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High-yield, Solid-phase Synthesis of Humanin, an Alzheimer's Disease Associated, Novel 24-mer Peptide which Contains a Difficult Sequence

ALEXANDRA EVANGELOU, CHRISTOS ZIKOS, EVANGELIA LIVANIOU* and GREGORY P. EVANGELATOS

Immunopeptide Chemistry Laboratory, Institute of Radioisotopes-Radiodiagnostic Products, NCSR 'Demokritos', Athens 153 10, Greece

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Abstract: Humanin is a novel, 24-mer residue bioactive peptide, which antagonizes Alzheimer's disease (AD) related neurotoxicity and offers a hope for developing new therapeutics against AD. Access to adequate amounts of pure humanin is a prerequisite for further, thorough, investigation of the pharmacological properties and therapeutic potency of the peptide. Until now, humanin has been obtained mainly by molecular biology techniques. In this work the Fmoc solid-phase synthesis of humanin on an in-house prepared 2-Cl-tritylamidomethyl polystyrene resin is described fully. Special precautions, i.e. prolonged deprotection steps, should be taken to achieve a high overall yield, since humanin seems to contain a 'difficult sequence' ($R^4G^5F^6S^7C^8L^9$) near its highly lipophilic, biologically important region $L^9L^{10}L^{11}L^{12}$.

Keywords: Alzheimer's disease; difficult sequence; Fmoc-deprotection; humanin; solid-phase peptide synthesis; 2-Cl-tritylamidomethyl polystyrene resin

INTRODUCTION

Humanin (HN) has been identified recently as a novel bioactive peptide consisting of 24 amino acid residues (MAPRGFSCLLLLTSEIDLPVKRRA) which antagonizes Alzheimer's disease (AD) related neurotoxicity in primary neurons and neuronal cell lines [1–3]. HN has been identified as the product of a cDNA, which suppresses neuronal cell death induced by various familial Alzheimer's disease genes, by neurotoxic Aβ peptides and by an anti-amyloid precursor protein antibody. As also reported, HN can suppress neuronal cell death induced by serum deprivation [4], while it can rescue human cerebrovascular smooth muscle cells from Aβ-induced toxicity [5]. The anti-apoptotic function of HN may result from HN interfering with Bax activation [6]. By using a specific antibody against HN, HN-like immunoreactivity has been detected in the testis and the colon of 3-week-old normal mice, but only in the testis of 12-week-old animals. HN-like immunoreactivity has also been unravelled in the neurons of the occipital lobe in the AD brain, but not in the age-matched control brain [7]. HN

Abbreviations: Aβ, β-amyloid peptide; Ac, acetyl; AD, Alzheimer's disease; Bax, B-cell leukaemia 2 associated X protein; Boc, *tert*-butoxycarbonyl; Bu^t, *tert*-butyl; cDNA, complementary deoxyribonucleic acid; DCM, dichloromethane; DIC, *N*, *N*′-diisopropylcarbodiimide; DIEA, *N*, *N*′-diisopropylethylamine; DMF, *N*,*N*-dimethylformamide; ESI-MS, electron-spray ionization mass spectrometry; Et_2O, diethyl ether; Fmoc, 9-fluorenylmethoxycarbonyl; HN, humanin; HOBt, 1-hydroxy-1,2,3-benzotriazole; ID, internal diameter; Pbf, 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl; PDA, photodiode array; RP-HPLC, reverse-phase high-performance liquid chromatography; SPPS, solid-phase peptide synthesis; TFA, trifluoroacetic acid; THF, tetrahydrofuran; TRIM, tripartite motif; Trt, trityl or triphenylmethyl; UV, ultraviolet.

*Correspondence to: Evangelia Livaniou, Immunopeptide Chemistry Laboratory, Institute of Radioisotopes-Radiodiagnostic Products, NCSR 'Demokritos', Aghia Paraskevi Attikis, Athens 15310, Greece; e-mail: livanlts@rrp.demokritos.gr
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is believed to be secreted from the cells through the endoplasmic reticulum–Golgi secretory pathway and to act from the outside of the cells presumably through a cell-surface receptor linked to tyrosine kinases [3]. The lipophilic region $L^9L^{10}L^{11}$ seems to be essential for HN secretion [8]. As reported in reference [9], the intracellular HN level is regulated by degradation through the TRIM11-linked proteasomal pathway. When administered *in vivo* [10], HN analogues are known to ameliorate the learning and memory impairment induced in mice treated with scopolamine. As can be concluded, humanin is a promising target for the development of therapeutics against AD. Clearly, having access to adequate amounts of the peptide is a prerequisite for carrying out relevant pharmacological studies. Until now, humanin as well as humanin analogues have been mainly prepared by molecular biology techniques [8]; on the other hand, the use of synthetic humanin has been mentioned [2,11], but the synthetic procedure has not been fully described, commented upon or discussed.

