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The effect of cyclization on the enzymatic degradation of herpes simplex virus glycoprotein D derived epitope peptide

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Abstract: One linear and three cyclic peptides corresponding to the 278–287 (^{278}LLEDPVGTVA^{287}) sequence of glycoprotein D (gD-1) of herpes simplex virus were synthesized for the analysis of the effect of cyclization on protection against enzymatic degradation. In this design, the turn-forming motif (^{281}DPVG^{284}) was positioned in the central part of the peptide and elongated by three amino acids at both termini. Cyclopeptide formation was achieved by the introduction of a peptide bond, a disulfide bridge or a thioether link. The stability of these peptides was compared in human serum and also in rat lysosomal preparations. The data obtained in 10% and 50% human serum show that all three types of cyclization enhanced the stability, but at different levels. Complete stability was only achieved by the introduction of a thioether link, while the presence of a disulfide or peptide bond resulted in improved, but partial resistance against hydrolytic decomposition. In lysosomal preparations the presence of cyclic primary structure provided full protection against enzymatic hydrolysis. Taken together, these findings indicate that by appropriate structural modification it is feasible to construct a synthetic antigen with high stability against enzymatic degradation in complex biological fluids. Further studies are in progress to identify enzymes responsible for degradation in diluted human sera as well as in the lysosomal preparations and to gain more detailed information on the mechanism of action.

Keywords: cyclopeptides; HSV gD-1; epitope; proteolytic stability; lysosomal degradation; human serum

INTRODUCTION

A major problem limiting the use of peptides as effective vaccine subunits is their instability, which is mainly due to the rapid degradation *in vivo* 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 the incorporation of non-proteinogenic amino acids such as β-Ala [4] or D-amino acids [5,6]. Cyclization, apart from enhancing the biological stability, can stabilize the conformation suitable for binding. Different strategies can be utilized for cyclization: for example, peptide bond formation between the amino group of the *N*-terminus and the carboxyl group of the *C*-terminus, i.e. head-to-tail cyclization [7], amide bond formation between the amino group of the *N*-terminus and a side-chain carboxy group [8], amide bond formation between two side chains, e.g. glutamic acid at the *C*-terminal and lysine at the *N*-terminal [9], or between two non-terminal side chains [10]. Another possibility for cyclic peptide formation is the introduction of a disulfide bridge after the incorporation of two cysteine residues at the two termini [11]. Furthermore, a ring can be established by a thioether link between the side chain of a *C*-terminally added cysteine residue and the α-carbon atom of an *N*-terminal acetyl group [12].

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 [13], and the resulting antibodies proved to be able to neutralize HSV-1 *in vitro* [13–16]. Another epitope of HSV gD-1 has been identified by monoclonal antibodies and located to the 268–287 region [17,18]. It was shown that the minimal epitope of this region in the HSV gD-1 was the ^{281}DPVG^{284} sequence [19] representing a turn structure that is important in the immunological recognition.

The aim of this study was the analysis of the effect of cyclization on enzymatic degradation. In addition, by the comparison of the enzymatic stability of linear and cyclopeptides, a correlation has been established between the type of linkage involved in cyclization and of the degradation profile. Therefore cyclopeptides, containing the epitope core (^{281}DPVG^{284})

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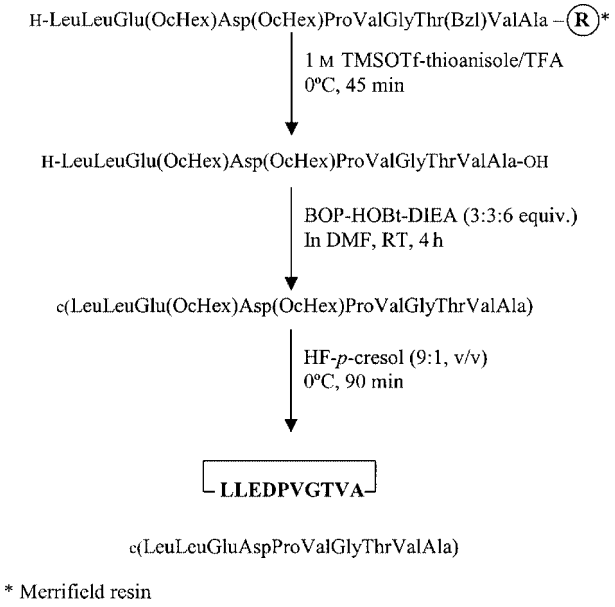


