

Activation of bee venom phospholipase A₂ through a peptide–enzyme complex

Ismael Mingarro^{*,***}, Enrique Pérez-Payá^{*,***}, Clemencia Pinilla, Jon R. Appel, Richard A. Houghten, Sylvie E. Blondelle*

Torrey Pines Institute for Molecular Studies, 3550 General Atomics Ct., San Diego, CA 92121, USA

Received 7 August 1995

Abstract Phospholipase A₂ activation by membrane-bound peptides was investigated in order to understand the role of the membrane-induced conformation on activation, and to examine the occurrence of a peptide–enzyme complex at the lipid/water interface. For the peptides studied, bee venom phospholipase A₂ was stimulated regardless of the membrane-bound conformation (α -helix, β -sheet or random coil). Using antisera raised against melittin, we were able to demonstrate the occurrence of a calcium-dependent complex involving the enzyme, phospholipid substrate, and peptide.

Key words: Phospholipase; Peptide–enzyme complex; Melittin

1. Introduction

Phospholipases A₂ (PLA₂) (EC 3.1.1.4) are a family of lipolytic enzymes that specifically catalyze the hydrolysis of the 2-acyl ester bond in 3-*sn*-phosphoglycerides in the presence of Ca²⁺ [1]. These enzymes are encountered both inside and outside the cell. The extracellular enzymes are relatively small proteins (ca. 14 kDa) containing multiple disulfide bridges, and are found in high concentrations in mammalian pancreatic juice as well as in snake and bee venoms. The water-soluble forms of these extracellular enzymes are the most thoroughly studied and have been used as model systems for the less tractable, membrane-bound forms, as well as for the study of enzyme catalysis at lipid/water interfaces [2]. The lipid homogeneity as well as the presence of charged groups at lipid/water interfaces were found to affect the PLA₂ enzymatic activity [3]. Such interfacial charges can be provided by detergents, ionized fatty acids, head-groups of phospholipids or, interestingly, by membrane-bound peptides [4,5]. Special interest in the mechanism of PLA₂ action stems from its role as a model system for the study of calcium-mediated enzymatic events at the surface of

membranes, especially those that release arachidonate and other second messengers.

Melittin, an amphipathic 26-amino acid peptide that is one of the main constituents of honey bee venom, has been reported to enhance the activity of venom PLA₂ [6–10], as well as to show modest calcium-dependent phospholipase-like activity [11]. The observed synergistic effect of melittin with PLA₂ was proposed in earlier studies to be caused by structural perturbations of the lipid bilayer induced by melittin. This was found to correlate with a reorientation of the phospholipid 2-acyl ester bond. Although the function of melittin is known to be involved in bee venom's lytic activity, melittin has also been shown to stimulate phospholipase activity in rat tissue homogenates and to cause a dose-dependent release of arachidonic acid from rat peritoneal mast cells and cultured human fibroblasts [8]. Moreover, the possibility that mammalian cells also contain small peptides which facilitate phospholipase activity has not been excluded.

The present investigation was carried out to further determine whether the presence of small peptides, which have high binding affinity to artificial and biological membranes, influence the regulatory mechanism of PLA₂ activation. The role of the induced conformation upon binding to lipids was also studied using melittin and eight synthetic peptides that cover different structural arrangements for the membrane-bound peptide state. Moreover, the occurrence of a PLA₂–peptide complex was demonstrated using melittin antisera.

2. Materials and methods

2.1. Peptide synthesis

Peptides were prepared by simultaneous multiple peptide synthesis using *t*-butoxycarbonyl chemistry as described elsewhere [12]. Final cleavage and deprotection were carried out using a 'low-high' hydrogen fluoride procedure and a 24-vessel cleavage apparatus [13,14]. The peptides were purified by preparative reversed phase-high performance liquid chromatography (RP-HPLC) using a DeltaPrep 3000 system combined with a Foxy fraction collector (Millipore, Waters Division, San Francisco, CA, USA). Analytical RP-HPLC and matrix-assisted laser desorption time-of-flight mass spectroscopy (Kompact Maldi-Tof mass spectrometer — Kratos, Ramsey, NJ, USA) were used to determine the final purity and identity of the peptides.

