Effect of Conjugation with Polypeptide Carrier on the Enzymatic Degradation of Herpes Simplex Virus Glycoprotein D Derived Epitope Peptide

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Two conjugates with epitope peptide ^{278}LLEDPVGTVA^{287} derived from glycoprotein D (gD-1) of Herpes simplex virus (HSV) were synthesized for analysis of the effect of conjugation on protection against enzymatic degradation. In this design, the turn-forming epitope core ^{281}DPVG^{284} was positioned in the central part of the peptide and elongated by three amino acids from the native sequence at both termini. Conjugation was achieved by the introduction of amide bond or thioether linkage between the C-terminal of the HSV peptide and the side chain of four lysine residues of the oligotuftsin derivative used as carrier molecule. We compared the proteolytic stability of the conjugates in diluted human sera as well as in rat liver lysosomal preparation. The data obtained in lysosomal preparation at two pH values (pH 3.5 and 5.0) show that the type of covalent bond between the carrier and the epitope peptide had no significant effect, as compared to the stability of the free, unconjugated peptide. Based on the identification of degradation fragments by mass spectrometry we found marked differences in the lengths and amounts of oligopeptides obtained. In contrast, in 10% and 50% human serum the conjugation provided full protection against enzymatic hydrolysis over 96 h, while the free peptide was decomposed quickly.

INTRODUCTION

One of the major limitations in the use of peptides as effective subunit vaccines is their instability in biological matrices, which is mainly due to the rapid degradation by proteases. To protect biologically active peptides from enzymatic decomposition there are various possible approaches, e.g., alteration of the peptide bond (*1*), cyclization (*2*), conjugation to carrier molecules (*3*), and incorporation of nonproteinogenic amino acids such as β-Ala (*4*) or D-amino acids (*5–7*). Conjugation could have several favorable effects on peptides: it can enhance the solubility, improve cellular uptake (*8*), provide prolonged blood circulation, alter biodistribution, and also in some cases it can diminish toxicity. Different strategies can be utilized for conjugation: the biologically active compound can be attached directly to the carrier molecule, or it is possible to use a spacer structure in between. This method is applied when the cargo has to be released on the effect of pH (for example by using a spacer sensitive to pH (*9*)) or enzymatic cleavage (e.g., by using a spacer sensitive to enzymes) and the biologically active compound can exercise its effect in the targeted compartment. When conjugated directly to a carrier molecule, different bonds could be considered: for example, amide bond formation between the amino group of the N-terminus of the cargo peptide and a side-chain carboxyl group (Glu/Asp) of the partner molecule, or between the carboxyl group of the C-terminus of the peptide and a side-chain amino group (Lys) of the carrier. A thioether link could be formed between the SH- side chain of the N- or C-terminal cystein residue of the cargo peptide and the chloroacetylated amino group of a side-chain of the carrier molecule (*10*); a disulfide bridge could be established between Cys residues present in both parties (*11*).

Literature studies mainly report on the drug release from conjugates on enzymatic effect; the enzymatic degradation of conjugates is not discussed as we know. Searle et al. studied the release of ellipticine derivative (6-(3-aminopropyl)-ellipticine) conjugated to *N*-(2-hydroxyprolyl)methacrylamide copolymer through GFLG and GG linker peptides in tritosomes (rat liver lysosomal enzymes) (*12*). Subr et al. used rat liver lysosomal preparation and cathepsin B to study the effect of Gly-Gly, Gly-Leu-Gly, and Gly-DL-Phe-Leu-Gly spacers on the release of methotrexate conjugated to hexamethylenediamine (*13*). Release of daunorubicin conjugated to succinylated serum albumin via one to four amino acids (L, AL, LAL, or ALAL) was studied in lysosomal preparation and in 95% calf serum (*14*).

Herpes simplex virus (HSV), with two closely related serotypes, HSV-1 and HSV-2, is one of the most common infectious agents in humans. Glycoprotein D (gD) of HSV type 1 or 2 is a major envelope protein, and appears to be the major target for the immune response. It has been shown that peptides from the N-terminal region of HSV-1 gD (gD-1) can induce both B and T cell responses (*14*), and the resulting antibodies are proven able to neutralize HSV-1 *in vitro* (*15–18*). Another epitope of gD-1 has been identified by monoclonal antibodies and located in the 268−287 region (*19, 20*). It was shown that the minimal epitope of this region in the HSV gD-1 was the