MATERIALS AND METHODS

Materials

The Fmoc-protected amino acids were purchased from Chemical and Biopharmaceutical Laboratories (Patras, Greece). DMF, DCM, THF, TFA and CH_3CN (analytical grade) were obtained from Merck or Sigma. The 2-Cl-tritylamidomethyl polystyrene resin was prepared in-house, as previously described [12,13].

General Analytical Methods

Analytical RP-HPLC was performed on a Waters HPLC System (pump 616E, detector 996 PDA). A LiChrospher RP C18 column (250 × 4.6 mm ID; 5 μm particle size, Merck) was used. The solvent system consisted of 0.05% TFA in 0.1 M NaCl (solvent A) and 0.05% TFA in CH_3CN (solvent B). Elution was achieved by applying a linear gradient from 80% A to 40% A in 25 min. The flow rate was 1.0 ml/min. Peptide peaks were detected spectrophotometrically (220 nm).

Semi-preparative RP-HPLC was performed on a Waters HPLC System (pump 600E, detector UV-484). A 10 Nucleosil 7 C18 column (250 × 12.7 mm ID; Macherey Nagel) was used. The solvent system consisted of 0.05% TFA in water (solvent A) and 0.05% TFA in CH_3CN (solvent B). Elution was achieved by applying a linear gradient from 100% A to 40% A in 52 min. The flow rate was 4.5 ml/min. Peptide peaks were detected spectrophotometrically (220 nm).

Amino acid analysis was performed using the Waters PICO-TAG amino acid analysis system, as described previously [13].

ESI-MS analysis was performed using an electrospray interface mass spectrometer (Finnigan AQA Thermoquest). Briefly, the peptide solution (2×10^{-5} M) in 50% aqueous methanol (containing 2% acetic acid) was infused into the spectrometer operating in positive mode, at a flow rate of 10 μl/min. The sample was transferred from AQA reservoir to ESI probe via a fused silica capillary by pressurizing the reservoir. The final mass spectrum was acquired across mass range 500–1400 m/z and was the result of the sum of 20 scans. The probe heater temperature was set at 140 °C and probe and cone voltages were held at 3500 and 35 V, respectively. Nitrogen gas obtained by a nitrogen generator (Nitrox, Dominick-Hunter UHPLCMS 12) was used for desolvation. The charge of each ion and molecular mass of the peptide were determined by deconvolution algorithms.

RESULTS

Synthesis of Humanin

Humanin was synthesized manually on an in-house prepared trityl-type resin, namely 2-Cl-tritylamidomethyl polystyrene resin [12], following the Fmoc strategy. The following side chain-protected amino acids were used: Fmoc-Arg(Pbf)-OH, Fmoc-Ser(Bu^t)-OH, Fmoc-Cys(Trt)-OH, Fmoc-Glu(OBu^t)-OH, Fmoc-Asp(OBu^t)OH, Fmoc-Lys(N^ε-Boc)-OH. Couplings were performed by dissolving an excess (4 mol equiv.) of Fmoc-protected amino acid and HOBt in DMF. The solution was cooled on ice and then DIC (4 mol equiv.) was added. The reaction mixture was left on ice for 10 min and then at 25 °C for another 10 min (Fmoc-Gly-OH was added to the resin immediately after remaining for 10 min on ice). Afterwards, the reaction mixture was added to the resin and allowed to react for 3 h. Coupling efficiency was monitored using the Kaiser ninhydrin test [14]. In the case of an intense, positive result, the coupling step was repeated (double coupling). Double coupling was necessary at positions L^{18}, L^9, C^8, F^6, G^5 and A^2. After *each* coupling (or double

coupling), the remaining free amino groups were acetylated (capping) using a mixture of Ac_2O/DIEA (2/1, v/v, 30 min). Removal of the *N*-terminal Fmoc group (deprotection) was performed using a 20% (v/v) solution of piperidine in DMF. The Fmoc-deprotection step was carefully monitored by UV absorption spectroscopy at 301 nm [15] and, when necessary, suitably elongated until a baseline optical absorbance was obtained. After peptide synthesis had been completed, the resin was extensively washed (DMF, DCM and Et_2O,) and then dried *in vacuo* (18 h). The peptide synthesized was cleaved from the resin using a mixture of TFA, thioanisole, ethanedithiol, phenol, H_2O and triisopropylsilane (81.5/5/2.5/5/5/1, v/v/v/v/v/v) for 3 h.

