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Chromatographic characterization of HSV-1 gD 268–284 and IL-6 179–185 synthetic oligopeptides by reversed-phase high-performance liquid chromatography, automated Edman degradation and mass spectrometric analysis

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Abstract

Two groups of synthetic oligopeptides ($n_{\text{amino acid}} = 7$ and 17) were prepared by solid-phase peptide synthesis using the Boc–polystyrene strategy. After deprotection, cleavage and gel permeation, the crude products were analysed by conventional RP-HPLC methods. Separation and isolation of major components were performed on a semi-preparative RP-HPLC column. In order to clarify the primary structure of these products, amino acid analysis, Edman degradation sequence determination and analytical RP-HPLC characterization were applied. The isolated fractions were further assessed by direct molar mass investigation utilizing the fast atom bombardment and ^{252}Cf plasma desorption mass spectrometry. The results with an interleukin-6 oligopeptide corresponding to the ^{179}LRALRQM^{185} sequence indicate that the single peak product obtained by RP-HPLC separation contains only one component, as verified by amino acid analysis and mass spectrometry. In contrast, the analysis of ^{268}LAPEDPEDSALLEDPVG^{284}-NH_2 from HSV-1 gD protein suggests that this large peptide amide showing a single peak after repeated purification by RP-HPLC contains microheterogeneities as revealed by mass spectrometry and sequencing, but not by amino acid analysis.

1. Introduction

During development, activation and functioning of the immune system, the cytokines play a prominent part in the realization of the cell to cell communication. Interleukin-6 (IL-6) as a multifunctional cytokine is produced by different cells (*e.g.*, monocytes, macrophages, fibroblasts, B- and T-lymphocytes) after appropriate stimulation. Further, IL-6 is the major regulator of the acute phase protein synthesis in human hepatocytes and in haematopoiesis. Several secondary structure prediction analyses of IL-6 and epitope mapping studies with IL-6 specific monoclonal antibodies and deletion mutants indicated that the C-terminal region (178–185 amino acids) is essential for biological activity [1–4]. Considering this observation, a number of C-terminal peptides including ^{179}LRALRQM^{185} were synthesized in our laboratory.

Herpes simplex virus (HSV), with two closely

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related serotypes, HSV-1 and HSV-2, is one of the most frequent infectious agents in humans. Glycoprotein D (gD) represents a major immunogenic glycoprotein component of the human virion envelope [5]. It is, therefore, a logical target for the construction of subunit vaccines against HSV infection. It has been shown that peptides from the N-terminal region of HSV-1 gD are able to induce both B- and T-cell responses [6–8]. Another epitope region has been predicted in our laboratory [9,10] and partially characterized by HSV gD specific monoclonal antibodies [11,12]. For inhibition studies and structural investigations, a peptide amide, ^{268}LAPEDPEDSALLEDPVG^{284}-NH_2, covering this sequence has been synthesized.

This study was aimed at verifying the primary structure of the ^{179}LRALRQM^{185} peptide and ^{268}LAPEDPEDSALLEDPVG^{284}-NH_2 peptide amide produced by solid-phase synthesis (SPS) and to compare the efficacy of routinely used (amino acid analysis, RP-HPLC) and newly introduced (mass spectrometry, automatic Edman degradation) analytical methods. These techniques and their combinations have been applied to the characterization of peptide mixtures from natural tissues [13,14] and for homogeneity studies of synthetic peptides derived from SPS strategy [13,15,16]. Both types of studies indicated that preparations showing a single RP-HPLC peak are not necessarily pure, single substances. In reported cases the chromatographic separation alone was found to be inadequate [13,15]. Our results suggest that with large peptides reliable results could be achieved only by a combination of analytical techniques including HPLC, sequencing and mass spectrometry.

