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Research paper

Synthesis and characterization of an acylated di-peptide (Myr-Trp-Leu) with modified transmucosal transport properties

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Abstract

In order to improve the buccal absorption of a dipeptide model compound, Tryptophan-Leucine (Trp-Leu), we have synthesised a lipophilic derivative by myristoylation of the N- terminal amino group of Trp-Leu. The acylated peptide (Myr-Trp-Leu) was characterized by HPTLC, purified and isolated by chromatography on a silica gel column. Its structure was confirmed by ^{13}C and ^{1}H NMR and mass spectroscopy. The increased lipophilicity of the Myr-Trp-Leu was compared to that of the native peptide by chromatography and by its partition coefficient between *n*-octanol and saline phosphate buffer. In addition, the sensitivity towards hydrolytic enzymes was studied. The interaction of Trp-Leu with liposomes as model membranes was also studied. The phase transition temperature of dipalmitoylphosphatidylcholine (DPPC) was lowered in the presence of Myr-Trp-Leu, while it was increased in the presence of native parent peptide. Permeation experiments performed in vitro with pig buccal mucosa showed that the Myr-Trp-Leu accumulated in the tissue at the various concentrations tested. In contrast, the native peptide was able to pass through the membrane at all concentrations used. Lipophilic modification of the peptide by acylation drastically changes its behaviour towards tissue systems.

Keywords: Tryptophan-leucine; Lipophilic derivative; Myristoylation; Chemical modification; Acylated peptide; Dipalmitoylphosphatidylcholine liposomes; Buccal mucosal permeation

1. Introduction

Peptides with therapeutic potential are a new and promising development. However, their administration to patients poses many problems due to their size and/or to their polar properties. Transepithelial permeation is a potentially interesting approach since it is relatively non-invasive and provides the possibility to control dosage over a long period of time. However, transdermal application can be difficult owing to the specific physico-chemical properties of these therapeutic agents. Because of their polarity, peptides are very poorly transported across biological membranes and since polypeptide size can also vary to a large extent, this can also hinder transport. Parenteral administration is also problematic because of the short half-lives of these drugs [1].

Various strategies can be considered in order to facilitate peptide penetration through physical (i.e. membrane) and metabolic barriers. One is the modification of the lipophilic properties of the compound since a certain degree of lipid solubility is known to be necessary for their passive transport across mucosal membranes.

Chemical modification of peptide drugs has been shown to result in an increase of their molecular weights [2] and lipophilicity [1,3–5]. Therefore, their in vivo pharmacokinetic behaviour has also been observed to be altered [6,7]. Covalent conjugation of lipidic amino-acids and peptides to poorly absorbed peptides and drugs can enhance the passage of pharmacologically active compounds across biological membranes and therefore promote activity [8]. Furthermore, it has been reported that chemically modified peptides can increase their resistance towards enzymatic degradation [9,10]. Hashizume et al. [11] have shown that chemical modification of insulin with palmitic acid not only increases the lipophilicity of insulin but also reduces its degradation.

In order to improve the buccal absorption of a model compound (Trp-Leu), we have synthesised a novel lipophilic derivative by acylation with myristic acid of the amino terminal of peptide and have compared its properties by different methods with those of the unacylated peptide. Furthermore, the UV absorption and the fluorescence properties of the tryptophan residue enable spectrophotometric

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determinations to be made. Myristic acid was selected as the acylating group because of its presence at the N-terminal end of many natural proteins. Its resistance to oxidation and its relatively high fluidity are also advantageous properties.

2. Materials and methods

2.1. Materials

2.1.1. Reagents

All reagents were of analytical grade. Tryptophan-leucine (Trp-Leu) and myristoyl chloride were purchased from Sigma Co (St Louis, MO). Carboxypeptidase A (EC 3.4.17.1) and aminopeptidase (EEC no. 232-874-6) were obtained from the same source. All other chemicals were of the highest purity grade and were purchased from Fluka (Buchs, Switzerland). Water was ion-exchanged, distilled and passed through a milli-Q water purification system before use (Millipore Corp., Bedford, MA).

