ELSEVIER

Journal of Chromatography B, 737 (2000) 55–61

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

Purification of the c-erbB2/*neu* membrane-spanning segment: a hydrophobic challenge

M. Goetz*, F. Rusconi, M. Belghazi, J.M. Schmitter, E.J. Dufourc

Institut Européen de Chimie et de Biologie, Ecole Polytechnique, Universités de Bordeaux I et II, B.P. 108, Avenue Pey-Berland, 33402 Talence Cedex, France

Abstract

High quality purification of membrane-spanning peptides and proteins remains a challenging problem. In this work we describe a tailored chromatographic purification of a synthetic 35-residue peptide corresponding to the transmembrane region of the tyrosine kinase receptor c-erb2/*neu*. Composed to over 70% by the amino acids alanine, isoleucine, leucine, phenylalanine and valine, this peptide presents a very hydrophobic character. Product isolation from the complex peptide mixture, obtained after acid cleavage of the resin matrix used during the solid-phase synthesis, represents a difficult task. We propose a three step strategy based on gel permeation and reversed-phase high-performance liquid chromatography, using aprotic polar solvent mixtures. The challenge consisted in obtaining a sufficient amount of an extremely pure sample, in order to allow structural analysis by NMR spectroscopy. Keeping trace of the synthetic peptide throughout the different purification steps was assured by MALDI TOF mass spectrometry, and the final product purity was checked by coupled liquid chromatography–ESI TOF mass spectrometry.

Keywords: Purification; Peptides

1. Introduction

Growth factor receptors of the tyrosine kinase family consist of two large, separately folded domains, one external and one cytosolic, connected by a single transmembrane segment. The sequence of the membrane spanning peptide appears to be important for function. For example, a single point mutation in the proto-oncogene c-erbB2/*neu*, resulting in a substitution of a valine residue to glutamic acid at position 664 within the transmembrane region, may transform it into an oncogene. The mutant receptor has constitutive tyrosine kinase activity in the absence of ligand, apparently as a result of greatly enhanced receptor dimerization [1–3]. Structural investigation by NMR spectroscopy of the corresponding synthetic peptides will allow us to study the relation between structure, dimer formation, tyrosine kinase activation and cell transformation. For this purpose, milligram amounts of highly pure synthetic peptide are needed.

High quality purification of membrane-embedded peptides and proteins remains a challenging problem. Strong intermolecular associations and the resulting insolubility in aqueous eluents, classically used in protein chromatography, have to be overcome. In this work we describe a three step purification protocol of a very hydrophobic 35-residue peptide issued from solid-phase synthesis: crude peptide

*Corresponding author. Tel.: +33-05-57-962228; fax: +33-05-57-962215.

E-mail address: michael.goetz@iecb-polytechnique.u-bordeaux.fr (M. Goetz)

0378-4347/00/$ – see front matter
PII: S0378-4347(99)00378-3

isolation from the reaction mixture by ether precipitation, intermediate purification by gel permeation chromatography, and polishing by reversed-phase HPLC.

The analytical part of this work, that means identification of the desired product and keeping its trace throughout the different purification steps, worked out to be another difficult task. MALDI TOF mass spectrometry was used to locate the synthetic peptide in the complex reaction mixture and to guide the elaboration of chromatographic conditions. Combined HPLC–ESI TOF mass spectrometry, where specific elution requirements had to be settled, allowed us to assess its final purity.

2. Experimental

2.1. Chemicals

Fmoc-L-Arg-Wang-resin and N-α-Fmoc-protected amino acids were purchased from Novabiochem (Läufelfingen, Switzerland); HOBt, HBTU and Piperidine from PE Biosystems (Courtaboeuf, France); DMSO from Sigma (St. Quentin, France); apomyoglobin, bovin insulin, histatin 5 and sinapic acid from Sigma (St. Louis, MO); acetic anhydrid, acetonitrile, dichloromethane, dimethylformamide, isopropanol, methanol, *N*-methylpyrolidone and trifluoroacetic acid (TFA) from SDS (Peypin, Paris, France).