2. Experimental

2.1. Materials

Abbreviations for amino acids follow the revised recommendations of the IUPAC–IUB Committee on Biochemical Nomenclature, entitled *Nomenclature and Symbolism for Amino Acids and Peptides* (recommendations of 1983). The IL-6 fragment ^{179}LRALRQM^{185} (H-^{179}Leu–Arg–Ala–Leu–Arg–Gln–Met^{185}–OH) and the HSV-1 gD fragment amide ^{268}LAPEDPEDSALLEDPVG^{284}–NH_2 (H–Leu–Ala–Pro–Glu–Asp–Pro–Glu–Asp–Ser–Ala–Leu–Leu–Glu–Asp–Pro–Val–Gly–NH_2) were synthesized manually by a solid-phase technique on Merrifield resin (Bachem, Bubendorf, Switzerland) with the Boc–polystyrene strategy in our laboratory. The solvents acetonitrile, methanol, acetic acid and trifluoroacetic acid (TFA) were of HPLC grade and others were of analytical-reagent grade from Chemolab (Budapest, Hungary). Chemicals were purchased from Reanal (Budapest, Hungary).

2.2. Gel permeation

After deprotection and cleavage from the resin, the crude products were first purified on a Sephadex G-25M (Pharmacia, Uppsala, Sweden) column using acetic acid–water (50:50) as eluent. A Bio-Rad, (Richmond, CA, USA) Econo instrument was applied and the peaks were detected by ninhydrin spot assay on Whatman (Maidstone, UK) paper.

2.3. High-performance liquid chromatography

After gel permeation, ^{179}LRALRQM^{185} was further purified by RP-HPLC. Analytical RP-HPLC was performed on a laboratory-assembled Knauer, (Bad Homburg, Germany) HPLC system using a Delta-Pak C_{18} column (300 × 3.9 mm I.D.) with 15-μm silica (300 Å pore size) (Milligen, Milford, MA, USA) as the stationary phase and linear gradient elution with eluent A = 0.1% TFA in water and eluent B = 0.1% TFA in acetonitrile–water (80:20, v/v) as the mobile phase at a flow-rate of 1 ml/min at ambient temperature and detection at 220 nm. A 20-μl volume of a solution of crude and purified peptide or peptide amide (1 mg/ml in eluent A) was injected.

Separation of major components was performed on a Delta-Pak C_{18} semi-preparative column (300 × 19 mm I.D.) with 15-μm silica

(300 Å pore size) (Milligen) as the stationary phase and linear gradient elution with eluent A = 0.1% TFA in water and eluent B = 0.1% TFA in acetonitrile–water (80:20, v/v) as the mobile phase and detection at 220 nm. The gradients were developed from 15 to 45% eluent B in 45 min for ^{179}LRALRQM^{185} and from 25 to 40% eluent B in 30 min for ^{268}LAPEDPED-SALLEDPVG^{284}–NH_2 with a flow-rate of 3 ml/min and the peptides were applied at a 40 mg/ml concentration. After collection of all the peaks, the relevant fractions were freeze-dried.

2.4. Amino acid analysis

The primary sequence of products from the peaks separated by RP-HPLC were investigated by amino acid analysis using a Beckman (Fullerton, CA, USA) Model 6300 amino acid analyser. Prior to analysis samples were hydrolysed in 6 *M* HCl in sealed and evacuated tubes at 110°C for 24 h.

2.5. Edman degradation of HSV-1 gD fragment

The sequence analysis of purified peptide samples was carried out on a Knauer (Bad Homburg, Germany) Model 910 sequencer equipped with an on-line HPLC gradient system to separate and determine the phenylthiohydantoin (PTH) amino acids at 269 nm. The peptide samples (about 300 pmol each) were applied on a poly(vinylidene difluoride) (PVDF) membrane. For HPLC, buffer A was 5% tetrahydrofuran–0.4% 3 *M* sodium acetate solution (pH 3.8)–0.025% 3 *M* sodium acetate solution (pH 4.6)–0.02% glacial acetic acid in water and buffer B was acetonitrile. The flow-rate was 280 μl/min and the gradient was developed from 15 to 45% B in 23 min.

