# TOBACCO MOSAIC VIRUS–ENKEPHALIN CONJUGATES: RESISTANCE AGAINST SOLUBLE PROTEASES

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## 1. Introduction

The covalent deployment of agonist molecules on the surface of a rigid carrier such as TMV represents a new concept for obtaining agonists and antagonists with strongly enhanced potency and receptor affinity in pharmacological assays and in biochemical binding tests (review [1]). A specific example (among others) is provided by TMV–enkephalin conjugates, in which the potency and the affinity calculated per single enkephalin molecule is multiplied 10–50-fold by the attachment to the virons [2].

One of several possible explanations for the observed superpotency and superaffinity is based on the assumption of a simultaneous interaction between more than one agonist molecule of the TMV conjugate and more than one receptor of the cell or vesicle surface (cooperative affinity enhancement) [1]. This explanation is not easily proved. It is supported, in the case of TMV–adrenocorticotropin conjugates, by the observation of drastically reduced dissociation rates of the conjugate–receptor complexes in comparison to those of the complexes between receptors and free adrenocorticotropin [3]. The hypothesis of 'cooperative affinity enhancement' would furthermore require that no superaffinity effects be observed when TMV conjugates interact with single, solubilized receptors.

To investigate this last point, we chose as models for such receptors soluble enzymes that can easily hydrolyse free enkephalin peptides. Using reversed-phase, high-pressure liquid chromatography (HPLC) as the analytical system, we were able to show that the covalent attachment to TMV-virions strongly inhibited the degradation of enkephalin peptides. This finding supported the 'cooperative affinity' hypothesis without, however, proving it. It also suggested that reduced rates of proteolysis might account for at least a part of the observed superpotency and superaffinity effects in pharmacological systems.

In vivo, however, the degradation of enkephalins appears to be caused by membrane-bound enzymes [4,5]. In this case, 'cooperative affinity enhancement' might even increase the degradation rates. Preliminary studies on enkephalinase [4] in collaboration with Professor Roques in Paris have not yet been conclusive. In this work, we therefore focus our attention on the protection against soluble enzymes provided by the TMV virion, which might in itself be of general applicability in pharmacological and pharmaceutical research.

*Abbreviations:* Amino acids and peptides, according to the tentative rules and recommendations of the IUPAC–IUB Commission of Biochemical Nomenclature (1972) Eur. J. Biochem. 27, 201–207; TMV, Tobacco mosaic virus wild strain; ala, D-alanine; Nip, L-*p*-nitrophenylalanine; Malex, 6-maleimidohexanoyl

## 2. Materials and methods

### 2.1. *Enzymes and substrates*

Aminopeptidase M (EC 3.4.11.1, 50 U/mg) was purchased from Röhm and Haas GmbH; thermolysin (EC 3.4.24.4, 17 U/mg) from Serva; angiotensin-converting enzyme (EC 3.4.15.1, B grade, 1.5 U/mg) from Calbiochem; and neutral protease (EC 3.4.24.4) from Miles Labs. [Met$^5$]-enkephalin (**1**) and [Leu$^5$]-enkephalin (**2**) were purchased from Bachem AG, and [D-Ala$^2$, Leu$^5$]-enkephalin (**3**) from Peninsula Labs. [D-Ala$^2$, L-adamantylalanine$^5$]-enkephalin amide (**4**) was the product described in [6]. $N^{\alpha}$-[D-Ala$^2$, Leu$^5$]-


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enkephalyl-$N^{\epsilon}$-(6-maleimidohexanoyl)-lysine amide (**5**) and $N^{\alpha}$-[D-Ala$^2$, Nip$^4$, Leu$^5$]-enkephalyl-$N^{\epsilon}$-(6-maleimidohexanoyl)-lysine amide (**6**) were synthesized via a classical approach by reaction in solution with purification and analysis of intermediates (to be published separately).

The TMV–enkephalin conjugates were prepared by addition of the sulfhydryl group of [Lys(mercaptosuccinyl)$^{68}$]-TMV (TMV ~ SH) [7] to the maleimide double bonds of **5** or **6** as in [8]. The resulting TMV ~ S-**5** and TMV ~ S-**6** contained ~150 enkephalin residues bound covalently to the surface of the virions (estimation, see [7,8]).

### 2.2. *Incubations*

The enzyme solutions contained 0.2 mg enzyme/ml buffer solution (0.01 M Tris–HCl + 0.01 M $CaCl_2$, (pH 7.8); the $Ca^{2+}$ stabilizes thermolysine and neutral protease against autolysis and assures good activity).

The enkephalin solutions were obtained by dissolving 0.5 mg (0.5–0.8 μmol) of compounds **2**, **3** and **5**/ml of the buffer used for preparing the enzyme solutions. Compounds **1**, **4** and **6** (0.5 mg/ml) were dissolved in the same buffer containing 20% by volume of ethanol.