2.2. Preliminary peptide isolation by filtration

The chemical synthesis of the *neu* peptide (primary sequence: ^{650}EQRASPVTFIIATVV^{664}-GVLLFLILVVVVGILIKRRR^{684}) was performed on an Applied Biosystems Peptide Synthesizer 433A (PE Biosystems, Courtaboeuf, France) using the Fmoc strategy [4] and will be described elsewhere [Goetz et al., in preparation]. Briefly: elongation was done on Fmoc-L-Arg-Wang-resin (0.1 m*M*; loading of starting resin: 0.4 mmol/g); DMSO was added during difficult coupling steps to increase the coupling efficiency by helping to break self-aggregation of growing peptide chains. At the end of each coupling step, unreacted amine positions were acetylated. The final peptide mixture was cleaved from the resin by TFA treatment, good splitting of the Pmc protection-group of arginine was achieved by allowing a reaction time of 180 min at room temperature. After separation of the solid resin, TFA was rapidly evaporated in vacuo and the crude peptide mixture precipitated in cold diethyl ether.

2.3. Hydrophobicity profile

Assaying the hydrophobicity of the chosen peptide segment was done with the Hopp Woods hydrophilicity values: Ala (−0.5), Arg (3.0), Gln (0.2), Glu (3.0), Gly (0.0), Ile (−1.8), Leu (−1.8), Lys (3.0), Phe (−2.5), Pro (0.0), Ser (0.3), Thr (−0.4) and Val (−1.5). Positive and negative values stand for relative hydrophilicity and hydrophobicity respectively [5]. A window of seven residues was used to plot the profile.

2.4. Gel permeation chromatography

The crude mixture was loaded onto a Superdex Peptide HR 10/30 column (Amersham Pharmacia Biotech, Orsay, France) using an ÄKTA explorer 100 liquid chromatography system (Amersham Pharmacia Biotech, Orsay, France). Elution of the product was done with 75% acetonitrile in ultrapure water (Milli Q, Millipore, St. Quentin, France) at a flow-rate of 0.5 ml/min and monitored at 215 and 280 nm.

2.5. Analytical and semi-preparative HPLC purification

The lyophilized product was dissolved in a mixture of 75% acetonitrile in Milli Q-water, both containing 0.08% TFA, and analyzed on a Waters Alliance HPLC station. Monitoring was performed at 215 and 280 nm with the Waters 2487 Dual λ absorbance detector (Waters S.A., St. Quentin, France). The elution was carried out with 0.05% aqueous TFA (=eluent A) and acetonitrile–isopropanol (80:20, containing 0.05% TFA=eluent B) at a flow-rate of 0.8 ml/min using a Waters DeltaPak C_4 column (5 μ, 100 Å, 3.9×150 mm). Maximum resolution of the synthetic peptide mixture was achieved with a 20 min linear gradient, where eluent B varied from 50 to 100%. Semi-preparative HPLC

was done with a Waters DeltaPak C_4 column (15 μ, 100 Å, 7.8×300 mm; Waters S.A., St. Quentin, France).

2.6. MALDI TOF mass spectrometry

MALDI TOF spectra were obtained on a Reflex III mass spectrometer (Bruker Daltonik, Bremen, Germany). Each chromatographic fraction was loaded on the target by the dried droplet method. Sinapic acid was chosen as matrix. Spectra were externally calibrated using the $[M+H]^+$ ions of two peptide standards, bovin insulin (average: 5734.56 rel. mol. mass) and histatin 5 (monoisotopic: 3035.52 rel. mol. mass). The amount of peptide deposited on each spot was typically 3–5 pmol. The analyses were run in the positive and reflector ion mode with an accelerating voltage of 20 kV and an extraction delay of 250 ns. Around 100 scans were averaged for each spectrum acquisition, using the XACQ Bruker acquisition software.

2.7. HPLC–ESI TOF mass spectrometry

HPLC–ESI-MS was performed on a LCT Electrospray Time Of Flight mass spectrometer (Micromass, Manchester, UK) equipped with a Z spray electrospray source. All data were acquired and analyzed using Micromass MassLynx NT 3.2 software. Instrument settings were as follows: source temperature: 150°C; desolvation temperature: 150°C; N_2 drying gas: 380–390 L/h; nebulizing gas: 70–80 L/h; capillary voltage: 3.2 kV; sample cone: 50 V. Acquisition was carried out from m/z 100–2000 with a scan time of 0.5 s in the continuum scanning mode. Calibration was done using a 1 pmol/μl solution of apomyoglobin by direct introduction into the electrospray source at 15 μl/min, using the previously described settings.