DISCUSSION

Pure HN was obtained using semi-preparative RP-HPLC and subsequently characterized with analytical RP-HPLC, amino acid analysis and ESI-MS. Under the above described conditions, the analytical RP-HPLC chromatogram of the crude product showed a clear main peak (Figure 1, I), while the yield obtained in pure peptide (Figure 2, I) was very high, exceeding 45%. Amino acid analysis data are shown in Table 1. The ESI-MS spectrum of pure HN is presented in Figure 3 (expected molecular mass: 2686.3; observed molecular mass: 2687.5; the main ions were the +4 and +3 charged states at *m/z* 672.9 and 896.73, respectively).

A previously described protocol was at first followed for the synthesis of HN. This protocol had been

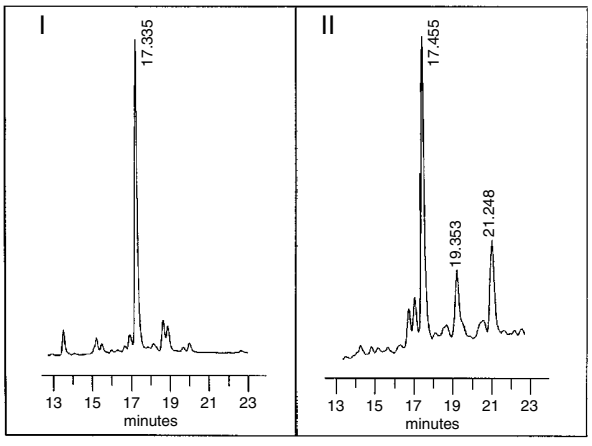

Figure 1 Analytical HPLC chromatograms of crude HN peptide obtained with the optimized (I) vs initial (II) synthetic protocol.

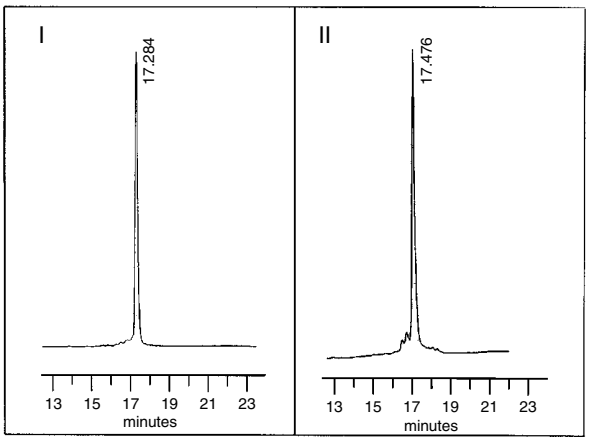

Figure 2 Analytical HPLC chromatograms of purified HN peptide obtained with the optimized (I) vs initial (II) synthetic protocol.

Table 1 Amino Acid Composition of Synthetic HN Peptide

Amino acid	Expected[a]	Analysed[a]
Met (M)	1	Trace
Ala (A)	2	2.39
Pro (P)	2	2.35
Arg (R)	3	3.23
Gly (G)	1	1.51
Phe (F)	1	0.70
Ser (S)	2	1.77
Cys (C)	1	0.73
Leu (L)	5	5.58
Thr (T)	1	0.91
Glu (E)	1	0.96
Ile (I)	1	1.28
Asp (D)	1	1.15
Val (V)	1	0.80
Lys (K)	1	0.94

[a] The data are presented as number of amino acid residues per peptide molecule.

applied successfully to the solid-phase synthesis of various peptides, such as thymosin β-4 [13], with a high overall yield. However, when applied to HN, this protocol led to large amounts of various by-products (presumably, deletion peptides) in the crude preparation (Figure 1, II), which were hardly removed by semi-preparative RP-HPLC purification (Figure 2, II), and this resulted in a very low yield (<10%).

The main difference between the two protocols is related to the deprotection time, which was fixed (30 min) in the protocol initially applied.