Scheme 1

were synthesized with peptide bond, disulfide bridge or thioether link for ring closure. For this a linear peptide with ^{278}LLEDPVGTVA^{287} sequence of gD-1 of HSV and its cyclic variants were prepared. Peptide bond formation was achieved between the amino group of the *N*-terminus and the carboxyl group of the *C*-terminus (c[LLEDPVGTVA]) (Scheme 1). Disulfide bridge was formed with the incorporation of two cysteine residues at the two termini (H-CLLEDPVGTVAC-NH_2) (Scheme 2). Thioether link was introduced between the side chain of a *C*-terminally added cysteine residue and the carbon of an *N*-terminal acetyl residue (CH_2CO-LLEDPVGTVAC-NH_2) (Scheme 3). Here we report on our findings on the enzymatic stability of peptides described in complex biological fluids. The enzymatic degradation was investigated in diluted human serum and also in rat liver lysosomal preparations at two pH values (pH 3.5 and pH 5). Our data show that cyclopeptide CH_2CO-LLEDPVGTVAC-NH_2 show high resistance against proteolytic degradation in diluted human serum as well as in lysosomal preparations.

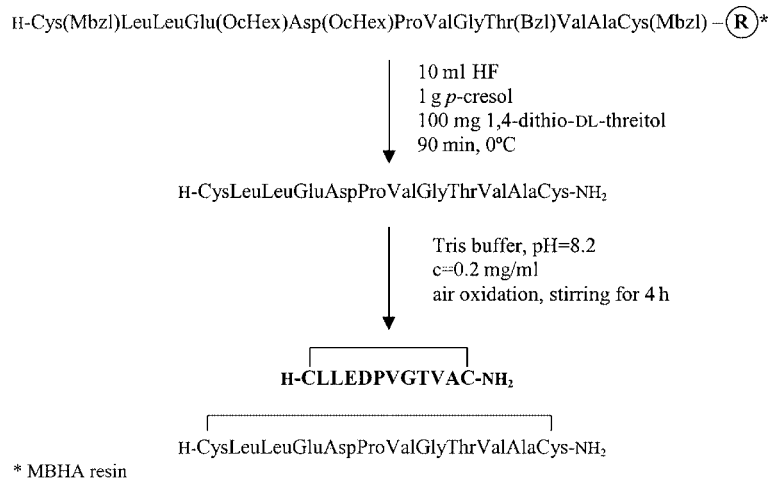
MATERIALS AND METHODS

Materials

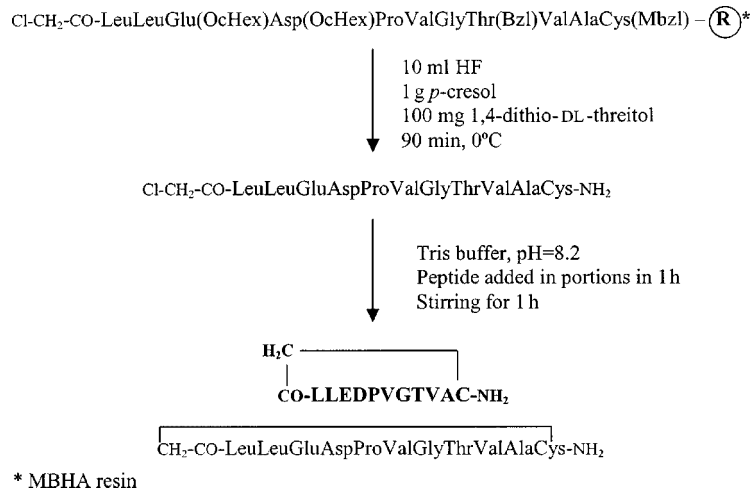
Amino acid derivatives were purchased from NovaBiochem (Laufelfingen, Switzerland) and Reanal (Budapest, Hungary). Merrifield resin was obtained from Reanal while 4-methylbenzhydrylamine (MBHA) resin was from NovaBiochem. All chemicals for coupling and cleavage (hydrogen fluoride (HF), thioanisole, *m*-cresol, *p*-cresol, *N*,*N*′-dicyclohexylcarbodiimide (DCC), benzotriazol-1-yloxy-*tris*-(dimethylamino) phosphonium hexafluorophosphate (BOP), trimethylsilyl trifluoromethanesulfonate (TMSOTf), 1,4-dithio-DL-threitol (DTT), 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.$3H_2O$ and acetic acid were from Reanal.