HPLC analysis was performed on a Smart HPLC system (Amersham Pharmacia Biotech, Orsay, France) equipped with a reverse phase Brownlee C_4 cartridge column (7 μm, 300 Å, 3.2×30 mm; Perkin Elmer, Courtaboeuf, France) at a flow-rate of 50 μl/min and a wavelength detection at 214 nm with 30% of the flow directed to the mass spectrometer through a fused-silica capillary. The eluents used were the same as described above.

3. Results

The synthetic 35-residue peptide, corresponding to the transmembrane region of c-erbB2/*neu*, is composed to almost 70% by non polar amino acids. Fig. 1 represents the hydrophobic profile, calculated by the method of Hopp and Woods [5], where the large domain of negative free energy values between residues 6 and 30 predicts membrane location. In order to minimize expected difficulties in peptide chain assembly during solid-phase synthesis, we allowed coupling times prolonged up to 50 min per residue, adding the aprotic polar solvent dimethylsulfoxide during the last 20 min for optimal solubilization of the growing peptide chains and the resin matrix [6]. Free amino groups, due to incomplete coupling, were protected by acetic anhydride capping to prevent the formation of deletion peptides (missing one or two residues inside the peptide chain), closely related in their physical properties to the target sequence and very difficult to separate.

The final crude peptide mixture was cleaved from the solid support by TFA treatment with phenol, thioanisol and ethanedithiol as scavengers. After separation of the resin, TFA was rapidly evaporated in vacuo and the peptide mixture precipitated in cold diethyl ether. In this first isolation step, most of the scavengers were eliminated by filtration through a fine-porosity, fritted glass funnel. The peptide mixture was solubilized in acetonitrile–trifluoroethanol–deionized water (1:1:1), prior to lyophilization.

The crude product was resolubilized in acetonitrile–deionized water (3:1) and submitted to gel permeation using a Superdex Peptide column. Smaller truncated peptide sequences, resulting from the acetylation blocking of the unreacted amino groups during the synthesis could be easily eliminated in this step. Fig. 2 shows the isocratic elution profile in acetonitrile–H_2O (3:1), monitored at 215 nm. A total of 14 fractions were collected and analyzed by MALDI TOF mass spectrometry. Among others, two MALDI matrices, α-cyano-4-hydroxycinnamic acid and sinapic acid were tested and the later chosen for sample preparation. The desired *neu* peptide was found most abundant in chromatographic fractions 1 to 4. These were pooled and lyophilized.

The next purification step, semi-preparative reversed-phase (RP) HPLC, lead to a large increase in