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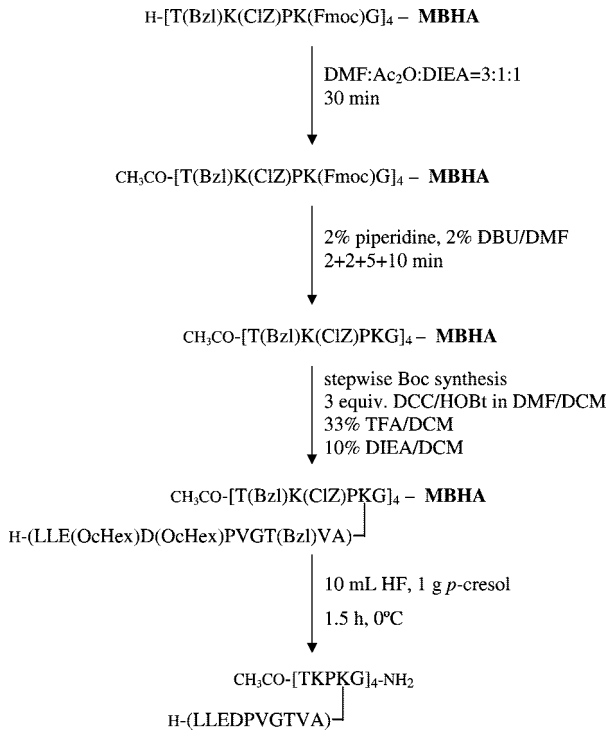
Scheme 1. Outline of the Synthesis of Amide Bond Containing Conjugate of LLEDPVGTVA of gD-1 of HSV



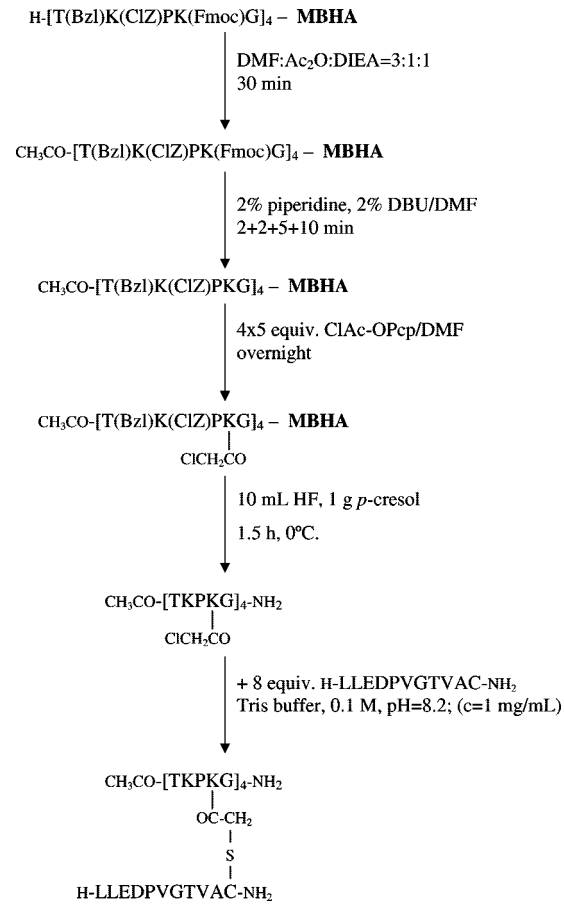
^{281}DPVG^{284} sequence (*21*) representing a turn structure that is important in the immunological recognition, as has been proven in the case of another sequence of the same protein (*22*).

The aim of this study was the analysis of the effect of conjugation on the enzymatic degradation of carrier attached epitope peptide. Therefore, conjugates containing oligotuftsin derivative as carrier and epitope peptide 278−287 with ^{281}DPVG^{284} in the central position were synthesized with amide bond or thioether link. Amide bond formation was achieved between the carboxyl group of the C-terminus of the oligopeptide and the ε-amino group of the side chain of the lysine residues in the carrier molecule (Scheme 1). For introduction of the thioether linkage, first we have elongated the peptide epitope by a C-terminally added cysteine residue. The carrier part of the conjugate was chloroacetylated and used for coupling (Scheme 2). Here we report on our findings on the enzymatic stability of the conjugated epitope peptide in complex biological fluids. The enzymatic hydrolysis of the constructs was investigated in diluted human sera (10% and 50%) and also in rat liver lysosomal preparation at two pH values (pH 3.5 and pH 5). Our data indicate that conjugates show high resistance against proteolytic degradation in diluted human sera but these compounds are degraded in lysosomal preparation.

EXPERIMENTAL PROCEDURES

Materials. Amino acid derivatives were purchased from NovaBiochem (Läufelfingen, Switzerland) and Reanal (Budapest, Hungary). 4-Methyl-benzhydrylamine (MBHA) resin was from NovaBiochem. All chemicals for coupling and cleavage (hydrogen fluoride (HF), thioanisole, *m*-cresol, *p*-cresol, *p*-thiocresol, *N,N′*-dicyclohexylcarbodiimide (DCC), 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU), 1-hydroxybenzotriazole (HOBt), trifluoracetic acid (TFA), *N*-ethyldiisopropylamine (DIEA)) were obtained from Fluka (Buchs, Switzerland). All solvents for the synthesis and purification as well as $Na_2HPO_4 \cdot 2H_2O$, KH_2PO_4, Na-acetate$\cdot 3H_2O$, and acetic acid were from Reanal.

