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High-performance liquid chromatographic separation of modified and native melittin following transglutaminase-mediated derivatization with a dansyl fluorescent probe

ENRIQUE PEREZ-PAYA, LORENZO BRACO and CONCEPCION ABAD*
Departamento de Bioquímica y Biología Molecular, Universidad de Valencia, Burjassot, E-46100 Valencia (Spain)
and
JEAN DUFOURCQ
Centre de Recherche Paul Pascal, CNRS, Château Brivazac, F-33600 Pessac (France)

ABSTRACT

The 26-amino acid linear, amphiphilic peptide melittin was enzymatically modified with the fluorescent probe monodansylcadaverine using guinea pig liver transglutaminase and a fluorescent derivative of stoichiometry 1:1 was obtained. Reversed-phase and size-exclusion high-performance liquid chromatographic modes were tested in order to resolve the labelled peptide and native species. The influence of several operational variables was analysed and the elution conditions were optimized so that a satisfactory resolution could be achieved in both instances in a rapid, easy manner. Both chromatographic modes offer the possibility of accurate monitoring of the time course of the enzyme-mediated conversion and, more interestingly, can be applied to the semi-preparative purification of the labelled peptide.

INTRODUCTION

We have been involved in the past in the investigation of protein–lipid interactions using hydrophobic or amphiphilic peptides [1–7]. Among other goals, our interest has recently been directed to the selective covalent modification of the cytolytic toxin melittin with dansyl fluorescent probes. This amphiphilic, 26-amino acid linear peptide has the sequence: Gly–Ile–Gly–Ala–Val–Leu–Lys–Val–Leu–Thr–Thr–Gly–Leu–Pro–Ala–Leu–Ile–Ser–Trp–Ile–Lys–Arg–Lys–Arg–Gln–Gln–CO–NH_2. Dansyl-labelled melittin can undoubtedly be regarded as a valuable tool for studies on the detailed mechanism of membrane lysis at a molecular level, on the basis of both its dramatically enhanced sensitivity to fluorimetric detection and its distinctive emission features relative to native membrane proteins.

In order to achieve a rational, selective modification of the peptide, conventional chemical procedures should in principle be disregarded, owing to their frequent lack of selectivity, if one needs to introduce a probe at a selected single position in the sequence. In contrast, the use of enzyme-mediated modification, in particular with

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transglutaminases, provides a unique strategy for the specific modification of glutamine residues in peptides [8,9] and proteins [10–12]. Transglutaminases (TGase; R-glutaminyl-peptide: amine γ-glutamyltransferase, E.C. 2.3.2.13) catalyse acyl transfer reactions in which the γ-carboxamide groups of glutamine residues act as acyl donors and primary amino groups from specific amines as acyl acceptors [13]. Evidence in this field has shown that the *in vitro* transglutaminase-mediated covalent modification of proteins and peptides generally leads to the incorporation of a single probe molecule per molecule of biopolymer [8,10]. However, as this exquisite selectivity minimizes the structural and chemical differences between modified and native species, it often becomes a source of difficulties in any separation and purification attempt.

Recently, using guinea pig liver transglutaminase, we have carried out a facile, selective modification of melittin in its C-terminal end with the probe monodansylcadaverine (DNC), yielding a derivative with a 1:1 stoichiometry [14]. This paper reports on the development of high-performance liquid chromatographic (HPLC) methods allowing a complete separation of DNC-melittin from the native peptide. It will be shown that both species can be succesfully resolved by using either size-exclusion (SE) or reversed-phase (RP) supports. In both chromatographic modes several experimental factors were tested in order to optimize peptide separation. Note that this separation is particularly complicated owing to the amphiphilic nature of melittin, which exhibits a highly positively charged C-terminal end and an amphipathic helical organization for the remainder of the molecule [15]. Finally, the advantages of each HPLC mode for the determination and semi-preparative isolation of labelled melittin are discussed.

EXPERIMENTAL

Materials

Melittin and monodansylcadaverine [N-(5-aminopentyl)-5-dimethylamino-1-naphthalenesulphonamide] were obtained from Serva (Heidelberg, Germany), guinea pig liver transglutaminase from Sigma (St. Louis, MO, USA) and dithiothreitol (DTT) and HPLC-grade solvents from Merck (Darmstadt, Germany). Doubly distilled water was purified by passing it through a Milli-Q purification system (Millipore, Bedford, MA, USA). All salts used were of analytical-reagent grade from Merck. The eluents were always filtered and degassed through a 0.45-μm regenerated cellulose filter (Micro Filtration System, Dublin, CA, USA) prior to use. Fluorescence spectroscopy-grade methanol from Merck was used in fluorimetric measurements.