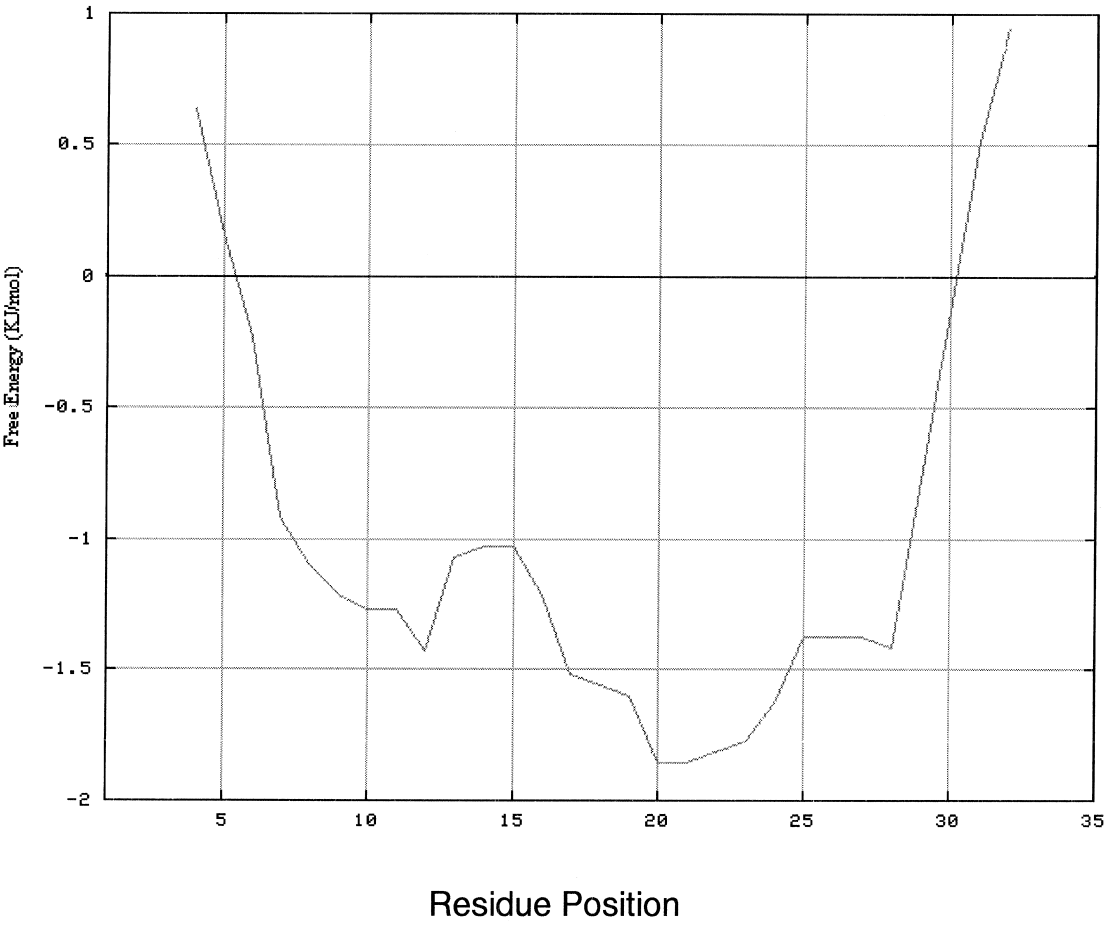

Fig. 1. Hydrophobic profile of the c-erbB2/*neu* transmembrane segment, using the method of Hopp and Woods [5]. Negative free energy values from residue 6 to 30 predict the membrane embedded region.

synthetic peptide purity. Elution was performed with 0.05% aqueous TFA (eluent A) and a solvent mixture with high eluotropic strength for eluent B: 0.05% TFA in acetonitrile–isopropanol (80:20). Elution conditions for semi-preparative purification were found optimal when eluent B varied in a 20 min linear gradient from 70 to 100%. Improved resolution was obtained by heating the column to 40°C. Fig. 3 shows the analytical HPLC profiles of the isolated, crude product (a), the size-exclusion prepurified pool, containing gel permeation chromatography fractions 1 to 4 (b), and the pure synthetic peptide obtained after RP-HPLC polishing (c).

Fig. 4 shows the MALDI TOF spectrum of the final product. The monoisotopic rel. mol. mass obtained on the spectrum was 3888.49 (calculated monoisotopic $[M+H]^+$ $m/z=3888.44$ rel. mol. mass). The high degree of purity of the synthetic *neu* peptide, as required for NMR analysis, was confirmed by combined HPLC/ESI TOF mass spectrometry. Five hundred picomoles of the peptide were solubilized in 50 μl acetonitrile–H_2O (30:70) and injected onto the chromatographic column. Eluent A and B composition were the same as described in the purification protocol. Here, elution conditions had to be carefully adapted in order to guarantee both, desorption of the hydrophobic peptide from the column and its protonation prior to mass analysis. We chose a gradient where B varied from 60–95% in 5 min, followed by an isocratic

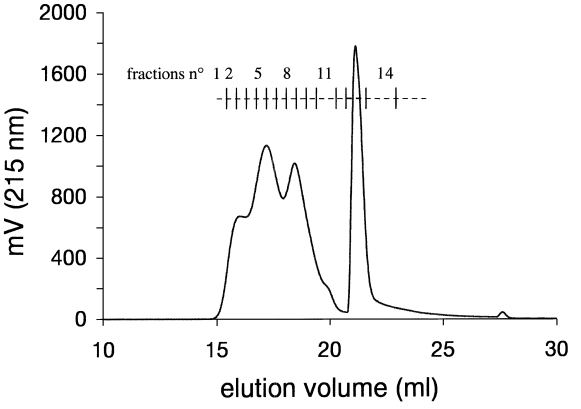

Fig. 2. Gel permeation elution profile of the crude synthetic peptide, monitored at 215 nm. Mass spectrometric analysis showed fractions 1 to 4 to contain the desired product.

elution at 95% B from 5 to 20 min. The *neu* peptide's retention time was approximately 15 min. ESI mass spectra were accumulated, combined across the top of the HPLC peak and the resulting spectrum was deconvoluted, showing an average rel. mol. mass of 3889.55 ± 0.22 (calculated average rel. mol. mass=3889.86), as presented in Fig. 5.