2.1.2. Chromatography

High performance, silica gel 60 thin-layer chromatography plates, 10 x 20 cm without a concentration zone, were obtained from Merck (Darmstadt, Germany). For preparative chromatography, a column of 20 cm with an internal diameter 1.2 cm was prepared using silica G 260, 100 mesh, purchased from Fluka (Buchs, Switzerland).

2.2. Experimental procedures

2.2.1. Synthesis of the tryptophan-leucine derivative

Trp-Leu (50 mg) was dissolved in 5 ml of sodium bicarbonate 1M. Myristoyl chloride (a five fold molar excess with respect to Trp-Leu) was dissolved in 0.5 ml of dioxane. Then, the two solutions were vigorously stirred, first at room temperature for 1 h, then, at 60°C for 30 min. The solution was adjusted to pH 4.4 with dilute acetic acid. The product was then extracted by chloroform. Centrifugation was used to separate the aqueous phase from the lower organic layer. Its purity was analysed by HPTLC with a $CHCl_3$/MeOH/H_2O (65:25:4) solvent mixture. The presence of unreacted myristic acid was shown by thin layer chromatography and detection with iodine vapour. The fatty acid was eliminated by chromatography on a silica gel G 260 column. Myristic acid was eluted by four volumes of a petroleum ether/ether (90:10) mixture and the acylated peptide was recovered with two volumes of mixture $CHCl_3$/MeOH (50:50). The solvent was evaporated under argon and the derivative was dried for two days under vacuum. It was stored at -20°C before use. At this stage, the extent of contamination of the product by myristic acid was found to be below 5% as assessed by HPTLC. The overall molecular yield after purification was about 70%.

2.2.2. Characterization of Myr-Trp-Leu

The structure of Myr-Trp-Leu was confirmed by ^{13}C and ^{1}H NMR spectra recorded using a Bruker AMX-400 spectrometer (9.4 Tesla) Romand High-Field NMR Facility and a Bruker AMX-2-600 (14.1 Tesla). Samples (each about 10 mg) were dissolved in 0.5 ml of $CDCl_3$ 99.8%. This solution was transferred to a NMR tube (Wilmad 507-PP) adding three drops of C_6D_6. Myr-Trp-Leu composition was also confirmed by mass spectroscopy (Finnigan SSQ 7000). Samples were dissolved in methanol and analyzed with a continuous flow injection technique. Electron spray mode was used.

2.2.3. Measurement of the apparent partition coefficient

The unacylated peptide (300 μg) was dissolved in 2 ml of 0.02 mM sodium phosphate buffer (PBS) pH 7.4. The same volume of *n*-octanol was added. The mixture was shaken for 24 h at room temperature. Due to the low water solubility of the acylated peptide, 300 μg was dissolved in 2 ml of *n*-octanol and 2 ml of PBS was added and the solution was shaken at room temperature. Phase separation was achieved by centrifugation and the concentrations in each phase were measured by spectrophotometry, at 274 nm for Trp-Leu and 280 nm for Myr-Trp-Leu.

2.2.4. Determination of Trp-Leu and Myr-Trp-Leu solubility

An excess of Trp-Leu or Myr-Trp-Leu was added to the medium used for permeation experiments [12] (ethanol: saline phosphate buffer pH 7.4 30: 70 v/v), and the mixture was shaken at 37 ± 1°C for 24 h. The suspension was centrifuged and the supernatant was filtered through a membrane filter (0.45 μm, polypropylene microcentrifuge tube filters, Whatman, Maidstone, UK). The concentration of Trp-Leu or Myr-Trp-Leu was determined by spectrophotometry at 274 and 280 nm, respectively. Solubility in other solvents was determined using the same procedure.