TGase-mediated modification of melittin by monodansylcadaverine

The procedure was basically similar to that described for the TGase-mediated modification of small peptides such as substance P [8] or β-endorphin [9] by polyamines. Briefly, melittin (100 μg) was incubated at 37°C for 3 h with 2 μM TGase in 50 mM Tris–HCl buffer (pH 8.0) containing 20 mM DTT, 20 mM $CaCl_2$ and 700 μM DNC (final volume 100 μl). The reaction was stopped by heating at 60°C for 5 min [16].

Apparatus

The HPLC instrumentation consisted of M-510 solvent-delivery systems, an

automated gradient controller, a U6K universal injector and an M-418 multi-wavelength detector, all from Waters Assoc. (Milford, MA, USA). Fluorescence of the effluent was monitored (λ_{ex} = 330 nm, λ_{em} = 520 nm) with an F-1050 fluorescence detector from Merck. Peak areas were digitized using an SP-4290 integrator (Spectra-Physics, San Jose, CA, USA).

Reversed-phase experiments were carried out using a μBondapak C_{18} column (30 cm × 0.78 cm I.D.) from Waters Assoc. A Guard-PAK (μBondapak C_{18}) precolumn, from Waters Assoc., was placed between the pump and the injector to protect the analytical column from mobile phase contaminants. When the system was operated under gradient conditions, the binary eluent consisted of 0.1% aqueous TFA (solvent A) and acetonitrile (solvent B).

For size-exclusion experiments, a Waters Assoc. Ultrahydrogel column (30 cm × 0.78 cm I.D.) packed with hydroxylated polymethacrylate-based gel of 120 Å nominal pore size (Ultrahydrogel-120) was used. This chromatographic support has been reported to bear some residual carboxyl groups, to be stable over a broad pH range (from 2 to 12) and to be compatible with mobile phases containing a high percentage of organic solvents [17]. All chromatographic measurements were conducted at room temperature. The flow-rate in all experiments under isocratic conditions was 1.0 ml/min. The injection volume was in the range 5–25 μl and up to 150 μl for analytical and semi-preparative purposes respectively, without any decrease in resolution. Good run-to-run reproducibility was observed in all instances and the recovery was always higher than 90%.

Corrected fluorescence spectra were obtained at 25°C on a Perkin-Elmer (Beaconsfield, UK) LS-5B spectrofluorimeter coupled to a Model 3700 data station using a 2.5-nm slit width.

RESULTS AND DISCUSSION

Reversed-phase chromatography

We first examined the ability of RP-HPLC to resolve modified and unmodified melittin, because this is by far the most widely used mode of HPLC of peptides [18]. The reaction products derived from TGase assay after 3-h incubation at a DNC:melittin ratio of 2 were analysed directly, without any sample pretreatment, on a μBondapak C_{18} column using either isocratic or gradient conditions. As illustrated in Fig. 1A, when elution was performed under isocratic conditions with a mobile phase containing 0.1% aqueous TFA–acetonitrile (55:45, v/v) four peaks were detected by UV detection at 283 nm, corresponding to DTT (2.9 min), free DNC (3.3 min), melittin (5.77 min) and DNC-melittin (10.1 min). Moreover, simultaneous fluorescence detection using excitation and emission wavelengths specific for the dansyl group showed only two peaks at retention times of 3.3 and 10.1 min (not shown). In fact, verification of the identity of the labelled melittin peak was carried our after collection, neutralization and lyophilization of the corresponding fraction, by using absorption or fluorescence spectroscopy, as described below for the SEC mode. The resolution between melittin and DNC-melittin was excellent, although under these conditions the separation was poor for DTT and free DNC.

When the volume fraction of acetonitrile in the eluent, φ, was varied from 0.44 to 0.50 at constant flow-rate under isocratic conditions, a plot of log k' (k' = capacity

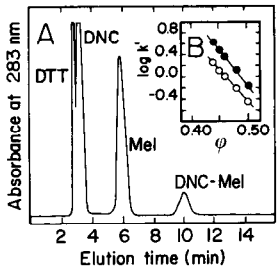

Fig. 1. Reversed-phase separation of melittin and DNC-melittin under isocratic conditions. (A) Elution profile obtained after direct injection, without any sample pretreatment, of an aliquot from the TGase reaction mixture incubated for 3 h. Column, μBondapak C18; mobile phase, 0.1% aqueous TFA–acetonitrile (55:45, v/v); flow-rate, 1.0 ml/min; detection, UV at 283 nm. (B) Effect of volume fraction of acetonitrile in the eluent, φ, on the retention of (○) melittin and (●) DNC-melittin.