Peptide synthesis and purification

Linear peptides were prepared by solid phase methodology using either 0.5 g Merrifield resin (capacity = 1.2 mmol/g) for the synthesis of homodetic 'head to tail' cyclopeptide or 0.5 g 4-methyl-benzhydrylamine resin (capacity = 1.04 mmol/g) for the synthesis of cyclopeptides with disulfide bridge or thioether link as well as for the synthesis of the linear peptide H-LLEDPVGTVA-NH_2. All amino acids were coupled as N^{α}-Boc-derivatives. 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. Coupling was carried out by using DCC/HOBt *in situ* active ester methodology in 4 : 1 (v/v) DCM/DMF mixture. Three equivalents of amino acid derivatives and coupling reagent were used. N^{α}-Boc groups were removed by 33% TFA in DCM. For neutralization



Scheme 2



Scheme 3

10% DIEA in DCM was applied. The success of the coupling was monitored by ninhydrin reaction [20] and/or isatine assay [21]. The following synthetic protocol was used: 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); ninhydrin or isatine reaction. The removal of semiprotected peptide (LLE(OcHex)D(OcHex)PVGTVA) from Merrifield resin was carried out in 1 M TMSOTf-thioanisole/TFA in the presence of *m*-cresol (2 v/v%) as scavenger. The peptide H-CLLEDPVGTVAC-NH_2 was cleaved from the MBHA resin with 10 ml HF containing 1 g *p*-cresol and 100 mg 1,4-dithio-DL-threitol. The peptide LLEDPVGTVAC was chloroacetylated before cleavage from the resin. The chloroacetyl group was introduced to the *N*-terminus using chloroacetic acid pentachlorophenyl ester in 5 equivalents excess in DMF after removal of the N^{α}-Boc group. The chloroacetylated peptide ($ClCH_2CO$-LLEDPVGTVAC-NH_2) was cleaved from the resin by the aid of anhydrous HF–*m*-cresol–*p*-thiocresol (1 ml:0.5 ml:0.5 g) for 1.5 h at 0 °C [22]. The cleaved peptides were precipitated with dry ether, filtered and washed several times with ether. The peptides were dissolved in 10% acetic acid and freeze-dried. The crude products were purified by reverse phase HPLC on a Phenomenex® Jupiter C_{18} column (10 μm, 300 Å 10 mm × 250 mm), using gradient elution, where eluent A: 0.1% TFA in water, while eluent B: 0.1% TFA in acetonitrile – water (80 : 20, v/v%). After sample application an isocratic elution with 15% or 25% B was applied for 2 min, then a linear gradient from 15% to 40% B or 25% to 50% 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.

Cyclization

Peptide bond formation (c(LLEDPVGTVA)). Purified semiprotected peptide (containing cyclohexyl ester protection at the ω-COOH group of Asp and Glu) was added in portions into DMF solution containing 3 equivalents of BOP/HOBt and 6 equivalents of DIEA during 1 h. The final peptide concentration in the solution was 0.5 mg/ml. The reaction was continued for 4 h at room temperature then the solvent was evaporated [23]. The product was solidified with water, filtered and washed with ether. The protecting groups from the dried peptide were cleaved by HF in the presence of *p*-cresol (Scheme 1). Crude peptide was purified by RP-HPLC on a Phenomenex® Jupiter C_{18} column (10 μm, 300Å, 10 mm × 250 mm) using gradient elution from 25% to 50% B over 25 min at room temperature with a flow rate of 4 ml/min.