2.2.5. The behaviour of Myr-Trp-Leu towards enzymes

Since the peptides were very poorly soluble in water the epithelial permeation experiments had to be performed in the presence of ethanol. Therefore, the sensitivity to hydrolytic enzymes had to be determined under the same conditions. A method suitably adapted from a protocol already described in the literature was used [13]. Trp-Leu and Myr-Trp-Leu were dissolved in 1 ml of a mixture of ethanol/tris-HCl pH 8.0, 0.1 M buffer 30/70 v/v. Each peptide was used at 1.6 mM and incubated for 10 and 20 min in the presence of three units of each enzyme in a total volume of 500 μl. The reaction was stopped by the addition of 150 μl of HCl. A phosphate buffer (50 mM pH 6.0) was chosen instead of citrate buffer in order to avoid any interference in the colorimetric assay method which consisted of using ninhydrin-cyanide to detect the amino groups fixed by hydrolysis. Readings were taken at 570 nm with a spectrophotometer (DU® 65 Beckman) of a solution without enzyme and compared to similar samples quenched with HCl before the addition of enzyme.

2.2.6. Interactions with liposomes

Two ml chloroform-methanol (1-1 v/v) solution containing dipalmitoylphosphatidylcholine (DPPC) 70 mg/ml was evaporated in a Corex tube under argon. One ml of phosphate buffer pH 7.4 100 mM was added and the resulting mixture subjected to sonication for 20 min at room temperature (MSE 100 W at 20 kHz) [14]. The opalescent suspension was centrifuged at 5000 g for 10 min and the supernatant was carefully collected. Control liposomes, liposomes with Trp-Leu and liposomes with Myr-Trp-Leu were analyzed by differential scanning calorimetry (DSC; SEIKO, Switzerland) in sealed sample chambers with a heating rate of 4°C/min. Myr-Trp-Leu or Trp-Leu were added to a molar reaction of 1 mole peptide for 10 moles of phospholipids.

Interactions of the peptides with liposomes were also studied by fluorimetry (Perkin–Elmer LS-5B, Switzerland) with a fixed excitation wavelength at 280 nm and emission recorded between 300 and 400 nm to follow the intrinsic fluorescence of tryptophan. For the fluorescence experiments, the phospholipid concentrations of the liposomes were adjusted to 100 μg/ml with the same liposome to peptide (w/w) ratio as for differential scanning calorimetry.

2.2.7. Permeation experiments

Permeation measurements of Trp-Leu and Myr-Trp-Leu were performed as described [12] using buccal (cheek) pig mucosa. Essentially, the tissue was mounted in a modified Franz diffusion cell [15] (area = 0.78 cm^2). The tissue was previously equilibrated for 30 min with the diffusion medium before addition of 0.5 ml of a solution of Trp-Leu or Myr-Trp-Leu at different concentrations (2; 4; 6 mg/ml in ethanol: saline phosphate buffer, pH 7.4, 30:70 v/v) in the donor compartment. The receptor solution, which was magnetically stirred, consisted of 2.2 ml of the same ethanol-buffer medium. The device was maintained at 37°C. After 24 h incubation, peptide concentration were assayed by HPTLC in the donor and the receptor compartments and in the tissue after extraction [12].

2.2.8. High performance thin-layer chromatography (HPTLC)

The contents of the various compartments were assayed using HPTLC on Silica gel 60 (Merck, Darmstadt, Germany) using the Camag Linomat IV (Camag, Muttenz, Switzerland). Calibration curves were obtained by application of known amounts of standards. Migration was performed in chloroform:methanol:water (16:6:1). The dried plates were scanned for UV absorption (in the Refl-Abs mode) with a CAMAG TLC Scanner II, Switzerland at 274 nm for Trp-Leu and at 280 nm for Myr-Trp-Leu. Quantifications were obtained by integration of the signals with a Camag SP4290 (Switzerland).

3. Results and discussion

3.1. Structure of product

The lipophilic derivative (Myr-Trp-Leu) was synthesised by covalent attachment of myristic acid to the terminal amino group. Myr-Trp-Leu was purified and isolated by chromatography on a silica gel column. The product was found to be more than 95% pure. Its structure was confirmed by ^{13}C and ^{1}H NMR analysis and by two-dimensional NMR analysis. The DQF-COSY experiments at 9.4 Tesla revealed all the proton spin systems which correspond to the Trp and Leu residues. The myristoyl chain was also identified using the same technique. N-terminal acylation was proved by the chemical shift of the amide carbon observed in ^{13}C spectroscopy. The full assigment of the carbon network was based on C,H heteronuclear correlation (HETCOR). The particular case of quaternary carbon assignment attribution was solved by GRASP-HMQC (Gradient Accelerated Spectroscopy) experiments optimized for the observation of C,H long range couplings over two or three bonds. Myr-Trp-Leu was also analysed by mass spectroscopy. Molecular ions of Myr-Trp-Leu were identified at *m/z* 550 and at *m/z* 566.