2.2. Preparation of phospholipid liposomes

A mixture of egg yolk phosphatidylcholine (EPC) and bovine brain phosphatidylserine (PS; Sigma, St. Louis, MO, USA) at a molar ratio of 92:8 was dissolved in a chloroform/methanol (9:1 (v/v)) mixture and dried by a stream of nitrogen gas. The dried lipid was hydrated in 10 mM Tris-HCl buffer, pH 8.0, to a 6.25 mM final concentration, with repeat vortex mixing for 15 min. To generate small unilamellar vesicles (SUVs), the cloudy suspension was sonicated in an ice-water bath for 20 min using an ultrasonic generator equipped with a microtip probe (Vibra cell, Sonics and Materials, Danbury, CT, USA).

*Corresponding author. Fax: (1) (619) 455-3804.

**Permanent address: Departament de Bioquímica i Biologia Molecular. Universitat de València, E-46100 Burjassot, València, Spain.

***These authors contributed equally to this work.

Abbreviations: CD, circular dichroism; EPC, egg yolk phosphatidylcholine; LPC, lysophosphatidylcholine; OD, optical density; PBS, phosphate-buffered saline; PLA₂, phospholipases A₂; PS, phosphatidylserine; RBC, red blood cell; RP-HPLC, reversed phase-high performance liquid chromatography; SUV, small unilamellar vesicle.

2.3. PLA2 assay

It should be noted that the use of synthetic peptides in the present study eliminates the possibility of venom PLA2 contamination as found in the commonly used natural melittins. The PLA2 reaction mixture contained 10 mM Tris-HCl buffer, pH 8.0, 1.2 mM CaCl_2 , 2.85 mM SUVs, 0.5 μg enzyme, and varying peptide concentrations (5–90 μM). The reaction was run at 25°C for 10 min. 20 μl aliquots of the reaction mixture were withdrawn and boiled to stop the reaction. The simultaneous disappearance of phospholipid and appearance of its lyso derivative were monitored by normal phase HPLC as described elsewhere [15] using a Waters Resolve column (90 Å normal pore size).

2.4. Circular dichroism measurements

All measurements were carried out on a Jasco J720 circular dichroism spectropolarimeter (CD; Eaton, MD, USA) in conjunction with a Neslab RTE 110 waterbath and temperature controller (Dublin, CA, USA). CD spectra were the average of a series of 3–7 scans made at 0.2 nm intervals. CD spectra of buffer containing SUVs without peptide were used as a baseline in all of the experiments. Ellipticity is reported as the mean molar residue ellipticity $[\theta]$; the limits of error of measurements at 222 nm were $\pm 500 \text{ deg} \cdot \text{cm}^2 \cdot \text{dmol}^{-1}$. Peptide concentrations were determined by UV spectrophotometry in buffer and in the presence of 7.2 M guanidine-HCl, at 280 nm in the case of melittin and melittin-related peptides using the reported extinction coefficient for melittin in buffer ($\epsilon_{280} = 5570 \text{ M}^{-1} \cdot \text{cm}^{-1}$ [16], at 276 nm in the case of tyrosine-containing peptides using $\epsilon_{276} = 1450 \text{ M}^{-1} \cdot \text{cm}^{-1}$ [17] or by quantitative amino acid analyses (Multiple Peptide Systems, San Diego, CA, USA) for peptides without aromatic residues.