Scheme 2. Outline of the Synthesis of Thioether Bond Containing Conjugate of LLEDPVGTVA of gD-1 of HSV



Synthesis of Conjugate with Amide Bond. Amide bond containing conjugate was prepared by solid phase methodology using 0.5 g 4-methyl-benzhydrylamine resin (1.04 mmol/g). The following side-chain protected amino acid derivatives were used: Boc-Thr(Bzl)-OH, Boc-Asp(OcHex)-OH, Boc-Glu(OcHex)-OH, Boc-Lys(Fmoc)-OH, and Boc-Lys(ClZ)-OH. Coupling was carried out by using DCC/HOBt *in situ* active ester method in 4:1 (v/v) DCM/DMF mixture. 3 equiv of amino acid derivatives and coupling reagents were used. N^{α}-Boc group was removed with 33% TFA in DCM. For neutralization 10% DIEA in DCM was applied. The success of the coupling was monitored by ninhydrine reaction (*23*) or isatine assay (*24*). The following protocol was applied: DCM washing (3 × 0.5 min); deprotection with 33% TFA/DCM (2 × 20 min); DCM washing (5 × 0.5 min); neutralization with 10% DIEA/DCM (4 × 0.5 min); DCM washing (4 × 0.5 min); coupling (60 min); DMF washing (2 × 0.5 min); DCM washing (2 × 0.5 min); ninhydrine or isatine reaction. After removal of the N^{α}-Boc group, the side-chain protected tetratuftsin derivative ([T(Bzl)K(ClZ)PK(Fmoc)G]$_4$) was acetylated at the N-terminus using a mixture of acetic anhydride and DIEA in DMF 1:1:3 (v/v/v) for 30 min. Then the Fmoc groups were removed with 2% piperidine, 2% DBU in DMF four times for 2 + 2 + 5 + 10 min. The sequence of LLEDPVGTVA decapeptide was built up stepwise on the ε-amino group of the Lys residues using suitable protected amino acids by Boc strategy. The conjugate was cleaved from the resin by the aid of anhydrous HF-*p*-cresol (10 mL/1 g) at 0 °C for 1.5 h, precipitated with dry diethyl ether, filtered, and washed several times with diethyl ether. The conjugate was dissolved in 10% acetic acid and freeze-dried.

Synthesis of Conjugate with Thioether Bond. The oligotuftsin derivative as well as peptide H-LLEDPVGTVAC-NH_2

was prepared by solid phase methodology using Boc strategy on 0.5 g 4-methyl-benzhydrylamine resin (1.04 mmol/g). The following side-chain protected amino acid derivatives were used: Boc-Cys(Mbzl)-OH, Boc-Thr(Bzl)-OH, Boc-Asp(OcHex)-OH, Boc-Glu(OcHex)-OH, Boc-Lys(Fmoc)-OH, Boc-Lys(ClZ)-OH. Synthesis was performed as described above. After the removal of the Fmoc group from the ε-amino group of the Lys residues tetratuftsin derivative was chloroacetylated at the side chain of the Lys residues. The chloroacetyl group was introduced by using chloroacetic acid pentachlorophenyl ester in 4 × 5 equiv excess (5 equiv per lysine residue) in DMF overnight at room temperature (*9*). The chloroacetylated peptide was cleaved from the resin by the aid of anhydrous HF in the presence of *m*-cresol-*p*-thiocresol (10 mL/0.5 mL/0.5 g) at 0 °C for 1.5 h (*25*).

RP-HPLC-purified chloroacetylated tuftsin oligopeptide derivative was dissolved in Tris buffer (0.1 M, pH = 8.2) at c = 1 mg/mL. Peptide H-LLEDPVGTVAC-NH_2 was added to this solution in 8 equiv excess little by little. Reaction was followed by RP-HPLC (Supelcosil C_{18} 5 μm; 4.6 mm × 250 mm column) using gradient elution. After sample application an isocratic elution with 20% eluent B was applied for 2 min, then a linear gradient from 20% to 45% eluent B was generated over 25 min at room temperature with a flow rate of 1 mL/min (eluent A, 0.1% TFA in water; eluent B, 0.1% TFA in acetonitrile−water (80:20, v/v %)). UV detection was performed at λ = 214 nm. Reaction was considered complete when no change in the position and intensity of peaks on the chromatogram was observed.

Purification by RP-HPLC. The crude products (peptides and conjugates) were purified by RP-HPLC on a Phenomenex Jupiter C_{18} column (10 μm; 300 Å, 10 mm × 250 mm), using gradient elution, where eluent A was 0.1% TFA in water, while eluent B was 0.1% TFA in acetonitrile−water (80:20, v/v %). After sample application an isocratic elution with 15% eluent B was applied for 2 min, then a linear gradient from 15% to 40% eluent B was generated over 25 min at room temperature with a flow rate of 4 mL/min. UV detection was performed at λ = 214 nm.