3.2. Properties and sensitivity to enzymes

Physicochemical properties of Trp-Leu and Myr-Trp-Leu were compared with the lipophilic characteristics of the product: partition coefficients were determined using *n*-octanol as the organic phase and phosphate buffer (pH 7.4). The partition coefficient of Trp-Leu was -0.68 whereas for Myr-Trp-Leu it was 1.04.

Myr-Trp-Leu was readily soluble in chloroform, soluble in propylene glycol and in a mixture of ethanol/phosphate buffer pH 7.4 30/70 v/v (4.34 mg/ml) and practically insoluble in phosphate buffer pH 7.4 (3.13×10^{-2} mg/ml). Chromatographic properties were studied by thin-layer-chromatography on silica gel. The R_f value of Myr-Trp-Leu was 0.81 as compared to 0.41 for the native peptide. These data indicated that myristoyl derivatization of Trp-Leu dramatically enhanced its lipophilicity. Muranishi et al. [16] and Yamada et al. [17] showed similar results with lauroyl thyrotropin-releasing hormone.

Lee and Yamamoto [18] showed that acylation modified the susceptibility of the derivative towards the action of proteolytic enzymes amino- and carboxy- peptidases. When Trp-Leu and Myr-Trp-Leu where incubated in the presence of purified carboxypeptidase A, they were found to be highly susceptible to hydrolysis in the ethanol containing buffer. As expected Myr-Trp-Leu was not degraded by aminopeptidase while the free peptide was broken down. The presence of ethanol was shown not to prevent the hydrolytic activity 'in vitro'. The acylamide group is not recognized by the aminopeptidase. Resistance to enzyme action has been observed with thyrotropin releasing

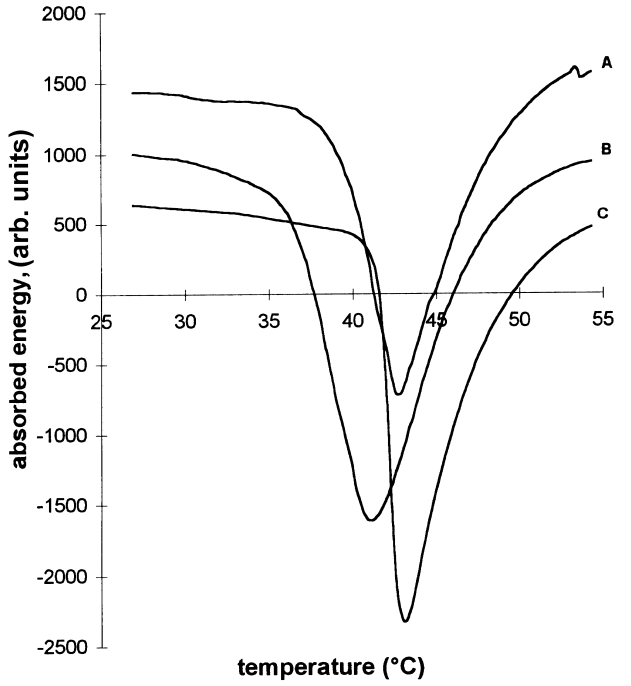

Fig. 1. Phase transition temperature of dipalmitoylphosphatidylcholine liposomes. (A): liposomes; (B): Myr-Trp-Leu + liposomes; (C): Trp-Leu + liposomes.

hormone [17], tetragastrin [19] and insulin [20]. Therefore, the acylated peptides, with an appropriate lipophilicity, improve passive transport [21] but may also improve stability against various enzymes.