Disulfide bridge formation (H-CLLEDPVGTVAC-NH_2). Purified deprotected peptide elongated by cysteines at both termini was dissolved in 0.1 M Tris buffer at pH = 8.2 (c = 0.2 mg/ml). The air oxidation under vigorous stirring was applied for 4 h (Scheme 2) [24] followed by freeze-drying and purification by RP-HPLC on a Phenomenex® Jupiter C_{18} column (10 μm, 300 Å 10 mm × 250 mm) using gradient elution from 15% to 40% B over 25 min at room temperature with a flow rate of 4 ml/min.

Thioether link formation (CH_2CO-LLEDPVGTVAC-NH_2). Purified *N*-terminally chloroacetylated peptide with Cys at the *C*-terminal was added in portions to Tris buffer at pH = 8.2 during 1 h. The reaction mixture was stirred at RT for 1 h more (Scheme 3), then freeze-dried [25]. Crude peptide was purified by RP-HPLC on a Phenomenex® Jupiter C_{18} column (10 μm, 300 Å 10 mm × 250 mm) using gradient elution from 25% to 50% B over 25 min at room temperature with a flow rate of 4 ml/min.

Characterization of the Purified Peptides

HPLC analysis. The homogeneity of each peptide was analysed by RP-HPLC on a Symmetry® C_{18}3.5 μm; 4.6 × 150 mm column. After sample application an isocratic elution with 15% or 25% B was applied for 2 min, then a linear gradient from 15% to 40% B or 25% to 50% B was generated over 25 min at room temperature with a flow rate of 1 ml/min. (Eluent A: 0.1% TFA in water, and eluent B: 0.1% TFA in acetonitrile – water (80 : 20, v/v%).) UV detection was performed at λ = 214 nm. The retention time values are summarized in Table 1.

Amino acid composition. The amino acid composition of peptides was determined by amino acid analysis using a

Table 1 Characteristics of the Linear and the Cyclopeptides

Peptide	Amino acid composition Found (calculated)									R_t (min)[b]	$[M+H]^{+c}$	
	Cmc[a]	Asp	Thr	Glu	Pro	Gly	Ala	Val	Leu		Calculated	Measured
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
H-CLLEDPVGTVAC-NH_2	—	1.0(1)	0.9(1)	1.2(1)	0.9(1)	1.2(1)	1.0(1)	1.8(2)	2.0(2)	21.5(1)	1218.5	1218.3
c[LLEDPVGTVA]	—	0.9(1)	0.9(1)	1.2(1)	0.9(1)	1.2(1)	1.1(1)	1.7(2)	2.1(2)	16.9(2)	996.2	996.2
CH_2CO-LLEDPVGTVAC-NH_2	0.8(1)	1.0(1)	0.9(1)	1.1(1)	1.0(1)	1.1(1)	1.1(1)	1.8(2)	2.2(2)	16.8(2)	1157.3	1157.4

[a] Cmc *S*-carboxymethyl cysteine.
[b] HPLC retention time, gradient: 0–2 min 15% B eluent, 2–22 min 40% B eluent (1), 0–2 min 25% B eluent, 2–22 min 50% B eluent (2), where eluent A: 0.1% TFA/water, eluent B: 0.1% TFA/acetonitrile – water 80 : 20 (v/v).
[c] ESI-MS.

Beckman Model 6300 analyser (Fullerton, CA). Prior to analysis samples were hydrolysed in 6 M HCl in sealed and evacuated tubes at 110 °C for 24 h.

Mass spectrometry. Molecular mass was determined by electrospray mass spectrometry on a Bruker Daltonics® esquire 3000 plus (Germany), ESI-MS spectrometer.

Enzymatic Digestion

Lysosome preparation. Livers from two male rats were collected and homogenized in 2 volumes of ice-cold 0.3 mol/dm^3 sucrose with 10 strokes at 300 rpm. The homogenate was diluted with 3 volumes of 0.3 M sucrose. The nuclei and cell debris were centrifuged at 2000 rpm for 10 min. The supernatant was centrifuged at 8000 rpm for 10 min to sediment the crude lysosomal-mitochondrial fraction. The sediment was rehomogenized in 10 ml 0.3 M sucrose, containing 1 mM $CaCl_2$. Homogenate was incubated at 37 °C for 5 min to Ca^{2+} uptake by mitochondria. Then 10 ml of 50% percoll solution was added and the homogenate was centrifuged at 8000 rpm for 10 min. The hard brown pellet at the bottom was the lysosomal fraction. For the best pipetting, it was diluted 1 : 2 by 0.3 M sucrose. The enzymatic activity of the preparation was determined with BSA as substrate according to Dingle [26].