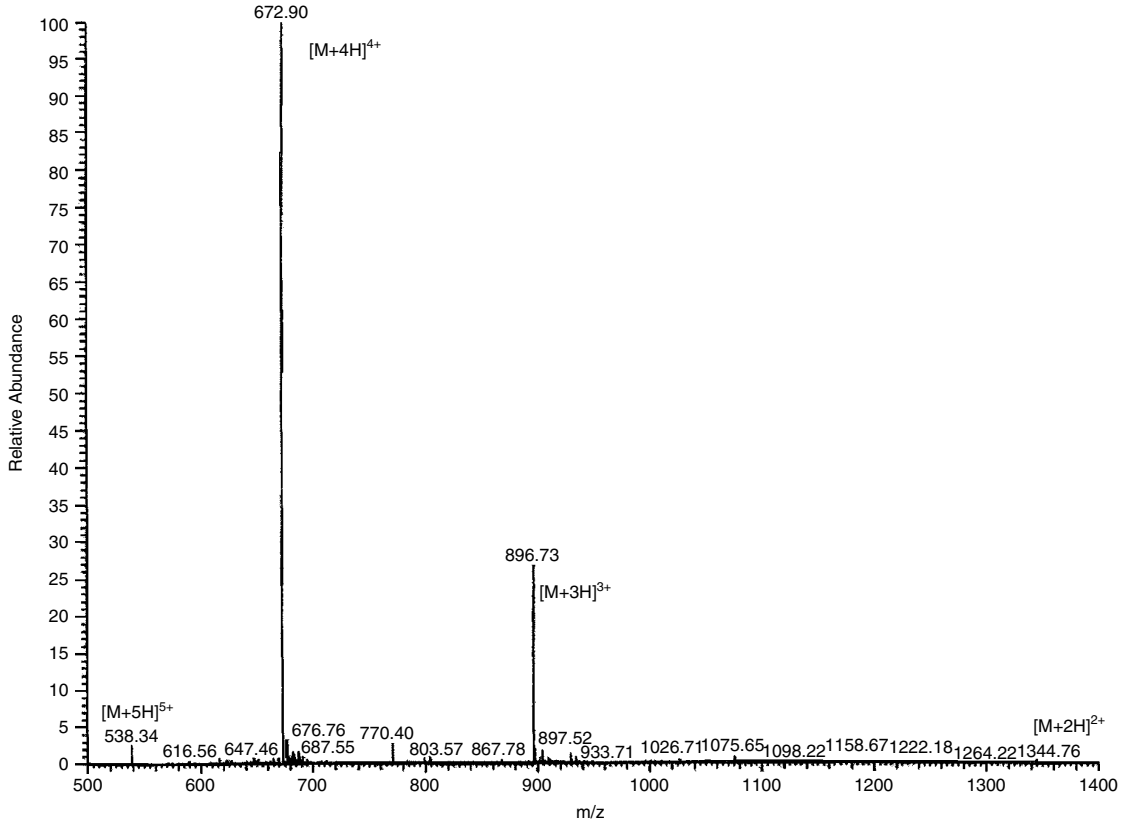

Figure 3 ESI-MS spectrum of synthetic HN peptide.

However, this 30 min deprotection time proved to be insufficient at certain steps, namely after coupling at positions L^9, C^8, S^7, F^6, G^5 and R^4, where deprotection could last as long as 150 min (Figure 4). Incomplete removal of the Fmoc groups, which can result in a low peptide yield [16], could be, in our opinion, the main reason for the very low amount of HN initially synthesized. Another difference between the two protocols was that capping followed *each* coupling (or double coupling) step in the improved version. The consequent increase in yield can thus be attributed to getting rid of incomplete couplings and, subsequently, of deletion peptides.

In conclusion, the pharmacologically active HN peptide was chemically synthesized in high overall yield following the Fmoc solid-phase peptide synthesis strategy (Fmoc SPPS). The most critical improvement in the synthetic procedure was to elongate the deprotection steps that followed coupling at positions L^9, C^8, S^7, F^6, G^5 and R^4. This finding may indicate that the region $R^4G^5F^6S^7C^8L^9$, which follows the highly lipophilic (and essential for HN secretion) sequence $L^9L^{10}L^{11}L^{12}$, is a 'difficult

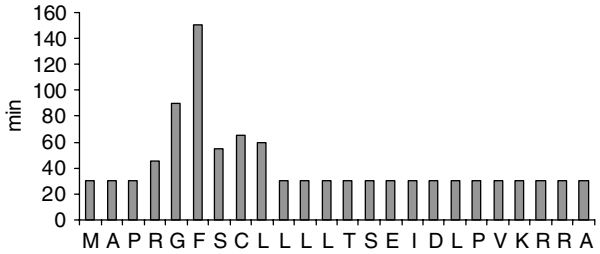

Figure 4 Optimal duration of each deprotection step during HN synthesis.

sequence', i.e. one that can cause intermolecular chain aggregation and therefore poor accessibility of the reagents [17,18]. The above conclusion is also in agreement with the observation that a double coupling step was necessary at positions L^9, C^8, F^6 and G^5. Another critical point in the overall synthetic procedure was to apply capping after *each* coupling or double coupling step. This might have acted as a 'preventive protecting procedure' for incomplete, difficult couplings, which were presumably not detected due to false-negative results in the Kaiser test. A false-negative Kaiser test is a problem often

reported in SPPS, which may be attributed to the low swelling ability of the polystyrene resins in ethanol, in which the ninhydrin solutions are prepared, when the resins are loaded with peptides containing difficult sequences that form chain aggregates [19]. The finally developed, improved protocol can be applied easily to any peptide synthesis in the laboratory and in particular can lead to the production of large amounts of pure HN. Such quantities are necessary for carrying out further investigations on the pharmacological potency of this peptide, which seems to offer a hope for developing novel therapeutics against Alzheimer's disease.

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