2.5. Hemolytic assay

The hemolytic activities of the peptides were determined using human red blood cells (RBCs). The blood was collected in heparin, maintained at 4°C, and used either the same day or the following day. The cells were washed three times with phosphate-buffered saline (PBS/35 mM phosphate buffer – 0.15 M NaCl, pH 7.0) and resuspended in PBS. The hemolytic activity of the peptides were determined as described [18] using 96-well tissue culture plates. In brief, 100 μl of a 0.5% RBC suspension was added to an equal volume of each peptide in PBS. The plates were incubated for 1 h at 37°C and the optical density (OD) of the supernatant was measured at 414 nm. The concentration of peptide necessary to lyse 50% RBCs (HD_{50}) was then determined for each peptide using a sigmoidal curve-fitting method (Graphpad, ISI, San Diego, CA, USA).

2.6. Melittin antisera and ELISA

New Zealand white rabbits were first injected with melittin (500 μg) in 1 ml PBS/complete Freund's adjuvant emulsion. Boosts were performed approximately every 30th day with melittin emulsified in incomplete Freund's adjuvant. Blood was collected 14 days after the fifth injection. Antisera were titrated against melittin by direct ELISA.

The direct ELISA used here is similar to the method previously described [19]. Briefly, melittin (10 $\mu\text{g}/\text{ml}$), PLA2, SUVs, lysophosphatidylcholine (LPC), as well as all combinations thereof, were adsorbed to 96-well polystyrene microtiter plates (A/2, Costar, Cambridge, MA, USA) in Tris buffer, pH 8.0, at the same relative concentrations used in the PLA2 assay mentioned above. Plates were incubated for 2 h at 37°C, washed with distilled water, and blocked with 1.5% BSA in PBS for nonspecific binding. Antisera were serially diluted two-fold and incubated for 1 h at 37°C. Goat anti-rabbit antibody-horseradish peroxidase conjugate (1:1000, Calbiochem, San Diego, CA, USA) was added to detect bound antisera. Plates were developed with OPD/ H_2O_2 (Sigma) for 10 min before stopping the reaction with sulfuric acid. Optical densities were measured at 492 nm on a Titertek Multiscan plate reader.

3. Results

3.1. Activation of bee venom PLA2 by melittin and melittin substitution analogues

The factors involved in the reported synergistic effect of melittin on PLA2 activity were investigated using two analogues of melittin, resulting from substitution of lysine-7 by

either a nonpolar amino acid (Ile, analog subK7I), or by a negatively charged amino acid (Glu, analog subK7E). Lysine-7 is the only positively charged amino acid not located at the C-terminus of the natural melittin, and was found earlier to be critical for both melittin's folding ability and biological activity [20]. Melittin and its two analogues were found to bind to artificial membranes with similar affinity [20], which was anticipated due to their identical charged C-terminal regions. Thus, we believe that the initial driving force for the interaction of melittin with phospholipid membranes involves electrostatic interactions between the highly positively charged C-terminus of melittin and the phospholipid head groups [21]. Conformational studies using CD spectroscopy showed that, upon increasing the ratio $[\text{SUVs}]/[\text{peptide}]$ (R_p), a gradual conforma-