factor) *vs.* φ was obtained as shown in Fig. 1B. The good linearity observed for both melittin and DNC-melittin demonstrates that the column is operating mainly through a reversed-phase mechanism. Moreover, the similarity between the slopes indicates that the perturbation introduced by the probe does not significantly alter the chromatographic behaviour of labelled melittin relative to its native species. Suitable selectivities (α = ratio of capacity factors) and reasonable analysis times were obtained for φ values in the range 0.44–0.50. On the other hand, the addition of 0.1% of TFA to acetonitrile in the mobile phase did not improve the resolution. Volatile buffers such as 0.1 *M* (pH 4.4) or 0.2 *M* (pH 3.0) ammonium acetate, which have often proved to be suitable for the separation of specific peptide mixtures [19,20], resulted in peak broadening and longer retention times in the present system.

A better resolution for all the components of the TGase reaction mixture was obtained when gradient elution conditions were used. In this instance, the effect of several operating factors on the retention behaviours of melittin and DNC-melittin was examined. For example, Fig. 2A shows resolution, R_s, as a function of the flow-rate at different gradient rates (for a constant range of φ from 0.3 to 0.7). R_s is defined as the ratio of the distance between the maxima of two adjacent peaks to the arithmetic mean of their base widths. As expected, R_s increases in all instances as the gradient rate decreases, whereas the influence of flow-rate is relatively small under the gradient conditions applied. Fig. 2B depicts the relationship between R_s and the average solute

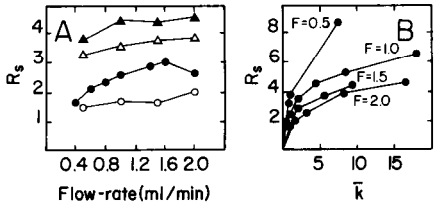

Fig. 2. Dependence of the resolution, R_s, between melittin and DNC-melittin under gradient conditions on the flow-rate, *F*, at different gradient rates: (▲) 0.5, (△) 1.0, (●) 2.0 and (○) 5.0% acetonitrile/min. (B) Plot showing the variation of R_s with $\bar{k}$ using different flow-rates. Column, μBondapak C_{18}.

capacity factor under gradient conditions for labelled melittin, $\bar{k}$, at different flow-rates, F; $\bar{k}$ is defined as [21]

$$\bar{k} = t_G\,(F)/1.15\,(\Delta\varphi)\,V_m\,S$$

where t_G is the gradient time, φ the fraction of organic solvent in the mobile phase, $\Delta\varphi$ the change in φ during the gradient, V_m the column void volume and S a solute parameter (slope of log k' *vs.* φ).

The interpretation of the data in Fig. 2B may not be immediately obvious and some comments are deserved. The figure is aimed at illustrating the different possibilities of variation of experimental conditions that can be used to obtain a given R_s value. For a given flow-rate, a given $\bar{k}$ value can be obtained by varying either t_G or $\Delta\varphi$. Note also that the pronounced variation of R_s with F does not contradict the data in Fig. 2A, because in this instance $\Delta\varphi$ was not always maintained constant. Thus, all the points in Fig. 2B correspond to points in Fig. 2A (for which $\Delta\varphi = 0.4$) except for the two R_s values higher than 6, for which $\Delta\varphi$ was 0.14 instead of 0.4. Although the highest value of R_s in the plot is achieved at $F = 0.5$ ml/min (for $\bar{k} = 7.4$), the long analysis time required (>200 min) is clearly not desirable and, therefore, for the best compromise between separation time and resolution the following elution conditions can be recommended: $F = 1.0$ ml/min and a 20-min linear gradient from 0.1% TFA–acetonitrile (70:30, v/v) to a final composition of 30:70 (v/v), which implies a total running time, including column recycling, of about 45 min.

Fig. 3 illustrates the elution profile corresponding to the same TGase reaction mixture as in Fig. 1 using the chromatographic gradient conditions recommended above. Note that in this instance the separation between DTT and DNC is improved relative to isocratic conditions (Fig. 1A), whereas the good resolution between native and modified peptide is maintained.