RP-HPLC Analysis. The homogeneity of peptides and conjugates was analyzed by RP-HPLC on a Symmetry C_{18} 3.5 μm, 4.6 mm × 150 mm column (for peptides) or on a Supelcosil C_{18} 5 μm, 4.6 mm × 250 mm column (for conjugates). After sample application an isocratic elution with 20% eluent B was applied for 2 min, then a linear gradient from 20% to 45% eluent B was generated over 25 min at room temperature with a flow rate of 1 mL/min: eluent A, 0.1% TFA in water; eluent B, 0.1% TFA in acetonitrile−water (80:20, v/v%). UV detection was performed at λ = 214 nm.

Amino Acid Analysis. The amino acid composition of compounds was determined by amino acid analysis using a Beckman model 6300 analyzer (Fullerton, CA). Prior to analysis, samples were hydrolyzed in 6 M HCl in sealed and evacuated tubes at 110 °C for 24 h.

Mass Spectrometry. Mass spectrometric experiments were performed on a Bruker Daltonics Esquire 3000plus (Germany) ion trap mass spectrometer, equipped with electrospray ionization source. Spectra were aquired in positive ionization mode, in the 50−2000 m/z region. Samples were dissolved in acetonitrile/water = 1:1 (v/v) mixture, containing 0.1% acetic acid. Samples were introduced into the mass spectrometer using a syringe pump, at a flow rate of 10 μL/min.

Enzymatic Digestion. *Lysosome Preparation.* For a detailed description of the lysosome preparation see Tugyi et al. (*7*). Briefly, rat livers were homogenized in 0.3 M sucrose, diluted with 0.3 M sucrose, and centrifuged. The supernatant was centrifuged again, and rehomogenized in sucrose containing 1 mM $CaCl_2$. After incubation at 37 °C for 5 min, 50% Percoll solution was added and homogenate was centrifuged again. The lysosomal fraction was obtained as a hard brown pellet. The enzymatic activity of the preparation was determined according to Dingle (*26*).

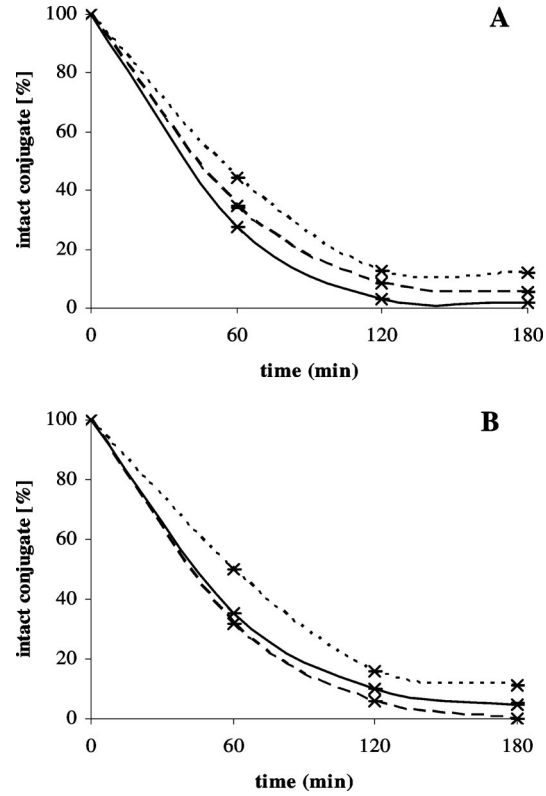


Figure 1. Degradation profile of the free peptide (H-LLEDPVGTVA-NH_2) (−x−), and its conjugates with amide (− −x− −) or thioether bond (---x---) in lysosomal preparation at pH = 3.5 (A) and at pH = 5.0 (B).

Digestion of Peptides and Conjugates in Lysosome Homogenates. Control peptide was dissolved at c = 2.5 mg/mL in 0.1 M acetate buffer, pH = 3.5 or pH = 5.0, while conjugate concentration was c = 3.76 mg/mL and c = 3.82 mg/mL for thioether and for amide conjugate, respectively. Afterward, the lysosome fraction was added ($c_{lysosomal}$ = 22 μL/mL), and peptide or conjugate samples were incubated at room temperature for 180 min. Aliquots of 100 μL were taken at 30 s and 60, 120, and 180 min. The enzymatic reaction was stopped by the addition of 5 v/v% perchloric acid. Samples were centrifuged at 4600 rpm for 5 min at 4 °C and the concentration of the intact peptide in the supernatant was determined by RP-HPLC (Supelcosil C_{18} 5 μm; 4.6 mm × 250 mm column), using calibration curves. For RP-HPLC analysis eluent A (0.1% TFA in water) and eluent B (0.1% TFA in acetonitrile−water (80:20, v/v)) were applied. A gradient 20−45% eluent B in 25 min was generated. Each sample was injected twice, and each digestion experiment was performed twice. Standard deviation of the twice-injected samples was negligible, while standard deviation for the independent studies was calculated and provided in Figure 1 and Table 2.