3.3. Interaction with liposomes

Liposomes prepared with a single phospholipid are known to be excellent model systems with which to study interactions of peptides with membranes [22,23]. The phase transition temperature of DPPC liposomes was observed to be 38.7°C under our experimental conditions. Addition of unacylated Trp-Leu (Fig. 1) at a molar ratio of 1 peptide to 10 phospholipids shifted the transition temperature up to 41.2°C indicating a rigidification of the bilayer membrane. Addition of the myristoylated peptide at a similar ratio decreased the transition temperature to 35.7°C. Such an effect may be due to the insertion of the myristoyl arm into the lipidic phase of the liposome bilayer [23].

The intrinsic fluorescence of tryptophan can be used as an indicator of the polarity of its environment. The fluorescence of Trp-Leu did not change when measured in the presence of liposomes, while the myristoylated derivative showed a strong increase of emission intensity accompanied by a 'blue' shift (Fig. 2). The results of differential scanning calorimetry and of tryptophan fluorescence are both consistent with the hypothesis that the unmodified peptide interacts mainly with the polar head group of the phospholipid while the acylated group is inserted into the hydrophobic part of the bilayer in agreement with the observation reported by Moscho et al. [22].

These results suggest that the nature of the interaction with the membrane is different depending on whether the free or the myristoylated peptide added to the liposome preparation. The opposite behaviour of the two peptides towards the lipid membranes shows the significance of the change in the properties introduced by acylation.

3.4. Permeation experiments

The difference in physico-chemical properties of the myristoylated- and native peptides resulted in large differences in behaviour when tested for permeation in the buccal epithelium system. The most striking observation with the Myr-Trp-Leu was its accumulation in the tissue at all concentrations of peptide tested (Fig. 3). The greater affinity of this peptide for the tissue is probably due to its high lipophilicity resulting in its dissolution into the lipidic phases of the biological membranes. The behaviour of Myr-Trp-Leu, with its long acyl chain towards buccal epithelium is consistent with the interactions with liposomes reported in the present paper.

In comparison, the native peptide (Trp-Leu) was able to pass into the receptor compartment (Fig. 4). But transport was greatest at lower concentration (2 mg/ml) where 55% of the dipeptide was found in the receptor compartment. In contrast, at the highest concentration (6 mg/ml) tested, transport was decreased and only 12% of Trp-Leu was found in the receptor. Further work is currently underway to explain these unexpected results.

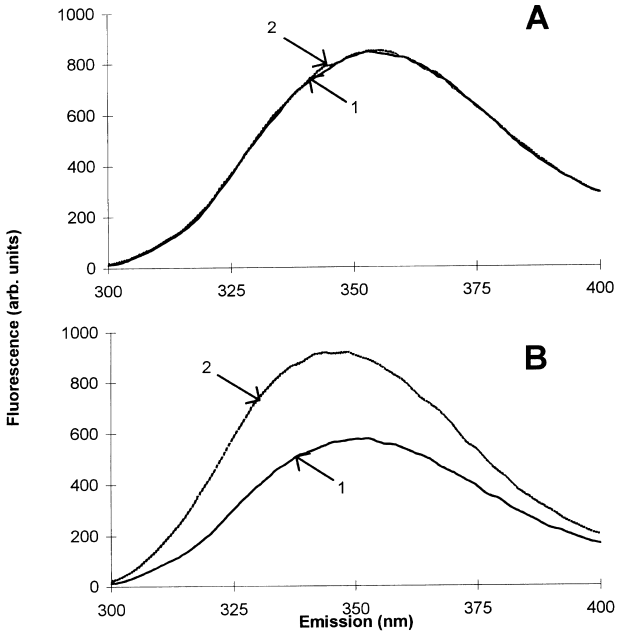

Fig. 2. Fluorescence spectra were recorded at 100 μg/ml at 280 nm excitation. (A) Trp-Leu; (B) Myr-Trp-Leu. (1) In the absence of liposomes; (2) in the presence of liposomes.