4. Discussion

To perform NMR structure analysis, large quantities of highly pure protein are requested. For the production of the c-erbB2/*neu* transmembrane segment (Glu^{650}-Arg^{684}), we decided to perform chemical synthesis, as the segment's shortness as well as its membranous location would make it difficult to realize by classic molecular biology techniques. Even with the well established solid-phase method, membrane protein synthesis is far from being routine. Hydrophobic interactions are expected to induce strong intra- and inter-chain associations within the peptide-resin matrix, affecting reaction rates and lowering coupling yields. Nevertheless, a modified protocol, especially adapted to hydrophobic proteins rendered the total chemical synthesis of the *neu* peptide possible.

The crude peptide mixture, cleaved and separated from its polymeric support by filtration, was submitted to gel permeation chromatography. This purification step, based on size-exclusion, allowed us to eliminate a major proportion of unwanted byproducts, as scavengers, salts and short peptidic contaminants. In the absence of any biological activity or specific labeling, only mass spectrometry did allow us to locate the desired product. The MALDI TOF technique is the most suitable when working with complex mixtures. As co-crystallization is a crucial step in sample preparation, the matrix has to be carefully chosen. Despite the *neu* peptide's rather

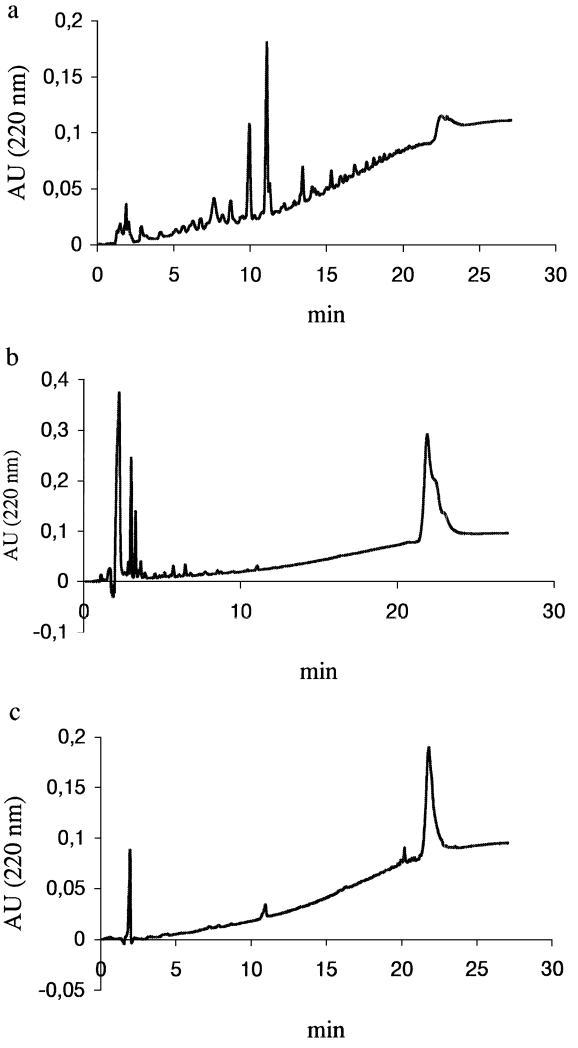

Fig. 3. Analytical HPLC profile of the isolated, crude product (**a**), the gel permeation prepurified pool (**b**) and RP-HPLC polished pure synthetic peptide (**c**), monitored at 220 nm. Elution conditions are described in the text.