Digestion of peptides in lysosome homogenates. Peptides were dissolved in 0.1 M acetate buffer, pH = 3.5 or pH = 5.0 at c = 2.5 mg/ml and the lysosome fraction was added ($c_{lysosomal}$ = 22 µl/ml). Peptides were incubated at room temperature for 180 min. Samples of 100 µl were taken at 30 s and 60, 120, 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 (Symmetry® C_{18} 3.5 µm; 4.6 × 150 mm column), based on calibration curves. For the RP-HPLC analysis A and B eluent were used where eluent A: 0.1% TFA in water, while eluent B: 0.1% TFA in acetonitrile – water (80 : 20, v/v). A gradient 15% B – 40% B in 25 min and 25% B – 50% B in 25 min was used. Each sample was injected twice, and each digestion was performed twice as well. Standard deviation of the twice-injected samples was negligible, while standard deviation for the independent studies was calculated.

Digestion of peptides in human serum. Human serum was purchased from National Institute of Haematology and Immunology, (Budapest, Hungary). Peptides were dissolved in 0.1 M phosphate buffer, pH = 7.2, containing 0%, 10% or 50% human serum at c = 2.5 mg/ml. Peptides were incubated for up to 96 h at 37 °C. Samples (100 µl for 0% and 10% human serum and 140 µl for 50% human serum) were taken after 0, 24, 48, 72 and 96 h. Further treatment of the samples was the same as that for the lysosomal digestion.

RESULTS AND DISCUSSION

In order to analyse the effect of cyclization on the enzymatic degradation of a peptide epitope derived from the glycoprotein D of herpes simplex virus envelope, three cyclic peptides with different linkages were designed. The stability of cyclopeptides with thioether link, disulfide bridge or peptide bond was studied in two complex biological mixtures possessing enzymatic activity. For the investigation of serum stability two concentrations of the human serum were used under aseptic conditions. Similarly the peptides were incubated with lysosomal preparations at two different pH values. In all experiments the amount of intact peptides was determined and by using these values their capability to resist against enzymatic degradation was documented.

Synthesis of Linear and Cyclic Peptides

The ^{278}LLEDPVGTVA^{287} region of HSV gD-1 contains the ^{281}DPVG^{284} epitope core [19] adopting turn structure under appropriate conditions [27]. For the present studies epitope peptides were prepared in which the turn region is stabilized by cyclization. Considering that cyclopeptides derived from short sequences (e.g. tetrapeptides) result in very rigid structure and usually provide no adequate representation of the conformation of the epitope in the native protein, the tetramer part was elongated by three amino acids at both termini.

These flanking regions are from the natural sequence of gD protein. In this design the turn forming motif (^{281}DPVG^{284}) is positioned in the central part of the peptide and this might be advantageous in cyclization.

Linear precursor peptide for preparation of homodetic 'head-to-tail' cyclic peptide was synthesized on Merrifield resin by Boc/Bzl strategy. After completion of the assembly of the sequence, 1 M TMSOTf-thioanisole/TFA cleavage mixture was used for detachment of the peptide from the resin resulting in a semiprotected derivative as outlined in Scheme 1. Under the conditions used the cyclohexyl ester bond is stable and the OcHex protecting group remains intact on the side chains of Asp and Glu [28]. In this way only the *C*-terminal carboxyl group was liberated that could react with the free *N*-terminus. BOP reagent in the presence of HOBt and DIEA was applied for amide bond formation in the cyclization step. The remaining protecting groups of the cyclopeptide were removed with anhydrous HF. The racemization of the *C*-terminal Ala was checked with the aid of Marfey's reagent [29] as described earlier [30]. Less than 1% D-Ala content in the cyclic peptide was detected (data not shown).