2.6. Mass spectrometry

Fast atom bombardment mass spectrometry (FAB-MS)

FAB mass spectra were obtained on a VG-ZA-2SEQ tandem mass spectrometer (Fisons, Loughborough, UK) equipped with a caesium ion gun (25 keV). For analysis, the peptide samples were dissolved in a 0.05 *M* NH_4HCO_3 buffer containing 1% of trifluoroacetic acid (TFA) and mixed with glycerol matrix.

Plasma desorption mass spectrometry (PD-MS)

PD-MS measurements were carried out using a Bioion 20 mass spectrometer (Applied Biosystems, Warrington, UK). A ^{252}Cf source was applied to ionize the molecules. The molar masses of the peptides were calculated by the time-of-flight technique. Thin layer chromatography was carried out on nitrocellulose (Mylar foil) which was wetted with 5 μl of a solution of

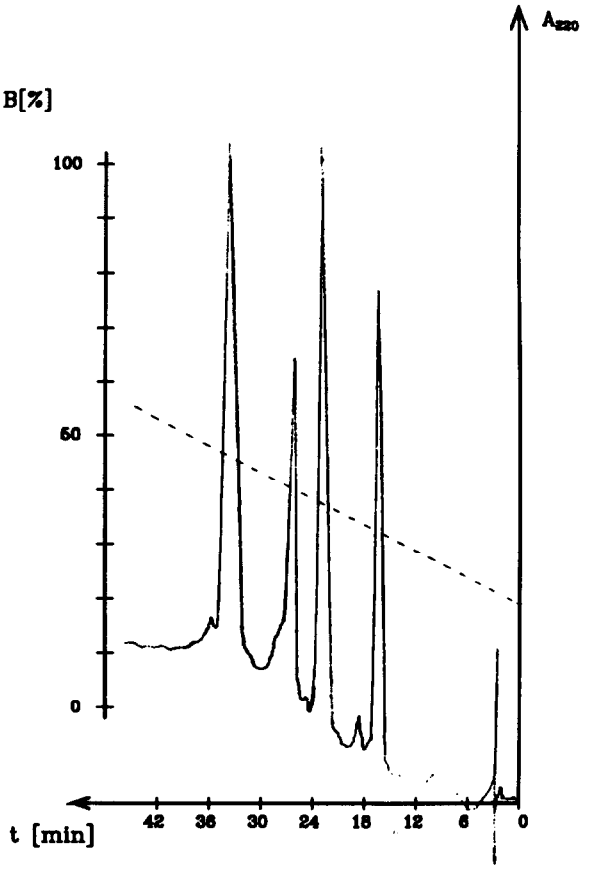

Fig. 1. Analytical RP-HPLC profile of the crude LRALRQM. Elution was performed at a flow-rate of 1 ml/min using a linear gradient from 15 to 55% eluent B in 45 min [eluent A = 0.1% TFA in water, eluent B = 0.1% TFA in acetonitrile–water (80:20, v/v)]. Detection at 220 nm. A 20-μl volume of a solution of crude peptide (2 mg/ml in eluent A) was injected.

1% TFA in methanol–water (50:50). The sample solution was applied to the wetted layer.

3. Results and discussion

3.1. Purification of the peptide and peptide amide

After gel permeation, the deprotected and cleaved material was analysed by analytical RP-HPLC. The chromatogram of the crude ^{179}LRALRQM^{185} preparation is shown in Fig. 1. Four major, easily separable peaks were detected by UV measurement at 220 nm after gradient elution. Fractions corresponding to these four peaks were collected, combined and isolated by freeze-drying after semi-preparative-scale separation. The first two peaks [LRALRQM/a, $R_F = 16$ min (Fig. 2a), and LRALRQM/b, $R_F = 22$ min] corresponded to

