constant value of peptide bond absorbance contribution was observed with glycine peptides both at 50°C; at 25°C no matter whether HPMC was present or not. With alanine peptides either a sharp or stepped increase between penta and octapeptide was followed by another drop in absorbance contribution which can be eliminated by increasing the run temperature or, on the contrary, stabilised by hydroxypropyl-methylcellulose addition to the background electrolyte. The final absorbance contribution per peptide bond was less with alanine peptides as compared to those containing glycine only.

While peptides of both investigated series (Gly and Ala) followed Offord's theory up to $(Gly)_{10}$ and $(Ala)_{12}$ (the peptide $(Gly)_{12}$ was not available to us), larger peptides exhibited a considerably longer retention time than predicted by the theory. It is proposed that in these cases 13 amino acids in the peptide is the critical length which results in discernible peptide sticking to the capillary wall.

This sticking interaction did not affect peptide bond absorbance contribution measured by peak area.

The hypochromicity of the peptide bond contribution observed with glycine and alanine pentapeptides is unlikely to be explained by the secondary structure attained by these peptides only as running the separation at an elevated temperature (50°C) did not introduce any increase in absorbance contribution. On the contrary the hypochromicity observed with larger alanine containing peptides has all the features of a structural alterations involving both the solutes as such and their interaction with the background electrolyte modifier (hydroxypropyl-methylcellulose).

Acknowledgements

This work was supported by the Grant Agency of the Czech Republic (Grants No. 203/96/K128 and 305/97/S070) and Grant Agency of the Charles University (Grant No. 265/95).

References

[1] I. Messana, D.V. Rosetti, L. Cassiano, F. Misiti, B. Giardina, M. Castagnola, J. Chromatogr. B 669 (1997) 149.
[2] R.R. Becklin, D.M. Desiderio, Anal. Lett. 28 (1995) 2175.
[3] H.G. Lee, D.M. Desiderio, J. Chromatogr. B 655 (1994) 9.
[4] I. Mikšík, J. Novotná, M. Uhrová, D. Jelínková, Z. Deyl, J. Chromatogr. A 772 (1997) 213.
[5] W.T. Simpson, D.L. Peterson, J. Chem. Phys. 26 (1957) 588.
[6] K. Imakori, J. Tanaka, J. Mol. Biol. 1 (1959) 359.
[7] K. Rosenheck, P. Doty, Biochemistry 47 (1961) 286.
[8] N.W. Draper, M.A. Rizack, R.B. Merryfield, Biochemistry 14 (1975) 2933.
[9] L. Juliano, A.C.M. Paiva, Biochemistry 13 (1974) 2445.
[10] H. Kawatani, H. Yajima, Chem. Pharm. Bull. (Tokyo) 22 (1974) 1872.
[11] P. Sieber, B. Kamber, K. Eisler, A. Hartmann, B. Riniker, W. Ritter, Helv. Chim. Acta 59 (1976) 1489.
[12] E.C. Jorgensen, I.C. Király Olah, T.C. Lee, G.C. Windridge, J. Med. Chem. 17 (1974) 323.
[13] O. Nishimura, C. Hatanaka, M. Fujino, Chem. Pharm. Bull. (Tokyo) 23 (1975) 1212.
[14] G. Jäger, R. Geiger, Justus Liebigs. Ann. Chem. (1973) 1928.
[15] Y.S. Klausner, M. Bodanszky, Bioorg. Chem. 2 (1974) 354.
[16] N. Fuji, H. Yajima, Chem. Pharm. Bull. (Tokyo) 23 (1975) 1596.
[17] P.A. D'Agostino, J.R. Hancock, R.L. Provost, J. Chromatogr. A 767 (1997) 77.
[18] Z. Deyl, J. Novotná, I. Mikšík, D. Jelínková, M. Uhrová, M. Suchánek, J. Chromatogr. B 689 (1997) 181.
[19] R.E. Offord, Nature 211 (1966) 591.
[20] Z. Deyl, V. Rohlicek, R. Struzinsky, J. Liq. Chromatogr. 12 (1989) 2515.
[21] E.C. Rickard, M.M. Strohl, R.G. Nielsen, Anal. Biochem. 197 (1991) 197.
[22] M. Castagnola, I. Messana, D.V. Rossetti, in: P.G. Righetti (Ed.), Capillary Electrophoresis in Analytical Biotechnology, CRC Press, Boca Raton, Ch. 8, 1996, pp. 239.