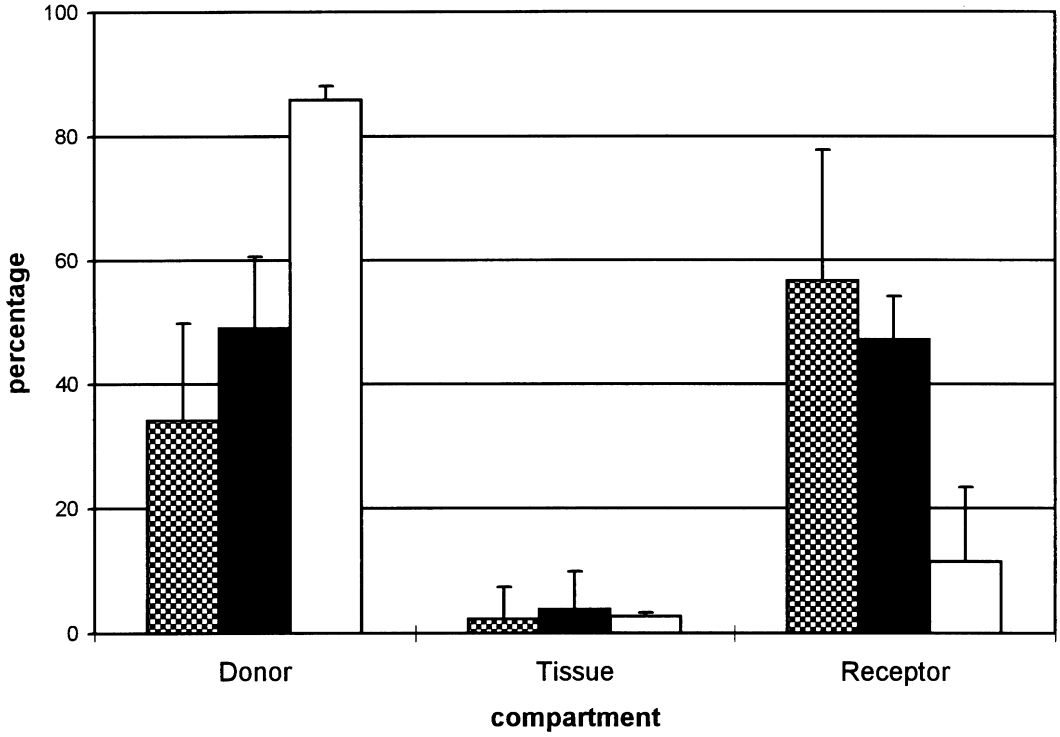

Fig. 3. Percentage of Trp-Leu determined in different compartments as a function of concentration after 24 h in permeation experiments with buccal mucosa ($n = 6 \pm$ SD): (▤) 2; (■) 4; (□) 6 mg/ml.