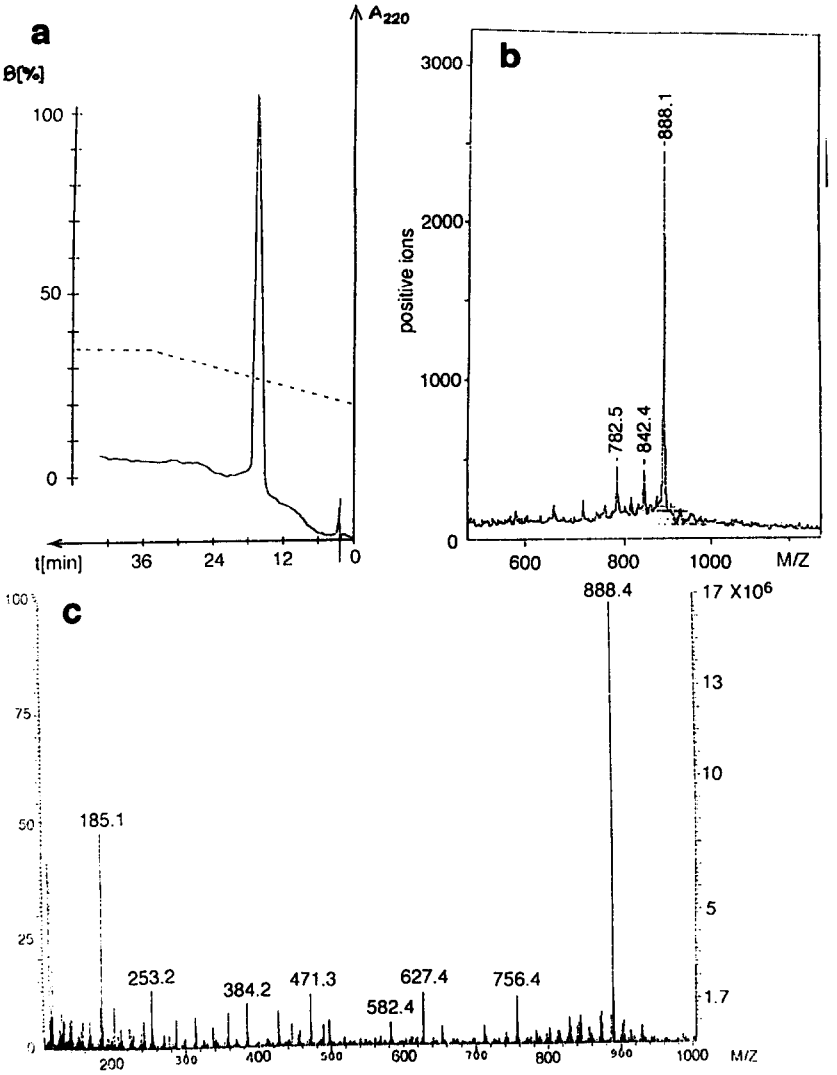

Fig. 2. (a) Analytical RP-HPLC profile and (b) ^{252}Cf PD and (c) monoisotopic FAB mass spectra of the purified LRALRQM/a peak of crude LRALRQM. RP-HPLC elution was performed at a flow-rate of 1 ml/min using a linear gradient from 15 to 35% eluent B in 30 min (eluents as in Fig. 1). Detection at 220 nm. A 20-μl volume of a solution of the purified first peak of the crude preparation (1 mg/ml in eluent A) was injected.

the required peptide sequence while the third and fourth peaks showed compounds with aberrant sequences. All four components were characterized by amino acid analysis and to check their purity were re-chromatographed by analytical RP-HPLC.

For the crude peptide amide ^{268}LAPEDPEDSALLEDPVG^{284}–NH_2 six different peaks were observed in RP–HPLC after gel permeation. The chromatogram of the crude peptide amide is shown in Fig. 3. After semi-preparative chromatography on the same packing material all peak-related material was characterized by analytical RP-HPLC, amino acid analysis and Edman degradation.