Digestion of Peptides and Conjugates in Human Serum. Human serum was obtained from National Institute of Haematology and Immunology (Budapest, Hungary). Control peptide was dissolved in 0.1 M phosphate buffer (pH = 7.2) containing 0%, 10%, or 50% human serum, at c = 2.5 mg/mL, while conjugate concentration was c = 3.76 mg/mL for compound with thioether linkage, and 3.82 mg/mL for the construct with amide bond. Conjugates were incubated at 37 °C up to 96 h. Samples (100 μL for 0% and 10% human sera and 140 μL for 50% human sera) were taken after 0, 24, 48, 72, and 96 h. All

Table 1. Characteristics of Conjugates and Peptide LLEDPVGTVA

	amino acid composition found (calculated)										R_t(min)[b]	[M+H]$^{+}$[c]	
peptide/conjugate	cmc[a]	Asp	Thr	Glu	Pro	Gly	Ala	Val	Leu	Lys		measured	calculated
H-LLEDPVGTVA-NH_2	-	0.9(1)	0.9(1)	1.1(1)	1.0(1)	1.2(1)	1.0(1)	1.8(2)	2.2(2)	-	16.5(*1*)	1013.2	1013.2
[TKPK(LLEDPVGTVA)G]$_4$	-	3.6(4)	7.6(8)	4.4(4)	8.0(8)	7.6(8)	4.0(4)	8.0(8)	7.6(8)	8.4(8)	24.9[2]	6084.4	6084.2
[TKPK(LLEDPVGTVAC)G]$_4$	2.8(4)	3.2(4)	3.6(4)	4.0(4)	7.6(8)	8.0(8)	4.0(4)	8.8(8)	8.4(8)	8.0(8)	24.7[2]	6724.4	6725.3

[a] cmc: measured as *S*-carboxymethyl cysteine. [b] HPLC retention time, gradient 0−2 min 15% eluent B, 2−22 min 40% eluent B (1), 0−2 min 20% eluent B, 2−27 min 45% eluent B (2), where eluent A is 0.1% TFA/water, eluent B is 0.1% TFA/acetonitrile−water 80:20 (v/v). See further details in Experimental Procedures. [c] ESI-MS measurements.

Table 2. Effect of Conjugation on the Stability of Epitope Peptide LLEDPVGTVA in Lysosomal Preparation at pH = 3.5 and pH = 5.0

	intact compound [%][a] after digestion with lysosomal preparation							
	pH = 3.5				pH = 5.0			
peptide/conjugate	0.5 min	60 min	120 min	180 min	0.5 min	60 min	120 min	180 min
H-LLEDPVGTVA-NH_2	100	28 ± 4	3 ± 4	2 ± 2	100	35 ± 2	10 ± 6	5 ± 3
[TKPK(LLEDPVGTVA)G]$_4$	100	44 ± 0,5	13 ± 0	12 ± 1	100	50 ± 0,5	16 ± 2	11 ± 3
[TKPK(LLEDPVGTVAC)G]$_4$	100	34 ± 5	9 ± 2	5 ± 1	100	31 ± 1	6 ± 0	0 ± 0

[a] Compound [%] = c_{intact}/c_{total}. The concentration of intact compound was calculated from the AUC (area under the curve) of the corresponding peak obtained after HPLC analysis as described in Experimental Procedures.

digestion experiments were performed under sterile conditions. Further treatment of the samples was identical to that described for the lysosomal digestion above.

Isolation and Identification of Degradation Fragments. Samples of the digests were analyzed by RP-HPLC using analytical column (Supelcosil C_{18} 5 μm; 4.6 mm × 250 mm column). For the RP-HPLC analysis eluent A (0.1% TFA in water) and eluent B (0.1% TFA in acetonitrile−water (80:20, v/v)) were applied. A gradient 20−45% eluent B in 25 min was generated. Fragments were collected in Eppendorf tubes and freeze-dried. Molecular mass of the fragments was detemined by mass spectrometry (see above).

RESULTS AND DISCUSSION

In order to study the effect of covalent coupling of epitope peptide on the enzymatic degradation, conjugates with an oligopeptide derived from the glycoprotein D of Herpes simplex virus were prepared. As carrier a tetratuftsin derivative composed of four tuftsin-like units, TKPKG, was used. Human tuftsin is a tetrapeptide (TKPR) present in the Fc receptor of the IgG molecule (*27*). TKPK is the canine tuftsin sequence (*28*) and the presence of the second Lys offers a new possibility to conjugate even two different entities. Both human and canine tuftsins exhibit immunostimulatory effect (*29*).

In conjugates prepared for the present study amide or thioether linkage was established between the carrier and the cargo epitope. In both cases oligopeptide ^{278}LLEDPVGTVA^{287} was attached to the tetratuftsin derivative through the C-terminal amino acid residue (Scheme 1). When a thioether bond was formed peptide 278−287 was elongated with a Cys at the C-terminal and its SH group was utilized in the conjugation (Scheme 2). The stability of conjugates with thioether link or amide bond was studied in two complex biological matrices possessing enzymatic activity. For the investigation of stability in serum we used two concentrations of the human serum (10% and 50%) under aseptic conditions. The conjugates and the free epitope peptide 278−287 were also incubated with lysosomal preparation at two different pH values (pH = 3.5 and 5.0). In experiments performed we determined the amount of intact compound. Based on the comparative analysis of these data we have documented the capability of conjugates to resist against enzymatic degradation.