Cyclic peptides with a disulfide bridge or thioether link were prepared on MBHA resin. First the linear versions (peptide H-CLLEDPVGTVAC-NH_2 and peptide $ClCH_2CO$-LLEDPVGTVAC-NH_2) were produced and cleaved from the resin by HF cleavage. The HPLC purified peptides were cyclized in 0.1 M Tris buffer at pH 8.2. In the case of disulfide bridge formation the linear peptide was dissolved in the buffer at 0.2 mg/ml peptide concentration, and air oxidation was applied under vigorous stirring (Scheme 2). The end of the oxidation was detected by the Ellman test [31]. In the case of the formation of a thioether linkage, the chloroacetylated linear peptide was added to the buffer solution in small portions in 1 h (Scheme 3) to eliminate the large volume of solution as well as to avoid the side reaction (e.g. dimer-, oligomer formation). The cyclization reactions were followed by HPLC purification. In our hands cyclopeptide with the thioether link provided the highest yield (approximately 70%), while the amide bond formation was less effective (yield approximately 25%). The disulfide bridge formation yielded about 50% product. Peptides used for the enzymatic studies were characterized by amino acid composition, relative molar mass and retention time values (Table 1).

Enzymatic Stability

The enzymatic stability of three cyclic peptides was studied with peptide bond, disulphide bridge or thioether linkage as well as the corresponding linear peptide derived from the LLEDPVGTVA of glycoprotein D of HSV in human serum, and in rat liver lysosomal preparations. The data are summarized in Tables 2 and 3 and the degradation profile of peptides in 50% serum (Figure 1) or in lysosomal preparations at pH 3.5 (Figure 2) are also shown.

For *in vitro* enzymatic stability studies mainly isolated enzymes such as carboxypeptidase A, aminopeptidase M, proteinase A, carboxypeptidase Y [32], α-chymotrypsin, carboxypeptidase Y [33] or complex biological fluids, such as human serum and urine [34], human plasma [35] or rat liver lysosomes [36] are in use. The present investigation used diluted human serum and rat liver lysosome preparations.

Stability in human serum. The enzymatic stability of the linear peptide H-LLEDPVGTVA-NH_2 and its cyclic derivatives were compared in 10% and in 50% human sera (Table 2 and Figure 1). The linear peptide degraded in 24 h even in 10% human serum. All three cyclic peptides were more stable, however, the rate of the degradation was different. 53% of peptide H-CLLEDPVGTVAC-NH_2 with disulfide bridge remained intact in 10% human serum after 96 h (Table 2). The rate of the decomposition was more pronounced (71%) in 50% human serum during the same period of time (Figure 1). The introduction of a peptide bond between the *N*- and *C*-terminal of H-LLEDPVGTVA-OH produced cyclic peptide with improved stability

Table 2 The Effect of Cyclization on the Stability of Linear and Cyclic Peptides Derived from LLEDPVGTVA in Human Serum

Peptide	Peptide [%][a]									
	In 10% human serum					In 50% human serum				
	0 h	24 h	48 h	72 h	96 h	0 h	24 h	48 h	72 h	96 h
H-LLEDPVGTVA-NH_2	100	0	0	0	0	100	0	0	0	0
H-CLLEDPVGTVAC-NH	100	90 ± 1	77 ± 3	69 ± 5	53 ± 13	100	67.5 ± 1	53 ± 1	40.5 ± 2	29 ± 1
c[LLEDPVGTVA]	100	96 ± 1	95 ± 4	93 ± 3	89 ± 5	100	81.5 ± 0	79 ± 3	75 ± 1	73 ± 3
CH_2CO-LLEDPVGTVAC-NH_2	100	100	100	100	100	100	100	100	100	100

[a] Peptide [%] = c_{intact}/c_{total}. The concentration of intact peptide was calculated from the AUC (area under the curve) of the corresponding peak obtained after HPLC analysis as described in Methods and Materials.

Table 3 The Effect of Cyclization on the Stability of Linear and Cyclic Peptides derived from LLEDPVGTVA in Lysosomal Preparation

Peptide	Peptide [%][a]							
	In lysosomes, pH = 3.5				In lysosomes, pH = 5.0			
	0.5 min	60 min	120 min	180 min	0.5 min	60 min	120 min	180 min
H-LLEDPVGTVA-NH_2	100	43 ± 5	14 ± 3	8 ± 4	100	63 ± 1	51 ± 0	33 ± 8
H-CLLEDPVGTVAC-NH_2	100	100	100	100	100	100	100	100
c[LLEDPVGTVA]	100	100	100	100	100	100	100	100
CH_2CO-LLEDPVGTVAC-NH_2	100	100	100	100	100	100	100	100

[a] Peptide [%] = c_{intact}/c_{total}. The concentration of intact peptide was calculated from the AUC (area under the curve) of the corresponding peak obtained after HPLC analysis as described in Methods and Materials.