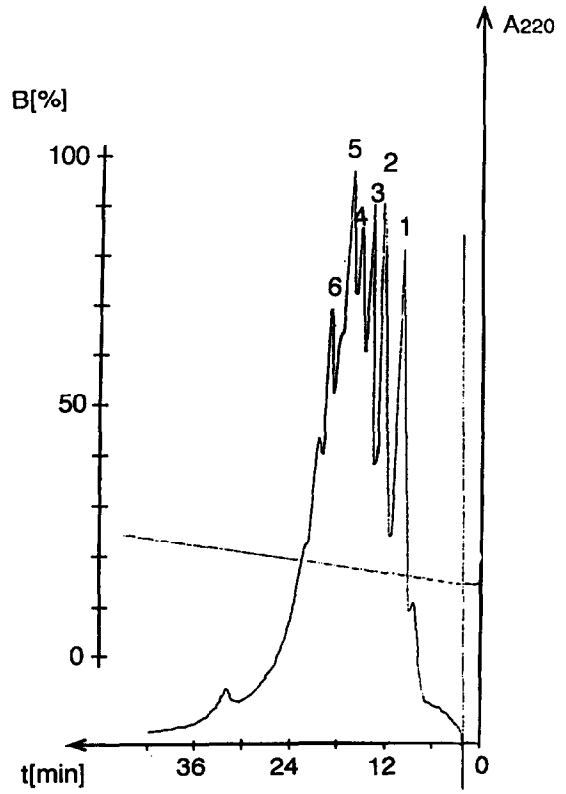

Fig. 3. Analytical RP-HPLC profile of crude LAPEDPEDSALLEDPVG–NH_2 peptide amide. RP-HPLC elution was performed at a flow-rate of 1 ml/ml using a linear gradient from 25 to 40% eluent B in 30 min (eluents as in Fig. 1). Detection at 220 nm. A 20-μl volume of a solution of crude peptide amide (1 mg/ml in eluent A) was injected.

3.2. Identification of the peptide and peptide amide

The amino acid composition of the isolated LRALRQM/a (R_F = 16 min, first peak in Fig. 1) and LRALRQM/b peptide (R_F = 22 min, second peak in Fig. 1) (Table 1) was essentially the same, but the R_F value of the LRALRQM/b peptide was much higher than that of the LRALRQM/a peptide (Fig. 2a). In order to understand this result, these two compounds were analysed by ^{252}Cf PD-MS and FAB-MS.

The relative molar mass (887.1) of the LRALRQM/a peptide (Fig. 2b and c) matches the calculated value (886.34) for ^{179}LRALRQM^{185}. In contrast, the mass spectrum of the LRALRQM/b peptide indicates the presence of an additional oxygen atom at the Met residue. This difference between the LRALRQM/a and LRALRQM/b peptide peaks was confirmed by both MS methods (Fig. 2b and c).

Results of the amino acid analysis of the peptide amide ^{268}LAPEDPEDSALLEDPVG^{284}–NH_2 peaks are given in Table 2. The analytical RP-HPLC profile of the first separated product (HSV/a) (Fig. 4a) shows only one peak but both PD-MS and FAB-MS show three components (Fig. 4b and c). The main peak (HSV/a) corresponds to the expected sequence of the 17-mer amide (calculated molar mass was 1765.93, observed mass 1766.2). The second and third components match the calculated values for the truncated derivatives of native or dehydrated peptide amides beginning either with ^{270}Pro or

Table 1
Amino acid analysis of ^{179}LRALRQM^{185} peptide

Amino acid	Calculated	Found[a]
Leu (L)	2	2.00
Arg (R)	2	1.93
Ala (A)	1	0.97
Gln (Q)	1	1.03[b]
Met (M)	1	0.87

[a] Acid hydrolysis (6 *M* HCl, 110°C, 24 h). The amino acid analysis was carried out on a Beckman Model 6300 analyser.

[b] The Q residue after acid hydrolysis was measured as E.