Synthesis of Conjugates. The ^{278}LLEDPVGTVA^{287} region of HSV gD-1 contains the ^{281}DPVG^{284} epitope core (*21*) adopting turn structure under appropriate conditions (*30*). For our studies we have elongated the tetramer core by three amino acids from the native sequence at both termini. The amide bond containing conjugate was prepared on MBHA resin by tandem solid phase peptide synthesis. After the cleavage of all four Fmoc protecting groups from Lys residues in the carrier molecule Ac-[T(Bzl)K(ClZ)PKG]$_4$-R, the synthesis was continued using the liberated ε-amino groups of the semiprotected carrier. Thus, four copies of the decapeptide ^{278}LLEDPVGTVA^{287} were assembled as branches using the Boc strategy (Scheme 1).

For the preparation of conjugate with thioether bond, first the epitope peptide with C-terminal Cys (LLEDPVGTVAC) and the semiprotected tetratuftsin derivative carrier Ac-[T(Bzl)K(ClZ)PKG]$_4$-R was synthesized also by solid phase synthesis on MBHA resin. In this case the free side chain of four Lys residues of the carrier molecule was chloroacetylated using chloroacetic acid pentachlorophenyl ester (*11*). Finally, the chloroacetylated carrier Ac-[T(Bzl)K(ClZ)PK(ClCH$_2$CO)G]$_4$-NH_2 as well as the Cys-containing peptide, H-LLEDPVGTVAC-NH_2, were cleaved from the resin by HF. After RP-HPLC purification the oligopeptide was conjugated with the purified carrier molecule under slightly alkaline condition (0.1 M Tris buffer, pH = 8.2). Tetratuftsin derivative was dissolved at c = 1 mg/mL, then peptide H-LLEDPVGTVAC-NH_2 was added in 8 equiv excess in small portions to avoid the risk of dimerization (Scheme 2). The conjugation reaction was followed by RP-HPLC analysis. Conjugates and the free peptide used for the enzymatic studies were characterized by amino acid composition, by relative molar mass determined by ESI-MS, and by RP-HPLC retention time values (Table 1).

Enzymatic Stability of Conjugates. For *in vitro* enzymatic stability studies of peptides authors have utilized in the literature mainly isolated enzymes like carboxypeptidase A, aminopeptidase M, proteinase A, carboxypeptidase Y (*31*), α-chymotrypsin, or carboxypeptidase Y (*32*), or complex biological fluids, like human serum and urine (*33*), human plasma (*34*), or rat liver lysosomes (*35*). It is interesting to note that only a limited number of studies on peptide conjugates has been reported so far and only the liberation of the free drug has been investigated.

We have studied the enzymatic stability of two conjugates with different linkages as well as the corresponding oligopeptide, H-LLEDPVGTVA-NH_2, as control, in diluted human sera and in rat liver lysosomal preparation. We have isolated and identified the degradation products and propose a hypothesis on the involvement of potential enzymes. The data are summarized in Tables 2 and 3. The degradation profiles of all three compounds in lysosomal preparation at pH 3.5 and pH 5.0 (Figure 1A,B) are also shown.

Table 3. Effect of Conjugation on the Stability of Epitope Peptide LLEDPVGTVA in Diluted Human Serum

	intact compound [%][a] after digestion with human serum									
	10%					50%				
peptide/conjugate	0 h	24 h	48 h	72 h	96 h	0 h	24 h	48 h	72 h	96 h
H-LLEDPVGTVA-NH2	100	0	-	-	-	100	0	-	-	-
$[TKPK(LLEDPVGTVA)G]_4$	100	100	100	100	100	100	100	100	100	100
$[TKPK(LLEDPVGTVAC)G]_4$	100	100	100	100	100	100	100	100	100	100

[a] Compound [%] = c_{intact}/c_{total}. The concentration of intact compound was calculated from the AUC (area under the curve) of the corresponding peak obtained after HPLC analysis as described in Experimental Procedures.

First we have analyzed the stability of the two conjugates and compared with that of the free linear peptide, H-LLEDPVGTVA-NH_2 in rat lysosomal preparation, containing various proteolytic enzymes. Considering the lack of consensus in the literature about the pH within the lysosome we have selected two pH values (pH 3.5 and pH 5.0) to perform the assay. Harada et al. reported on a value of pH 4 (*36*); Bach et al. observed that the pH in lysosomes varies between 4.3 and 4.5 (*37*). Other groups found a different level of acidity between pH 4.5 and pH 5.0 (pH 4.5 (*38, 39*), 4.67 (*40*), 4.75 (*41*), 5.0 (*42*)). It is also known that the optimal pH for the main lysosomal enzyme, Cathepsin D, is pH 3.5 (*43*).