The above results show not only that both isocratic and gradient RP modes allow an excellent separation between melittin and DNC-melittin, but also that easy, accurate monitoring of the progress of the enzymatic labelling can be achieved, especially taking into account that the reported reaction times for transglutaminase-mediated conversions are long, from 1 to 24 h [10,11]. In fact, we have observed that as

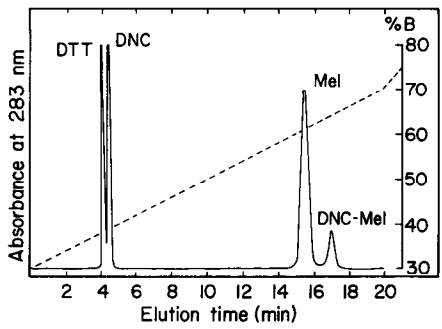

Fig. 3. Reversed-phase separation of melittin and DNC-melittin under optimized gradient conditions (see text), corresponding to the same sample as in Fig. 1A. Column, μBondapak C_{18}; flow-rate, 1.0 ml/min; solvent A, 0.1% aqueous TFA; solvent B, acetonitrile.

reaction takes place a gradual increase in the DNC-melittin peak area occurs at the expense of a concomitant decrease in the melittin and DNC peaks (results not shown).

Size-exclusion chromatography

The effectiveness of the Ultrahydrogel-120 column for melittin analysis was next investigated using 0.1 *M* sodium acetate buffer (pH 4.4) as eluent, on the basis of our recent optimization study on the elution of polyanions and polycations on Ultrahydrogel supports [22]. When melittin was injected under these conditions a strong retention was observed, much longer than the total permeation volume of the column (V_t = 11.0 ml), as shown in Fig. 4A. This anomalous behavior is not surprising if the amphiphilic nature of melittin is taken into account. In fact, the peptide hydrophobic (residues 1–20) and highly positively charged (residues 21–26) moieties are likely to be responsible for the superimposition of hydrophobic and electrostatic secondary effects, causing a dramatic deviation from ideality. The electrostatic interactions between melittin and the matrix residual carboxyl groups may have relatively little importance because at this eluent pH poly-L-lysine standards are not significantly retained [22]. Therefore, if hydrophobic interaction is indeed predominant, addition of a miscible organic modifier to the eluent should diminish the retention of melittin. This is demonstrated in Fig. 4A, where it is shown that a gradual increase in the acetonitrile content of the mobile phase caused a drastic reduction of the peptide elution volume concomitant with pronounced peak sharpening. A similar behaviour has been reported by Mant *et al.* [23] for the elution of a polymer series of synthetic peptides when using a similar range of acetonitrile concentrations in the eluent. Note that even for the highest acetonitrile proportion applied (20%, v/v) the melittin elution volume does not strictly correspond to that expected from its molecular weight (*ca.* 2840). This implies that even under these conditions elution does not occur through a pure size-exclusion mechanism. Anyway, a linear relationship was observed between peak area and injected sample concentration, as shown in Fig. 4B. Higher organic modifier compositions were not tested according to manufacturer's directions.

Fig. 5 shows a typical elution profile of the TGase reaction mixture after incubation for 3 h under the same experimental conditions as in Fig. 1A using 0.1 *M*

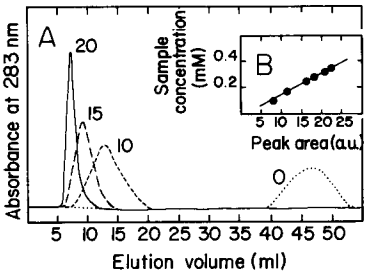

Fig. 4. (A) Elution profiles of melittin on Ultrahydrogel-120 as a function of the percentage of acetonitrile in the eluent (as indicated). Flow-rate, 1.0 ml/min. (B) Correlation between injected melittin concentration and peak area using 0.1 *M* acetate buffer (pH 4.4) containing 20% of acetonitrile as eluent.

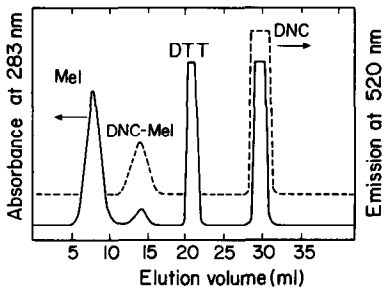

Fig. 5. Separation of melittin and DNC-melittin on Ultrahydrogel-120. Eluent, 0.1 *M* acetate buffer (pH 4.4) containing 20% of acetonitrile; flow-rate, 1.0 ml/min. Simultaneous detection by UV at 283 nm and fluorescence emission at 520 nm on excitation at 330 nm.