Table 2
Amino acid analysis of the ^{268}LAPEDPEDSALLEDPVG^{284}–NH_2 peptide amide fractions

Amino acid	Calculated	Found[a]					
		HSV/a	HSV/b	HSV/c	HSV/d	HSV/e	HSV/f
Leu (L)	3	3.03	2.98	2.98	3.02	2.94	2.85
Ala (A)	2	1.96	1.99	1.98	2.00	2.00	1.46
Pro (P)	3	2.88	2.89	2.93	2.92	2.89	2.89
Glu (E)	3	3.22	3.19	3.20	3.19	3.17	3.01
Asp (D)	3	2.98	2.98	2.92	2.92	2.91	3.41
Ser (S)	1	1.03	1.01	0.98	0.97	1.02	1.31
Val (V)	1	0.94	0.94	0.97	0.97	0.97	0.89
Gly (G)	1	1.04	1.02	1.02	1.02	1.10	1.18

[a] Acid hydrolysis (6 *M* HCl, 110°C, 24 h). The amino acid analysis was carried out on a Beckman Model 6300 analyser.

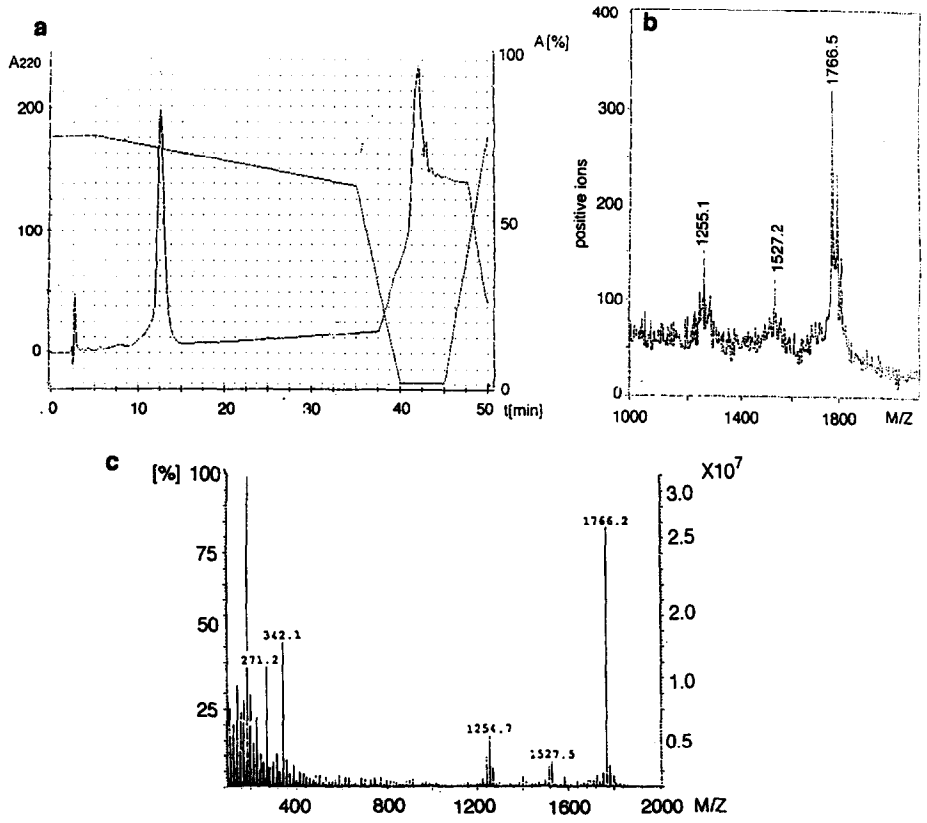

Fig. 4. (a) Analytical RP-HPLC profile and (b) ^{252}Cf PD and (c) monoisotopic FAB mass spectra of the purified HSV/a (first) peak of the crude LAPEDPEDSALLEDPVG–NH_2 peptide amide. Elution was performed at a flow-rate of 1 ml/min using a linear gradient from 25 to 40% eluent B in 30 min (eluents as in Fig. 1). Detection at 220 nm. A 20-μl volume of a solution of the purified first peak of the crude preparation (1 mg/ml in eluent A) was injected.

with ^{273}Pro [observed mass for 270–284–NH_2 1582.5 and for 273–284–NH_2 1240.2 and their derivatives, dehydrated (three times dehydrated 1527.2 and otherwise modified species)] (Fig. 4b and c).