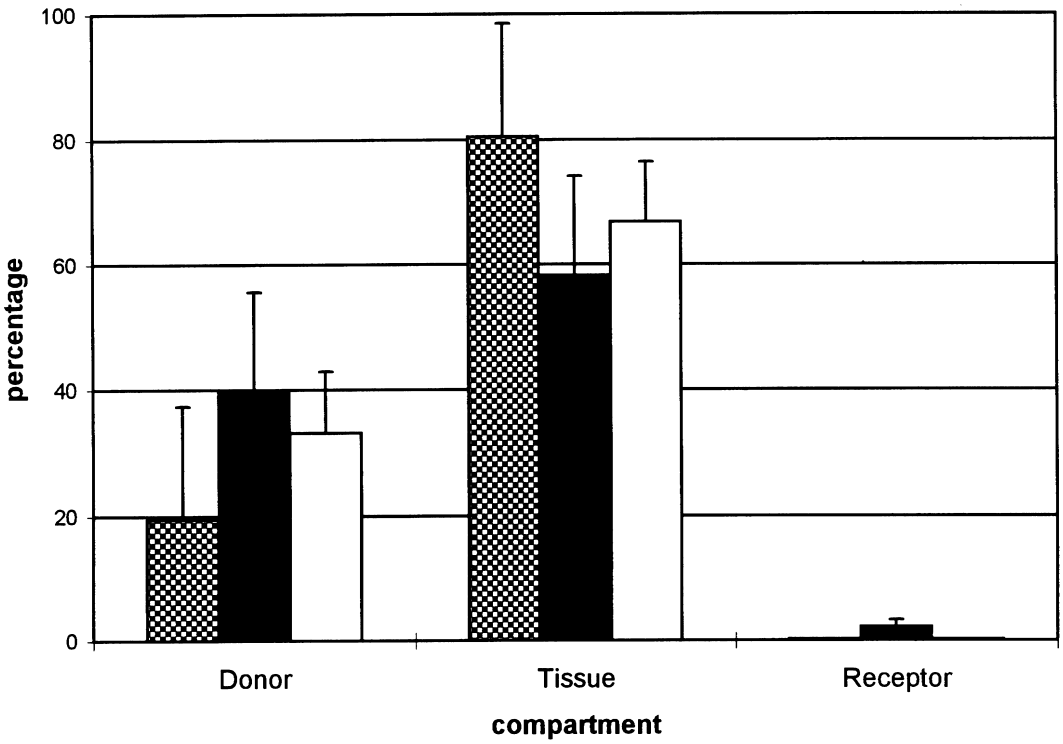

Fig. 4. Percentage of Myr-Trp-Leu determined in different compartments as a function of concentration after 24 h in permeation experiments with buccal mucosa ($n = 6 \pm$ SD): (▤) 2; (■) 4; (□) 6 mg/ml.

4. Conclusion

The acylation procedure presented here is relatively straightforward as compared with other methods such as those reported by Hashimoto et al. [1] or Muranishi et al. [16,24]. The method of the reaction is sufficiently general so that it could be applied to other peptides. A good yield was reproducibly obtained. Myr-Trp-Leu was lipophilic and showed a strong interaction with liposomes and buccal epithelium. As expected N-terminal acylation made the peptide resistant towards degradation by aminopeptidase while its sensitivity to carboxypeptidase was not improved despite its high lipophilic properties.

Our primary interest was to examine the effect of peptide modification on buccal delivery. In a preceeding study [12] and here, we have demonstrated that acylation of Trp-Leu with myristic acid increased the lipophilicity of the product, which resulted in a greater affinity of Myr-Trp-Leu to the membrane components and its accumulation in the tissue. Accumulation in the tissue could however present, in some cases, new properties that could be exploited.

References

[1] M. Hashimoto, K. Takada, Y. Kiso, S. Muranishi, Synthesis of palmitoyl derivatives of insulin and their biological activities, Pharm. Res. 6 (1989) 171–176.

[2] T. Fujita, H. Yasuda, Y. Takakura, M. Hashida, H. Sezaki, Alteration of biopharmaceutical properties of drugs by their conjugation with water-soluble macromolecules: uricase-dextran conjugates, J. Control. Rel. 11 (1990) 149–156.

[3] H. Bundgaard, J. Moss, Prodrugs of peptides (Part 6): bioreversible derivatives of thyrotropin-releasing hormone (TRH) with increased lipophilicity and resistance to cleavage by the TRH-specific serum enzyme, Pharm. Res. 7 (1990) 885–892.

[4] J. Moss, A. Buur, H. Bundgaard, In vitro study of intestinal metabolism and penetration of thyrotropin-releasing-hormone (TRH) and its prodrug, Int. J. Pharm. 66 (1990) 183–191.

[5] T. Temna, E. Yodoya, S. Tashima, T. Fujita, M. Murakami, A. Yamamoto, S. Muranishi, Development of new lipophilic derivatives of tetragastrin: physicochemical characteristics and intestinal absorption of acyl-tetragastrin derivatives in rats, Pharm. Res. 10 (1993) 1488–1492.

[6] T. Fujita, M. Nishikawa, Y. Ohtsubo, Y. Ohno, Y. Takakura, H. Sezaki, M. Hashida, Control of in vivo fate of albumin utilizing combined chemical modification, J. Drug. Targeting. 2 (1994) 157–165.

[7] Y. Takakura, T. Fujita, H. Furitsu, M. Nishikawa, H. Sezaki, Pharmacokinetics of succinylated proteins and dextran sulfate in mice. Implications for hepatic targeting of protein drugs by direct succinylation via scavenger receptors, Int. J. Pharm. 105 (1994) 19–29.

[8] R.A. Hughes, I. Toth, P. Ward, S. Ireland, W.A. Gibbons, Lipidic peptides. III: lipidic amino acid and oligomer conjugates of morphine, J. Pharm. Sci. 80 (1991) 1103–1105.