The reference TMV suspension contained 35 mg/ml (2.00 μmol) TMV protein. The TMV ~ S-**5** and TMV ~ S-**6** suspensions contained 30.2 mg/ml (1.73 μmol) TMV protein and 0.12 μmol/ml covalently attached **5** or **6**.

Solutions (50 μl) containing **1**–**6**, or 100 μl of the solutions containing TMV, TMV ~ S-**5** or TMV ~ S-**6**, were incubated with 20 μl (40 μl for the TMV series) of enzyme solution for 16 h at 37°C.

Samples (4 μl) of the incubations of **1**–**6** were injected into the HPLC column system without further treatment. The incubation mixtures containing TMV, TMV ~ S-**5** or TMV ~ S-**6** were freed of protein and nucleoprotein by micropartition ultrafiltration in the Amicon Micropartition System MPS-1. The ultrafiltrate (20 μl samples) was then subjected to HPLC.

As a control of the possible adsorption of the degradation products to either TMV or filtration membranes of the MPS-1 system, incubations were also made with 100 μl TMV solution, 30 μl enkephalin solutions (**1**, **3**) and 20 μl enzyme solution.

### 2.3. *High-pressure liquid chromatography*

HPLC was carried out with a gradient system composed of two Model 110-00 Altex pumps, a Rheodyne Model 712-20 sample injection valve with a 20 μl loop, a Model 1601 Gradient Master controller (Laboratory Data Control), and a Uvikon Model LCD 725 spectrophotometer.

The column was 25 cm long with an inner diameter of 3.2 mm. It was filled with LiChrosorb RP-8 (10 μm particles) by a balance-density packing technique.

The mobile phase was composed of 0.025 M phosphate buffer (pH 6.8) and acetonitrile (HPLC grade, Fluka). The detection was at 220 nm (0.02 absorbance units = full scale).

The solutions of the enkephalins **1**–**4**, before and after incubation with enzymes, were chromatographed at room temperature, ~20°C, with a linear gradient from 5–20% acetonitrile in 10 min (gradient I), at a flow rate of 2 ml/min. The solutions of the enkephalin **5** and **6** and the ultrafiltrates from the TMV suspensions were analysed before and after enzymic degradation with gradients from 5–30% acetonitrile in 10 min (gradient II) at the same flow rate.

The gradient elution program was followed by a 3 min washing step with 50% acetonitrile and, finally, the column was equilibrated with 5% acetonitrile.

### 2.4. *Degradation products*

The products obtained by enzymic hydrolysis were identified by comparison of their chromatographic retention times (HPLC) with those of analytically pure synthetic peptides and amino acids available in our laboratory. The reference compounds had the following retention times (min): Tyr (0.5), Leu (1.0), Met (1.1), Tyr–Gly–Gly (1.1), Phe (1.8), Tyr–ala–Gly (1.8), Phe–Met (6.6), Gly–Gly–Phe–Leu (8.0), Phe–Leu (8.6), **1** (9.6), **2** (11.1), **3** (11.7) in gradient I, and **4** (17.8), Lys(Malex)-$NH_2$ (6.3), Tyr–ala–Gly–Phe (6.4), Tyr–ala–Gly–Nip (7.3), Leu–Lys(Malex)-$NH_2$ (8.3), **5** (12.6), **6** (19.5) in gradient II. Possible ambiguities resulting from identical retention times, e.g., Met and Tyr–Gly–Gly, Phe and Tyr–ala–Gly, were usually easily resolved by taking into account the presence or absence of other, complementary degradation products. Thus, unambiguous assignments could be made for all major peptides and amino acids obtained by enzymic hydrolysis of our educts.

## 3. Results

The results are summarized in fig.1 and illustrated in fig.2.

Aminopeptidase cleaved the $Tyr_1$–$Gly_2$ peptide bond of **1** and **2**, but not the $Tyr_1$–$ala_2$ bond of **3**–**6**. Fig.2A shows the release of Tyr and Gly–Gly–Phe–Leu as main products from **2**. A minor compound with a retention time of 1.1 min was tentatively identified as Tyr–Gly–Gly, because it arose from both **1** and **2**; compounds with retention times corresponding to Phe–Met and Phe–Leu (here as a shoulder of the Gly–Gly–Phe–Leu peak) were also discerned.

With thermolysin, the identified products were Tyr–Gly–Gly (from **1** and **2**), Tyr–ala–Gly (from **3**), and Phe–Met or Phe–Leu, depending on the educt. Fig.2B shows a typical result obtained with **3** after 16 h incubation; with shorter incubation times (e.g., 6 h), substantial amounts of educt still emerged at 11.7 min and the product peaks were smaller.