Results from MS analysis correspond to the data obtained by the Edman degradation, which indicated the presence of all three sequences (each *ca.* 10% of the total amount) (data not shown).

The other five separated peaks were also characterized by analytical RP-HPLC, PD-MS, FAB-MS and Edman degradation. These fractions proved to be heterogenic even by analytical RP-HPLC and in most instances more than one peak was detected (data not shown).

All these results indicate that in certain instances it is necessary to investigate even the RP-HPLC-purified product by different MS methods (PD-MS, FAB-MS) and possibly by Edman degradation because sometimes the perfect-looking results of the analytical RP-HPLC and amino acid analyses do not guarantee that the synthetic peptide or peptide derivative is entirely pure.

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5. References

[1] J.F. Bazan, *Proc. Natl. Acad. Sci. U.S.A.*, 87 (1990) 6934.
[2] J.F. Bazan, *Immunol. Today*, 11 (1990) 350.
[3] S.C. Bischof, *Lymphokine Cytokines Res.*, 11 (1992) 33.
[4] J.P.J. Brackenhoff, *J. Immunol.*, 145 (1990) 561.
[5] M.J. Hall and K. Katrak, *Vaccine*, 4 (1986) 138.
[6] B. Dietzschold, R.J. Eisenberg, M. Ponce de Leon, E. Gulob and F. Hudecz, *J. Virol.*, 52 (1984) 431.
[7] E. Heber-Katz, M. Hollósi, B. Dietzschold, F. Hudecz and G.D. Fasman, *J. Immunol.*, 135 (1985) 1385.
[8] W.J. Weijer, J.W. Drijfhout, H.J. Geerlings, W. Bloemhoff, M. Feijbrief, A.B. Cees, P. Hoogerhout, K.E.T. Keerling, T. Popken-Boer, K. Slopsema, J.B. Wilterdrink, G.W. Welling and S. Welling-Wester, *J. Virol.*, 62 (1988) 501.
[9] G. Mezö, M. Szekerke, I. Kurucz and F. Hudecz, in E. Bayer and G. Jung (Editors), *Peptides 1988*, Walter de Gruyter, Berlin, 1989, p. 701.
[10] F. Hudecz, A. Hilbert, G. Mezö, I. Mucsi, J. Kajtár, Sz. Bösze, I. Kurucz and É. Rajnavölgyi, *Pept. Res.*, 6 (1993) 263.
[11] R.J. Eisenberg, D. Long, M. Ponce de Leon, J.T. Matthews, P.G. Spear, M.G. Gibson, L.A. Lasky, P. Berman, E. Gulob and G.H. Cohen, *J. Virol.*, 53 (1985) 634.
[12] V.J. Isola, R.J. Eisenberg, G.H. Siebert, C.J. Heilman, W.C. Wilcox and G.H. Cohen, *J. Virol.*, 63 (1989) 2325.
[13] G. Grübler, E. Yildiz, H. Zimmermann, M. Mihelic, H. Echner, S. Stoeva, S. Spyropoulos and W. Voelter, in N. Yanaihara (Editor), *Peptide Chemistry*, ESCOM, Leiden, 1993, p. 208.
[14] E. Wünsch, in W. Voelter and G. Weitzel (Editors), *Structure and Activity of Natural Peptides*, Walter de Gruyter, Berlin, 1981, p. 3.
[15] E. Bayer, W. Rapp and L. Zhang, in G. Jung and E. Bayer (Editors), *Peptides 1988*, Walter de Gruyter, Berlin, 1989, p. 390.
[16] G. Jung, A.G. Beck-Sickinger, N. Zimmermann, J. Metzger, R. Spohn, S. Stevanovic, K. Deres and K.-H. Weismuller, in R. Epton (Editor), *Innovation and Perspectives in Solid Phase Synthesis*, Intercept, Andover, 1992, p. 227.