acetate buffer (pH 4.4) containing 20% of acetonitrile as mobile phase. Four well resolved peaks were obtained by UV detection at 283 nm at 7.6, 12.8, 21.0 and 30.7 ml, corresponding to melittin, DNC-melittin, DTT and free DNC, respectively. As expected, only the two peaks corresponding to DNC-melittin and free DNC were detected on monitoring the effluent fluorescence emission at 520 nm. As with melittin, labelled melittin and also DNC and DTT exhibited non-ideal behaviour, all eluting beyond the total permeation volume of the column. Note that this non-ideal behaviour was particularly advantageous for our purposes, and that otherwise a satisfactory separation between modified and native peptide based only on molecular weight differences would probably have been impossible. It is worth emphasizing in this regard that, as has been described previously [18,23], the non-ideal properties of size-exclusion columns, rather than being a limitation, may in many instances become a useful analytical tool.

The identity of the peak eluting at 12.8 ml (putative DNC-melittin) was checked by both absorption and fluorescence spectroscopy after peak collection, desalting and lyophilization of this fraction. Fig. 6 shows the UV absorption spectrum of the powder after dissolution in methanol. This spectrum differed markedly from that corresponding to a single tryptophan (native melittin), as a result of the attached fluorescent probe. In fact, the spectrum was similar to that obtained for a 1:1 mixture of melittin and free DNC or for the model peptide dansyl–Gly–Trp (see Fig. 6). Moreover, from the molar absorptivities of melittin and DNC in methanol [ε_{280} (melittin) = 5900 l mol^{-1} cm^{-1}; ε_{332}(DNC) = 3860 l mol^{-1} cm^{-1}], a 1:1 stoichiometry was deduced for the DNC-melittin derivative. In addition, Fig. 6 also shows the fluorescence emission spectra in methanol upon excitation at 280 and 350 nm. As expected, emission bands were obtained corresponding to Trp (maximum at 340 nm) and dansyl (maximum at 530 nm) groups. The position of the emission maxima was coincident with that deduced using a 1:1 melittin–DNC mixture or dansyl–Gly–Trp under the same experimental conditions. Further, the labelled melittin yielded a single peak at 12.8 ml when rechromatographed on Ultrahydrogel-120. All the above observations demonstrate that this peak indeed corresponds to a 1:1 monodansylcadaverine-melittin covalent derivative. The same conclusions were drawn after a similar analysis of the modified melittin peak emerging from the reversed-phase support.

Finally, volatile buffers such as 0.1 *M* ammonium acetate (pH 4.4), were also

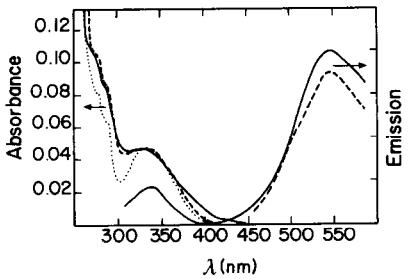

Fig. 6. Left-hand axis: absorption spectra in methanol of (solid line) DNC-melittin obtained from the corresponding fraction in the Ultrahydrogel-120 effluent (see text), (dashed line) an equimolar mixture of native melittin and free DNC and (dotted line) the model dipeptide dansyl–Gly–Trp. Right-hand axis: fluorescence emission spectra in methanol of DNC-melittin on excitation at (solid line) 280 and (dashed line) 350 nm. Peptide concentration: 11 μM.

tried as mobile phases in the SEC mode and a similar resolution was obtained as with the above-mentioned conditions. Hence these buffers can be used for semi-preparative purposes avoiding the need for desalting of the collected sample.

CONCLUSIONS

The amphiphilic peptide melittin was enzymatically modified with the fluorescent probe monodansylcadaverine using guinea pig liver transglutaminase and a fluorophoric derivative of stoichiometry 1:1 was obtained. It has been shown that reversed-phase and size-exclusion HPLC modes can be successfully used for the complete separation, directly from the reaction mixture, of the labelled peptide and its native species. When operating under isocratic conditions both modes provide an easy, rapid, convenient means of accurately monitoring the time course of the enzymatic reaction and of isolating the labelled product for semi-preparative purposes.

A good resolution of all the components of the TGase enzymatic assay was achieved in both size-exclusion and gradient reversed-phase modes, the former being more appropriate in the present case based on its simpler operation and especially its shorter time of analysis. However, if larger peptides or even proteins were labelled in a similar manner with a dansyl-containing probe, reversed-phase gradient conditions might be the method of choice owing to the greater versatility of operation.

Finally, the behaviour observed for both melittin and dansyl-labelled melittin on Ultrahydrogel-120 is a clear example of how a non-ideal SEC mechanism can turn out to be advantageous for an *a priori* compromised separation.

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