Data obtained are summarized in Table 2 and Figure 1. We observed that both conjugates degraded in lysosomal preparation with a fairly similar degradation profile, in a nearly identical way to that the free peptide. However, it is interesting to note that the rate of the enzymatic hydrolysis was slightly dependent on the type of linkage present in conjugates: 88% and 89% of the amide bond containing conjugate degraded at pH = 3.5 and 5.0, respectively, while 95% and 100% of the thioether containing conjugate disappeared at pH = 3.5 and 5.0, respectively.

In the next series of experiments we have studied the enzymatic degradation of the compounds in 10% and in 50% human sera. Data, summarized in Table 3, showed no degradation when peptide was conjugated to oligotuftsin derivative as carrier under the conditions applied. However, the control linear peptide degraded along the amide bonds in 24 h even in 10% human serum.

Identification of Fragments in Lysosomal Preparation. In order to gain insight into the degradation process, fragments present in the reaction mixture were identified by mass spectrometry. We found that the fragmentation of the two conjugates differ from each other and also from that of the free peptide.

The main fragment of peptide H-LLEDPVGTVA-NH_2 is H-DPVGTVA-NH_2, but we have also detected the presence of smaller fragments like H-LLE-OH, H-DPVG-OH, and H-EDPVG-OH. The change of the pH from 3.5 to 5.0 has no significant effect on the number and composition of fragments formed.

Similarly, the pH has essentially no effect on the fragmentation profile of the amide bond containing conjugate. The quantity, number, and composition of the fragments are almost identical at the pH values used. The predominant fragment appearing at the highest quantity is a truncated conjugate missing one N-terminal LLE tripeptide unit (M: 5729.8) (Figure 2A). In marked quantity we found conjugates without (a) two LLE trimer (M: 5374.4), (b) one LLE and one LLEDPVG units (M: 5006.0), or (c) three LLE tripeptides (M: 5018.7). Accordingly we have isolated the tripeptide H-LLE-OH (M: 373.9) from the digests (Figure 2A).

In the case of the thioether conjugate the degradation was faster and the number of fragments formed was higher. In addition we observed pH-dependent fragmentation. The degradation rates of the different fragments were also pH-dependent. At pH = 3.5 the quantity of most of the fragments present after

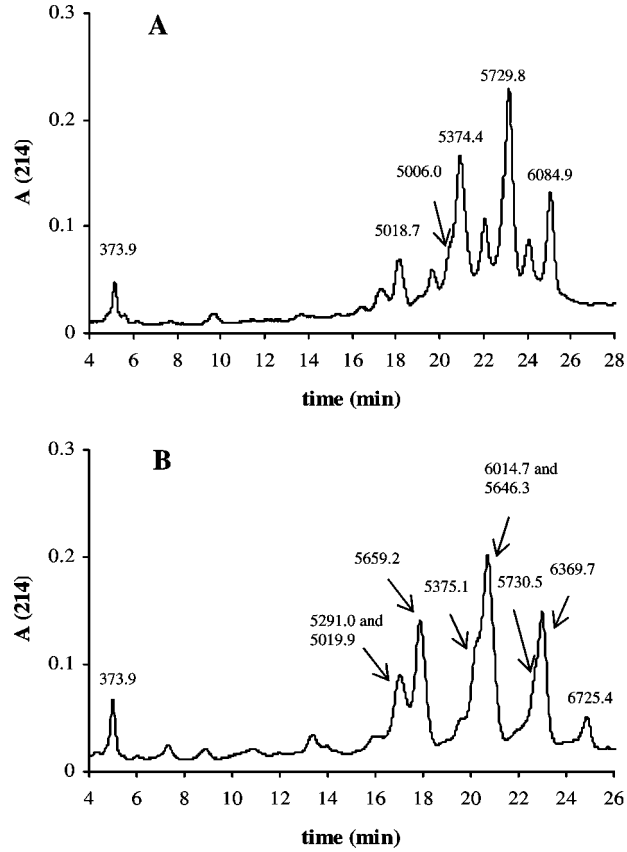


Figure 2. RP-HPLC chromatogram of digest obtained after treatment with lysosomal preparation of conjugate with amide bond at pH = 5.0 after 120 min (A); and conjugate with thioether linkage at pH = 3.5 after 120 min (B). Molecular masses of fragments identified are shown on the peaks.

60 min was increased after 120 and 180 min (Figure 3A). In contrast at pH = 5.0 the amount of the majority of the fragments present at 60 min was decreased until the end of the period studied (Figure 3B). However, the predominant fragments were found to be identical at both pH values. These are the following (see also Figure 3B): conjugate missing one, two, or three LLE tripeptides (M: 6369.7, 6014.7, and 5659.2, respectively) or one LLE tripeptide plus one LLEDPVG unit (M: 5646.3) or two LLE plus one LLEDPVG unit (M: 5019.9). The H-LLE-OH tripeptide (M: 373.9) is detectable at both pH as well as in the case of the amide bond containing conjugate. At pH = 5.0 fragments missing a full LLEDPVGTVA sequence (one LLEDPVGTVA, M: 5730.5; one LLEDPVGTVA and one LLE, M: 5375.1; one LLEDPVGTVA and two LLE, M: 5019.9) were also detectable in minor quantity. Interestingly at this pH the fragment H-LLEDPVGTVA-OH was also detectable (data not shown). The cleavage of the bond between the E and D amino acids observed in all three cases (the two conjugates and free peptide) is probably due to the presence of tripeptidyl peptidase