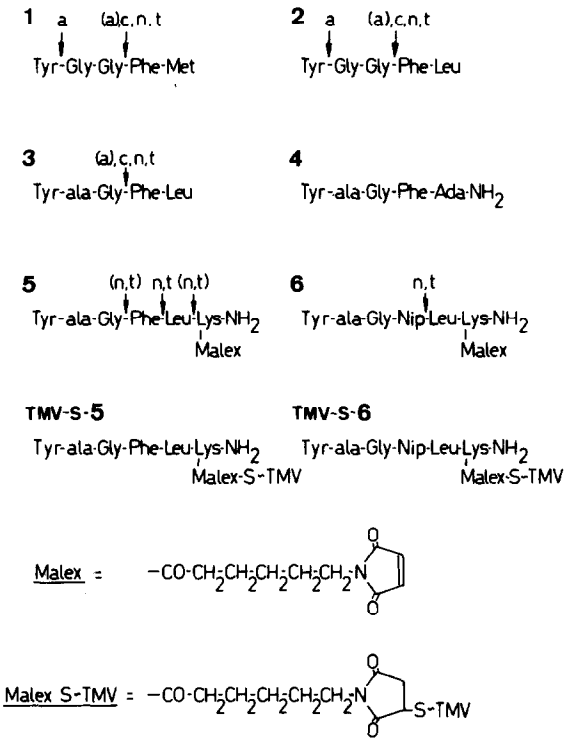


Fig.1. Enzymic degradation of enkephalins and their analogues. The points of hydrolysis are indicated by arrows: a, aminopeptidase; t, thermolysin; n, neutral protease; c, angiotensin-converting enzyme. Minor degradation is indicated by parentheses, e.g. (a).

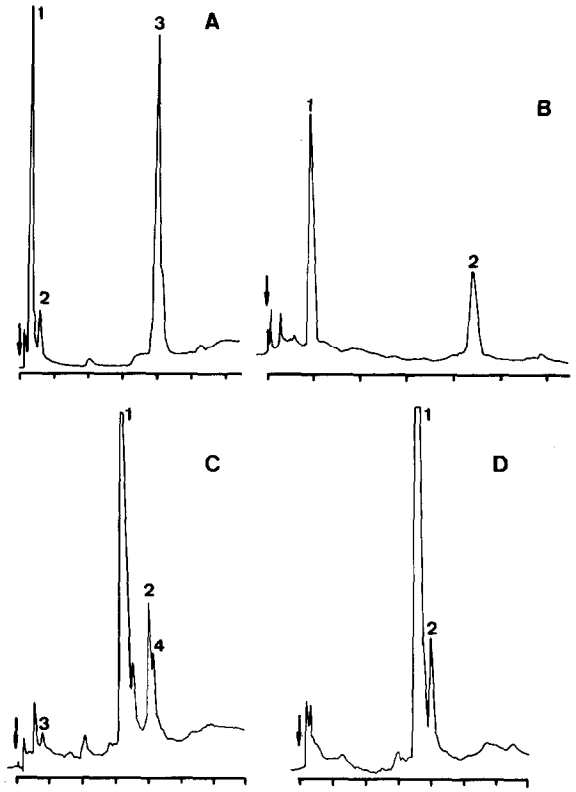


Fig.2. Typical HPLC tracings obtained from: **2** + aminopeptidase [(A) 1 = Tyr, 2 = Tyr–Gly–Gly, 3 = Gly–Gly–Phe–Leu + Phe–Leu]; **3** + thermolysin [(B) 1 = Tyr–ala–Gly; 2 = Phe–Leu]; **5** + thermolysin [(C) 1 = Tyr–ala–Gly–Phe, 2 = Leu–Lys(Malex)-$NH_2$, 3 = Tyr–ala–Gly, 4 = Tyr–ala–Gly–Phe–Leu]; **6** + thermolysin [(D) 1 = Tyr–ala–Gly–Nip, 2 = Leu–Lys(Malex)-$NH_2$].

Neutral protease and angiotensin-converting enzyme both produced essentially the same pattern with **1**–**3** as did thermolysin. Catalysis by angiotensin-converting enzyme was much weaker, however, due to the low activity of the preparation: after 16 h incubation, ~30–50% of **3** remained unchanged, whereas with thermolysin and neutral protease, only Tyr–ala–Gly and Phe–Leu were identified.

Adamantylalanine in position 5 completely inhibited the hydrolysis of **4** by all enzymes tested, as already demonstrated with thin-layer chromatography [6].

The hexapeptides **5** and **6** were both hydrolysed by neutral protease and thermolysin (angiotensin-converting enzyme was not tested), but in a different manner: **6** yielded only Tyr–ala–Gly–Nip and Leu–Lys(Malex)-$NH_2$ (fig.2D), whereas **5** yielded Tyr–

ala–Gly–Phe and Leu–Lys(Malex)-$NH_2$ as the major and Tyr–ala–Gly and Tyr–ala–Gly–Phe–Leu as minor products (fig.2C; Lys(Malex)-$NH_2$ was not resolved).