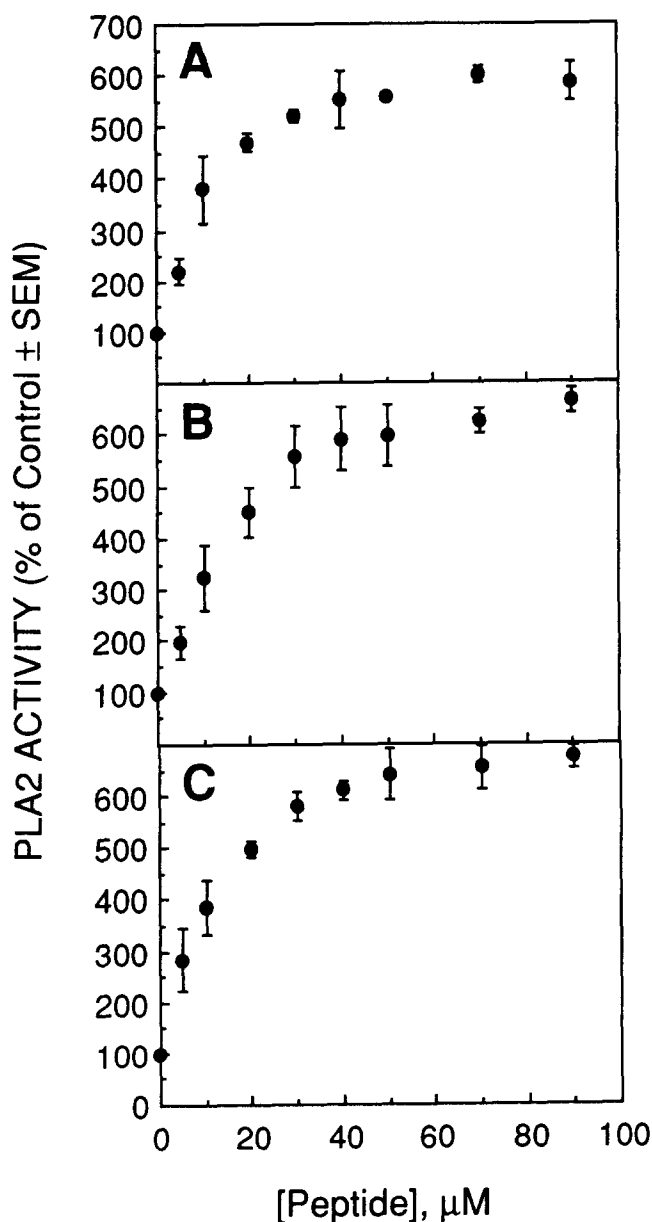


Fig. 1. Activation of bee venom phospholipase A_2 hydrolysis of SUVs by melittin (A), subK7E (B), and subK7I (C). The reaction was performed and monitored as described in section 2. Each point is the mean $\pm$ S.E.M. of three experiments made in duplicate.

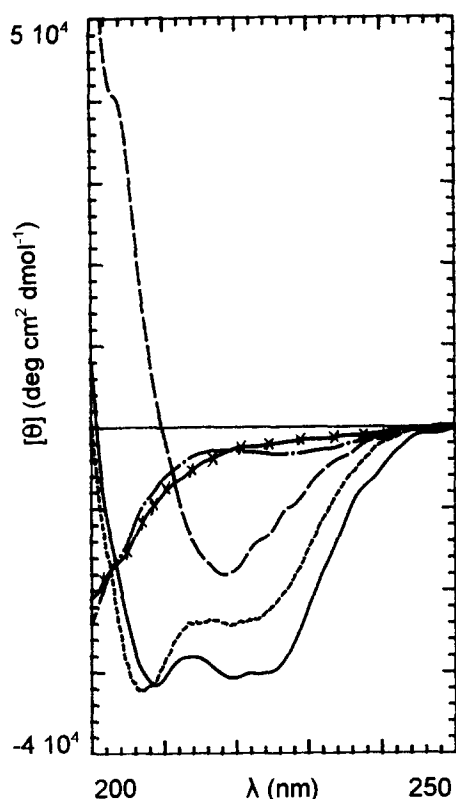


Fig. 2. CD spectra of representative peptides in the presence of SUVs. The CD spectra were recorded at a peptide concentration of 50 μ M in 5 mM MOPS buffer in the presence of EPC/PS SUVs at a ratio R_{ip} = 50. The mean residue ellipticities ($[\theta]$ in $\text{deg} \cdot \text{cm}^2 \cdot \text{dmol}^{-1}$) are shown for melittin (solid line), YLK (dotted line), KL (dashed line), GKP (crossed line), and GEP (centered line).

tional change occurred in which a random conformation converted to an α -helix for melittin and subK7E. In contrast, different degrees of β -structure were observed for subK7I at a low SUV content, while subK7I was predominantly α -helical at R_{ip} values higher than 30 [20]. These differences in folding ability were found to correlate with variations in hemolytic and antimicrobial activities [20].