[9] L. Laster, J.H. Walsh, Enzymatic degradation of C-terminal tetrapeptide amide of gastrin by mammalian tissue extracts, Fed. Proc. 27 (1968) 1328–1330.

[10] M. Haga, K. Saito, K. Ikegaki, F. Sato, N. Yokoo, Hypoglycemic effect of intestinally administered monosaccharide-modified insulin derivatives in rats, Chem. Pharm. Bull. 38 (1990) 1983–1986.

[11] M. Hashizume, T. Douen, M. Murakami, A. Yamamoto, K. Takada, S. Muranishi, Improvement of large absorption of insulin by chemical modification with palmitic acid in rats, J. Pharm. Pharmacol. 44 (1991) 555–559.

[12] F. Veuillez, A. Ganem-Quintanar, J. Deshusses, F. Falson-Rieg, P. Buri, Comparison of the ex-vivo oral mucosal permeation of tryptophan-leucine (Trp-Leu) and its myristoyl derivative, Int. J. Pharm. 170 (1998) 85–91.

[13] S.S. Tate, Microvillus membrane peptidases that catalyse hydrolysis of cysteinylglycine and its derivatives, in: S.P. Colowick, N.O. Kaplan (Eds.), Methods in Enzymology, Academic Press, New York, 1985, pp. 471–480.

[14] J. Gruenberg, D. Coral, A.L. Knupfer, J. Deshusses, Interactions of liposomes with trypanosoma brucei plasma membrane, Biochem. Biophys. Res. Comm. 88 (1979) 1173–1179.

[15] D.R. Friend, In vitro skin permeation techniques, J. Control. Rel. 18 (1992) 235–248.

[16] S. Muranishi, A. Sakai, K. Yamada, M. Murakami, K. Takada, Y. Kiso, Lipophilic peptides: synthesis of lauroyl thyrotropin-releasing hormone and its biological activity, Pharm. Res. 8 (1991) 649–652.

[17] K. Yamada, M. Murakami, A. Yamamoto, K. Takada, S. Muranishi, Improvement of intestinal absorption of thyrotropin-releasing hormone by chemical modification with lauric acid, J. Pharm. Pharmacol. 44 (1991) 717–721.

[18] V.H.L. Lee, A. Yamamoto, Penetration and enzymatic barriers to peptide and protein absorption, Adv. Drug. Del. Rev. 4 (1990) 171–207.

[19] E. Yodoya, K. Uemura, S. Tashima, T. Fujita, M. Murakami, A. Yamamoto, S. Muranishi, Enhancement permeability of tetragastrin across the intestinal membrane and its reduced degradation by acylation with various fatty acids in rats, J. Pharmacol. Exp. Ther. 271 (1994) 1509–1513.

[20] H. Asada, T. Douen, Y. Mizokoshi, T. Fujita, M. Murakami, A. Yamamoto, S. Muranishi, Stability of acyl derivatives of insulin in the small intestine: relative importance of insulin association characteristics in aqueous solution, Pharm. Res. 11 (1998) 1115–1120.

[21] S. Muranishi, A. Yamamoto, Modification of peptides for intestinal delivery, in: D.J.A. Crommelin, K.K. Midha, T. Nagai (Eds.), Topics in Pharmaceutical Sciences, Medpharm Scientific, Stuttgart, 1994, pp. 373–382.

[22] A. Moscho, O. Orwar, D.T. Chiu, B.P. Modi, R.N. Zare, Rapid preparation of giant unilamellar vesicles, Proc. Natl. Acad. Sci. USA (1996) 11443–11447.

[23] N. Muranishi, Y. Nakajima, M. Kinugawa, S. Muranishi, H. Sezaki, Mechanism for the inducement of the intestinal absorption of poorly absorbed drugs by mixed micelles II. Effect of the incorporation of various lipids on the permeability of liposomal membranes, Int. J. Pharm. 4 (1980) 281–290.

[24] S. Muranishi, M. Murakami, M. Hashizume, K. Yamada, S. Tajima, Y. Kiso, Trials of lipid modification of peptide hormones for intestinal delivery, J. Control. Rel. 19 (1992) 179–188.