TMV ~ S-5 and TMV ~ S-6 were not hydrolysed in the presence of the different enzymes: no degradation products could be detected with the 2 gradient systems even after 24 h. Mixtures of TMV with **1** or **3**, however, yielded the expected degradation products of **1** and **3** with no decrease in peak strength. This indicates that the produced peptides are not significantly adsorbed to the virus or to the filtration membrane of the MPS-1 system.

## 4. Discussion

Reversed-phase HPLC was found to be a reliable and sensitive method for studying the enzyme-catalysed hydrolysis of the synthetic enkephalins (**1** and **2**) and their analogues and derivatives (**3**–**6**) on the 2.5 μg scale. The incubation mixtures containing 10 μg quantities of enzyme protein were directly injected into the columns. Mixtures containing tobacco mosaic virus in mg amounts were applied to the columns after removal of high-$M_r$ compounds by ultrafiltration/micropartition without loss of hydrolysis products.

The hydrolysis of [$Met_5$]-enkephalin (**1**), [$Leu_5$]-enkephalin (**2**), [$ala_2$, $Leu_5$]-enkephalin (**3**), and [$ala_2$, $Ada_5$]-enkephalin amide (**4**) as test compounds with the exopeptidase, aminopeptidase M, and the endopeptidases, thermolysin, neutral protease, and angiotensin-converting enzyme gave the expected, clear-cut results.

A minor cleavage of **1** and **2** between $Gly_3$ and $Phe_4$ by amino-peptidase to produce products with the retention times of Tyr–Gly–Gly and Phe–Met or Phe–Leu, respectively, is essentially unexplained. It probably reflects the presence of endopeptidase activity in the enzyme preparation and a small contamination of the commercial educts with [D-$Tyr_1$]-enkephalins. D-Tyrosine would inhibit the cleavage by amino peptidase, and the D-Tyr–Gly–Gly produced by endopeptidase activity would have the same retention time as L-Tyr–Gly–Gly, because of the enantiomeric, not diastereomeric, relationship between the two.

The same $Gly_3$–$Phe_4$ bond was the major point of hydrolysis of **1**–**3** by thermolysin (*Bacillus thermoproteolyticus*), neutral protease (*Bacillus subtilis*), and angiotensin-converting enzyme. These metalloendopeptidases thus behave like the enkephalinases $A_1$ and $A_2$ [4,5] which also appear to be Zn-containing enzymes [9]. Our observation supports the hypothesis of the Paris group that the active sites of the enkephalinases and of thermolysin have essential features in common [10,11]. The commonality of the active sites of thermolysin and of neutral protease has already been demonstrated by structural studies [12,13].

The reason for the comparatively weak action of angiotensin-converting enzyme in our examples was not studied in detail: it could be the low specific activity of the preparation and the fact that, in order to keep the chromatographic conditions constant, the pH and chloride concentrations were not optimal for this enzyme (pH 8.3 and 300 mM [14]). Clearly, the enzyme cleaved **1**–**3** in the same manner as thermolysin, neutral protease, and the enkephalinases [11].

We confirmed that the very hydrophobic and bulky side chain of adamantylalanine inhibits the hydrolysis of the $Gly_3$–$Phe_4$ peptide bond of **4** [6,16].

Having thus proved the usefulness of HPLC for our work, we turned to the study of compounds **5** and **6**, and of their TMV conjugates, TMV ~ S-5 and TMV ~ S-6.

Introduction of the C-terminal Lys(Malex)-$NH_2$ group had profound effects on the point of enzymic hydrolysis by thermolysin and neutral protease. In **5**, it moved the specificity of hydrolysis from the $Gly_3$–$Phe_4$ bond to the $Phe_4$–$Leu_5$ and, to a minor extent, the $Leu_5$–$Lys_6$ bonds. The *p*-nitrophenylalanine$_4$ of **6** so strongly favored the $Nip_4$–$Leu_5$ cleavage that hydrolysis at other points was not detected.

The attachment of **5** and **6** to [Lys(mercaptosuccinyl)$_{68}$]-TMV virions completely inhibited the enzyme-catalysed hydrolysis of the peptides. Control experiments with **1** and **3** in the presence of TMV showed that neither the catalytic action of the enzymes nor the recovery of the products of hydrolysis were impaired by the presence of the virus.

The observed resistance of TMV–enkephalin conjugates, which might partially explain their enhanced affinity and potency, raises new questions. In particular, why is the productive recognition of TMV-bound hormones by their receptors still possible whereas that by proteolytic enzymes is very strongly hindered? The study of such questions might provide new insights into receptor mechanisms and provide clues

for devising and preparing agonists with prolonged effects at the target-cell level [1].

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