Melittin, subK7E, and subK7I stimulated bee venom PLA2 equally (on a molar basis) in a dose-dependent manner, reaching a plateau at a common 40 μ M peptide concentration (Fig. 1). At this concentration, an increase in PLA2 activity of up to

approximately 500% of control values (i.e. without peptide added) was observed in the presence of any of the three peptides. It should be noted that under these experimental conditions (peptide concentration up to 80 μ M and R_{ip} values higher or equal to 35), the three peptides all adopt an α -helical conformation. This suggests that the presence of a positive charge at position 7 has no specific role in the peptide-induced enhancement of PLA2 activity.

3.2. Activation of bee venom PLA2 by peptides having different structural motifs

In order to study the importance of peptide conformation on the activation of PLA2, peptides were designed to adopt different structural motifs (α -helix, β -sheet and random coil – Table 1) upon binding to SUVs (phospholipid substrate used on the PLA2 activity experiments). The ellipticity values at 222 nm as determined by CD spectroscopy (Fig. 2) in the presence of saturating concentrations of SUVs, as well as the induced structural motifs for all the peptides tested, are described in Table 1. When assayed for PLA2 activation, only GEP, and the two truncation analogues of melittin had little effect on PLA2 activity. These results indicate that the enhancement of the catalytic activity of PLA2 is independent of the structural motif adopted by the peptide upon binding to the membrane. The weak effect seen for GEP may be explained by the fact that GEP, in contrast to the other peptides, does not bind to the SUVs. This is due to electrostatic repulsions between the negatively charged side chain of glutamic acid residues and the negative charge density of the SUVs. Although melittin's truncation analogues were found to have strong binding affinity to SUVs, resulting in the adoption of an α -helical conformation (Table 1), their weak PLA2 activation activity may be due to their short length as compared to the other peptides studied. Interestingly, no correlation was found between the hemolytic activity of these peptides and their ability to activate the enzyme (Table 1). Thus, peptides having very weak hemolytic activity, such as subK7I, KL and GKP, were found to enhance PLA2 activity to the same extent as the highly hemolytic peptides melittin, subK7E, and YLK. These results indicate that membrane disruption does not appear to be a necessary criterion for activation of PLA2. In contrast, perturbation of the membrane homogeneity resulting from the peptide binding to SUVs was found in this study to facilitate the activity of PLA2. Finally, it is noteworthy that none of the peptides were able to replace Ca^{2+} as a PLA2 effector, since no PLA2 activity could be detected in the presence of EDTA.

Table 1
Lipid-induced structural motifs in the presence of SUVs^a and biological activities of the peptides studied

Name	Peptide sequence	Motif	$[\theta]_{222}$ ($\text{deg} \cdot \text{cm}^2 \cdot \text{dmol}^{-1}$)	PLA2 Activity (% of control)	HD ₅₀ ($\mu\text{g/ml}$)
melittin	GIGAVLKVLTTGLPALISWIKRKRQQ-NH ₂	α	-27630	470 $\pm$ 50	2.55
subK7E	GIGAVLEVLTTGLPALISWIKRKRQQ-NH ₂	α	-31700	490 $\pm$ 30	4.00
subK7I	GIGAVLIVLTGLPALISWIKRKRQQ-NH ₂	α	-29460	460 $\pm$ 40	> 500
mel(1–13)	GIGAVLKVLTTGL-NH ₂	α	-30710	270 $\pm$ 60	> 500
mel(14–26)	Ac-PALISWIKRKRQQ-NH ₂	α	-26690	230 $\pm$ 30	> 500
YLK	YKLLKLLKLLKLLKLL-NH ₂	α	-23700	420 $\pm$ 40	4.08
KL	KLKLLKLLKLLKLLKLL-NH ₂	β	-14890	430 $\pm$ 30	> 500
GKP	KGKPGKPGKPGKPGKPGK-NH ₂	random	- 2400	400 $\pm$ 30	> 500
GEP	KGEPGEPGEPGEPGEPK-NH ₂	random	- 3040	120 $\pm$ 20	> 500

^a The CD spectra were recorded at a peptide concentration of 50 μ M and R_{ip} = 50.