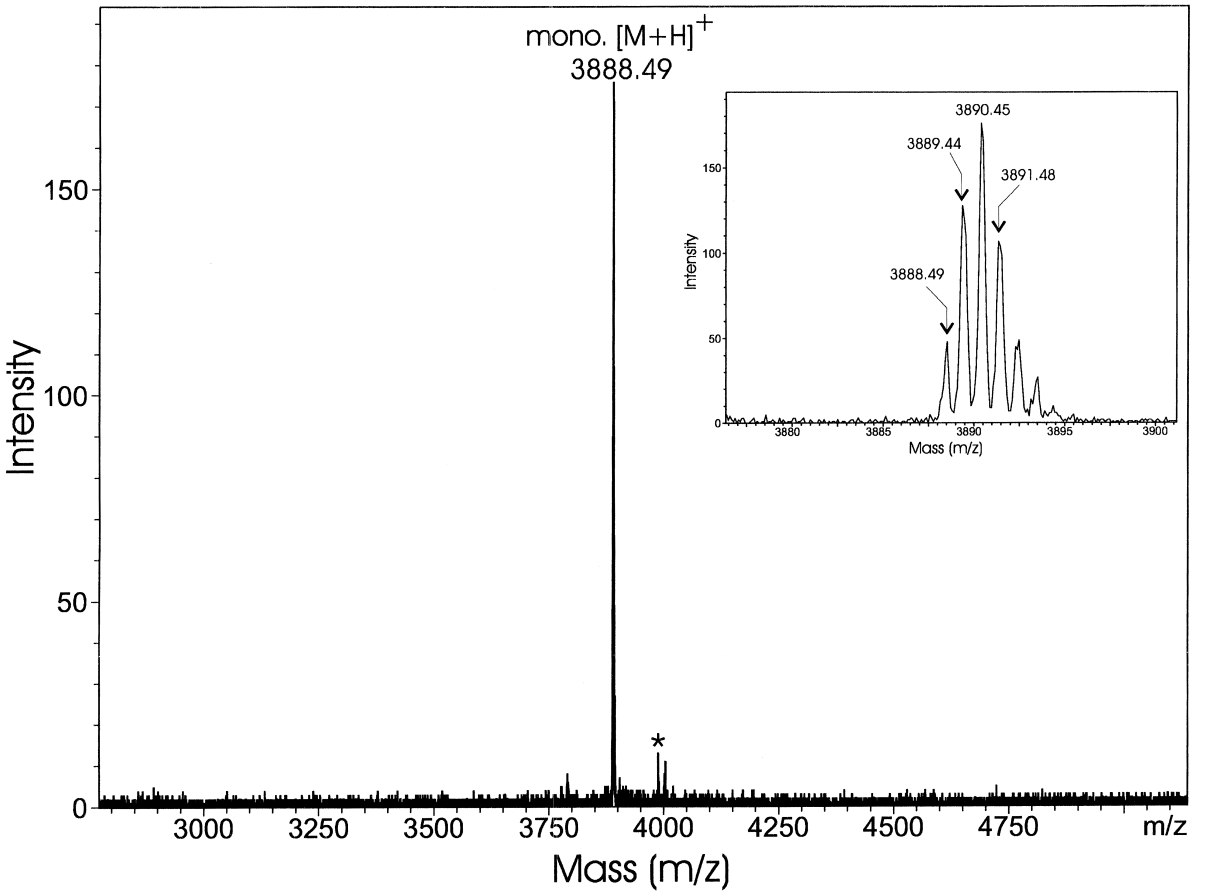

Fig. 4. MALDI TOF mass spectrum of the pure peptide. An expansion of the major peak is shown in the upper right corner. Peaks are labeled with the monoisotopic $[M+H]^+$ m/z. The asterisk indicates an unidentified molecular species, which could be attributed to incompletely deprotected peptide.

low molecular mass, sinapic acid, classically used for larger polypeptides, proved to give better results than α-cyano-4-hydroxycinnamic acid, and was thus retained for further structural analyses of the individual chromatographic fractions. Those containing the *neu* peptide were pooled and submitted to reversed-phase HPLC. Here again, MALDI TOF mass spectrometry assisted product identification as well as gradient elaboration. In order to improve chromatographic resolution, we made use of an aprotic polar solvent mixture at a slightly elevated temperature. ESI TOF mass spectrometric analysis clearly shows the synthetic peptide's high degree of purity, that we obtained with this purification methodology. Thus, line broadening in the HPLC profiles seems rather due to conformational diversity or possible oligomerization of this protein segment than to side-product contamination.