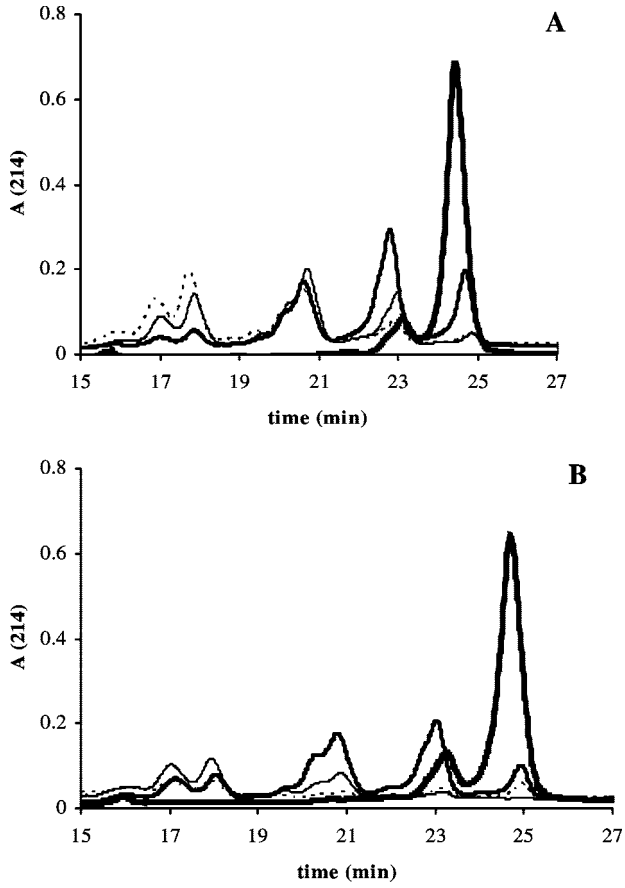


Figure 3. RP-HPLC chromatogram of digest obtained after treatment with lysosomal preparation of amide bond containing conjugate (A) and thioether linkage containing conjugate (B) after 0 (thick line), 60 (-), 120 (-), and 180 min (---).

I (*44*). The split of GT as well as AC bonds could be due presumably to the enzyme Cathepsin D (*45*).

CONCLUSION

In this study we reported on the synthesis and on proteolysis of two ^{278}LLEDPVGTVA^{287} conjugates with amide or thioether bond. We compared the stability of these conjugates in diluted human sera and in rat lysosomal preparation. The data obtained in human sera show that conjugation enhanced the stability of the epitope peptide as compared to its free form. It should be noted that the type of linkage between the peptide and the carrier part of the conjugate essentially did not influence the degradation pattern. These results also show that in diluted human sera the conjugation of an oligopeptide could provide full protection against enzymatic hydrolysis. Comparing the degradation of the two conjugates in lysosomal preparation, we found only modest differences. In the case of the conjugate with an amide bond the rate of the degradation was somewhat lower and the number of fragments were also limited as compared to the conjugate with a thioether bond. The fragment without an LLEDPVGTVA sequence appears only in the digest of the thioether conjugate. This difference could be explained by the fact that for the formation of this fragment in the case of the amide conjugate, the cleavage of the amide bond between the α-carboxyl group of Ala residue of the epitope and the ε-amino group of the Lys of the carrier molecule would be required. This cleavage probably is prevented by the fact that this is an isopeptide bond and perhaps also by steric restriction, while in the thioether conjugate the amide bond between the Ala and the Cys residue is a peptide bond and it could be more accessible because the Cys residue enhances the distance between the carrier and the peptide side chain. It is interesting to note that there is no detectable fragment from the carrier backbone of the conjugate. This observation could be explained by the conformation of the tetratuftsin derivative used as carrier (*46*), but it is more likely that the attachment of four copies of the epitope peptide to the side chains of the carrier limits the accessibility of backbone amid bonds for proteolytic enzymes.

Taken together, our results indicate that conjugation of the epitope peptide studied fully protected its degradation in diluted human sera as compared to the free linear epitope peptide, which decomposed quickly. This observation might be useful for the design of artificial immunogens. We have also demonstrated that the fragments derived from the thioether conjugate can possess the full-size epitope peptide in contrast to the amide conjugate from which only one fragment with the epitope core (DPVG) was produced. It is attractive to speculate that complete epitope peptide released from the thioether conjugate in the lysosome might be available for MHC for antigen presentation. Considering this finding, one can propose to design an amide conjugate in which a spacer unit could provide an appropriate distance between the epitope peptide and the carrier. Thus endopeptidases could have access to amide bonds and release the "full" epitope sequence.

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