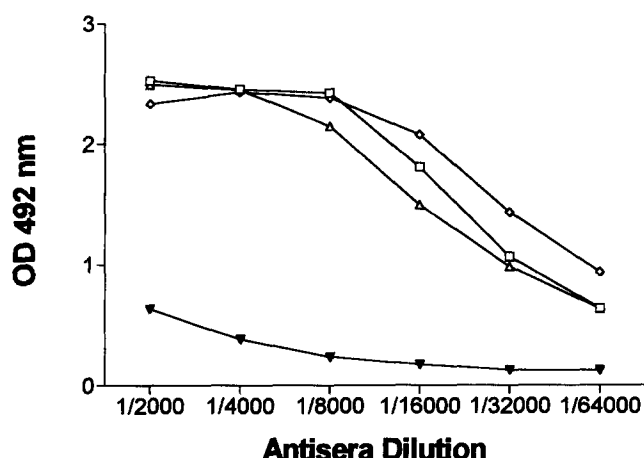


Fig. 3. Immunological evidence for melittin-PLA2 complexation. Melittin recognition by different dilutions of melittin antisera was determined under the same experimental conditions used in PLA2 activation experiments. The OD readings at 492 nm are plotted for melittin in the presence of SUVs (□), PLA2 (Δ), both SUVs and PLA2 (▼), and SUVs, PLA2 and EDTA (◇).

3.3. Immunological evidence of the formation of a peptide-PLA2 complex

To investigate the possible occurrence of a peptide-PLA2 complex in the presence of phospholipid membranes, immunological studies were carried out using melittin as a representative peptide model. Thus, melittin antisera were used to detect free melittin by ELISA. Fig. 3 shows the recognition of melittin at different dilutions of antisera under the same experimental conditions used in the PLA2 activation experiments. Excellent recognition was found for melittin in the presence of the substrate of the enzymatic reaction (SUVs) or in the presence of PLA2 (Fig. 3). However, a small decrease in recognition was observed in the presence of the hydrolysis product (LPC), with or without PLA2 (data not shown). In contrast, no significant recognition was found when PLA2 was added to melittin in the presence of SUVs under the initial conditions used for the PLA2 activation experiments, even at the highest antiserum concentration (dilution 1/2000 – Fig. 3). These results indicate that the formation of a complex between melittin and PLA2 occurs only in the presence of SUVs. Interestingly, when PLA2 was inactivated with EDTA, melittin was strongly recognized in the presence of SUVs and enzyme (Fig. 3). This later result supports the hypothesis that Ca^{2+} -activated PLA2 interacts with melittin. Using truncation analogues of melittin, the antisera were found to recognize the last four residues at the C-terminal region of melittin (data not shown). The loss of recognition observed for melittin may be explained by interactions between PLA2 and the C-terminal region of melittin.

4. Discussion and conclusion

The present studies resulted in several clarifying observations concerning the role of membrane-bound peptides in PLA2 activation. Despite the differences in their primary structure, or membrane-induced secondary structure, the peptides melittin, subK7E, subK7I, YLK, KL, and GKP were found to equally activate bee venom PLA2 only when the enzyme was activated

with Ca^{2+} . Membrane-active peptides such as melittin and mastoparan have been reported to facilitate phospholipase activity [6–10], which is known to be enhanced by alterations in lipid substrate packing caused by the presence of such peptides. However, the present studies show that nonlytic membrane-bound peptides may also enhance phospholipase activity regardless of their membrane-induced conformation. As was shown by the weak PLA2 activation observed for mel(1–13) and mel(14–26), the binding ability of peptides to SUVs, while essential, does not appear to be sufficient in itself for activation to occur.