5. Conclusion

The very hydrophobic character of the 35-residue peptide corresponding to the transmembrane region of the tyrosine kinase receptor c-erb2/*neu* demanded especially adapted protocols for synthesis and purification. We chose Fmoc based solid-phase synthesis to realize the sequence. Prolonged coupling times adding the aprotic polar solvent dimethylsulfoxide proved to diminish the expected intra- and inter-

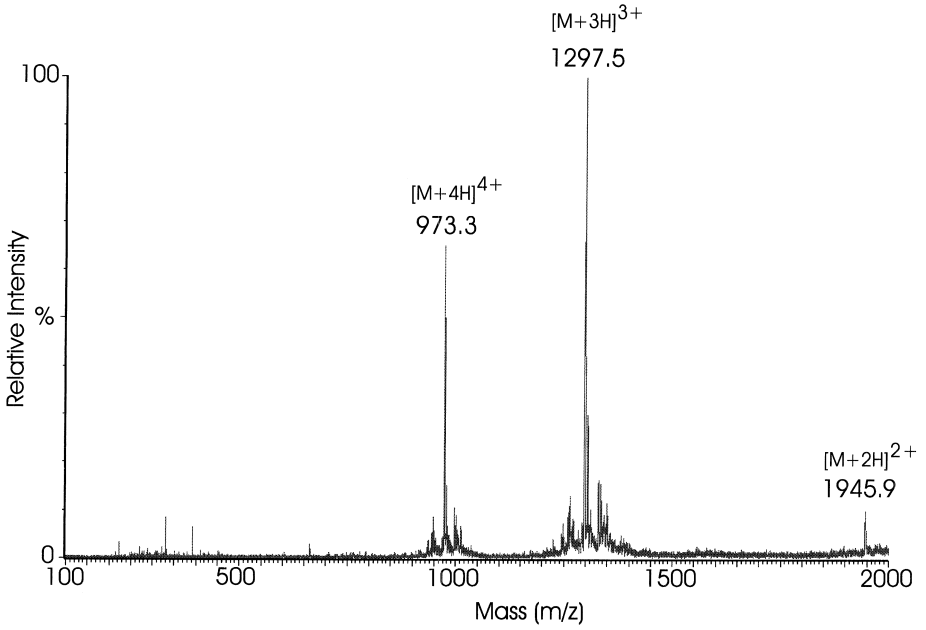

Fig. 5. Reversed-phase HPLC–ESI TOF mass spectrum of the purified peptide, corresponding to a sum of 350 scans. The peaks are labeled with their protonation state.

chain associations inside the peptide-resin matrix. After peptide assembly, cleavage of the resin and isolation of the crude peptide mixture by ether precipitation, a chromatographic purification protocol was tailored in order to obtain a sample sufficiently pure for structural analysis.

The first chromatographic purification step involved gel permeation. Smaller, truncated synthetic byproducts as well as most of the scavengers used during the acidic final cleavage were eliminated. Subsequent semi-preparative reverse phase HPLC allowed to separate closely related peptide products. In this step, the use of isopropanol was of much benefit for solubilization and peptide desorption of the chromatography column. In this work, MALDI TOF mass spectrometry was used to locate chromatographic fractions containing the *neu* peptide. The quality of the final purified peptide was assessed by combined HPLC–ESI TOF mass spectrometry. These optimized conditions could certainly be generalized for the synthesis and purification of other protein sequences containing large hydrophobic domains.

References

[1] H. Cao, L. Bangalore, B.J. Bormann, D.F. Stern, EMBO J. 11 (1992) 923.
[2] S.O. Smith, C.S. Smith, B.J. Bormann, Nature Struct. Biol. 3 (1996) 252.
[3] L.I. Chen, M.K. Webster, A.N. Neyer, D.J. Donoghue, J. Cell Biol. 137 (1997) 619.
[4] L.A. Carpino, G.Y. Hahn, J. Am. Chem. Soc. 92 (1970) 5748.
[5] T.P. Hopp, K.R. Woods, Proc. Natl. Acad. Sci. USA 78 (1981) 3824.
[6] M. Goetz, J.M. Schmitter, S. Geoffre, E.J. Dufourc, J. Peptide Sci. 5 (1999) 245.