Self-complexation of PLA2 from different sources has been reported to dramatically increase its catalytic activity [22]. In the present study, we have shown that peptide-induced activation of PLA2 correlates with the formation of a complex that involves not only the enzyme and peptide, but also the phospholipid substrate and calcium. Although the nature of this complex must be investigated further, one can envision that such peptide-enzyme complexes have properties similar to those resulting from self-complexes of PLA2.

Acknowledgements: The authors wish to thank Eileen Silva for editorial assistance. This work was supported by: National Institutes of Health Grant GM-45583; a Spanish Ministry of Science and Education post-doctoral fellowship to E.P.P. and short-term fellowship to I.M.; and Houghten Pharmaceuticals Inc., San Diego, CA.

References

- [1] Dennis, E.A. (ed.) (1991) Phospholipases, Methods in Enzymology, vol.197, Academic Press, San Diego, CA.
- [2] Dennis, E.A. (1983) The Enzymes 56, 307–353.
- [3] Burack, W.R. and Biltonen, R.L. (1994) Chem. Phys. Lipids 73, 209–222.
- [4] Conricode, K.M. and Ochs, R.S. (1989) Biochim. Biophys. Acta 1003, 36–43.
- [5] Rao, N.M. (1992) Biochem. Biophys. Res. Commun 182, 682–688.
- [6] Mollay, C. and Kreil, G. (1974) FEBS Lett. 46, 141–144.
- [7] Yunes, R., Golkhammer, A.R., Garner, W.K. and Cordes, E.H. (1977) Arch. Biochem. Biophys. 183, 105–112.
- [8] Argiolas, A. and Pisano, J.J. (1983) J. Biol. Chem. 258, 13697–13702.
- [9] Nishiya, T. (1991) J. Biochem. 109, 383–388.
- [10] Fletcher, J.E. and Jiang, M.-S. (1993) Toxicon 31, 669–695.
- [11] Pérez-Payá, E., Houghten, R.A. and Blondelle, S.E. (1994) Peptide Res. 7, 286–288.
- [12] Houghten, R.A. (1985) Proc. Natl. Acad. Sci. USA 82, 5131–5135.
- [13] Tam, J.P., Heath, W.F. and Merrifield, R.B. (1983) J. Am. Chem. Soc. 105, 6442–6455.
- [14] Houghten, R.A., Bray, M.K., De Graw, S.T. and Kirby, C.J. (1986) Int. J. Peptide Protein Res. 27, 673–678.
- [15] Mingarro, I., Abad, C. and Braco, L. (1994) Biochemistry 33, 4652–4660.
- [16] Quay, S.C., Condie, C.C. and Minton, K.W. (1985) Biochim. Biophys. Acta 831, 22–29.
- [17] Marqusee, S., Robbins, V.H. and Baldwin, R.L. (1989) Proc. Natl. Acad. Sci. USA 86, 5286–5290.
- [18] Blondelle, S.E., Simpkins, L.R., Pérez-Payá, E. and Houghten, R.A. (1993) Biochim. Biophys. Acta 1202, 331–336.
- [19] Appel, J.R., Pinilla, C., Niman, H. and Houghten, R.A. (1990) J. Immunol. 144, 976–983.
- [20] Pérez-Payá, E., Houghten, R.A. and Blondelle, S.E. (1995) J. Biol. Chem. 270, 1048–1056.
- [21] Batenburg, A.M., Hibbeln, J.C.L. and de Kruijff, B. (1987) Biochim. Biophys. Acta 903, 155–165.
- [22] Cordella-Miele, E., Miele, L. and Mukherjee, A.B. (1990) J. Biol. Chem. 265, 17180–17188.