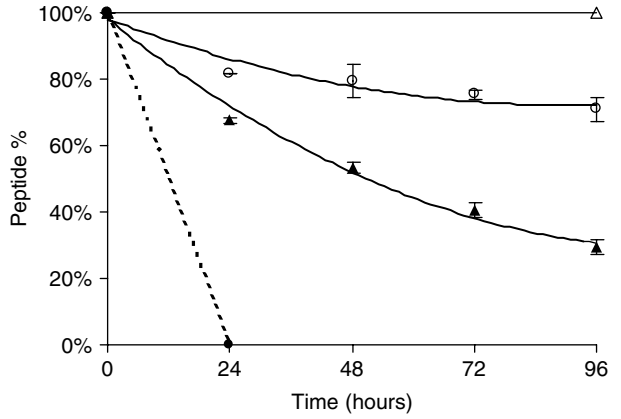


Figure 1 Decomposition of linear (H-LLEDPVGTVA-NH_2) (●), and cyclic peptides with peptide bond (c[LLEDPVGTVA]) (○), disulfide bridge (H-CLLEDPVGTVAC-NH_2) (▲) or thioether link (CH_2CO-LLEDPVGTVAC-NH_2) (Δ) ring closures, in 50% human serum.

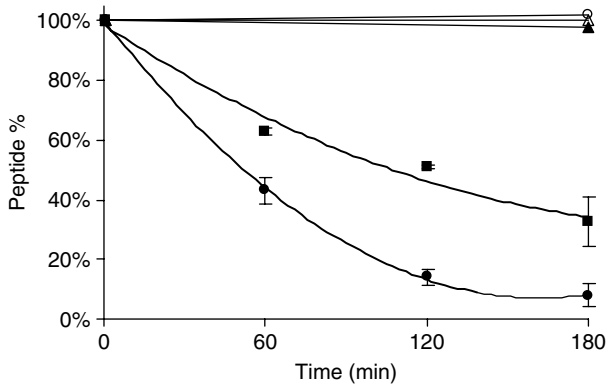


Figure 2 Decomposition of linear peptide (H-LLEDPVGTVA-NH_2) in lysosomal preparations at pH 3.5 (●) and at pH 5.0 (■), and cyclic peptides with peptide bond (c[LLEDPVGTVA]) (○), disulfide bridge (H-CLLEDPVGTVAC-NH_2) (▲) or thioether link (CH_2CO-LLEDPVGTVAC-NH_2) (Δ) ring closures, in lysosomal preparations at pH 3.5.

exhibiting 73% and 89% starting material after 96 h in 10% and 50% human sera, respectively. On the other hand, peptide CH_2CO-LLEDPVGTVAC-NH_2 with a thioether linkage was found to be completely stable under both conditions even after 96 h of the assay. Taken together, based on data described above the following order of stability could be established for cyclic peptides in human serum: CH_2CO-LLEDPVGTVAC-NH_2 > c[LLEDPVGTVA] > H-CLLEDPVGTVAC-NH_2.

Stability in lysosomal preparations. The next series of experiments studied the stability of the peptides in rat lysosomal preparations, containing various proteolytic enzymes. Considering the lack of consensus in the literature about the pH within the lysosome, two pH values (pH 3.5 and pH 5.0) were selected to perform the assay. Harada *et al.* reported on a value of pH 4 [37]; Bach *et al.* observed that the pH in lysosomes varies between 4.3 and 4.5 [38]. Other groups found different levels of acidity between pH 4.5 and 5.0 (pH 4.5 [39,40]; 4.67 [41], 4.75 [42], 5.0 [43]). It is also known that the optimal pH for the main lysosomal enzyme, cathepsin D is active at pH 3.5 [44].

Data of the present experiments are summarized in Table 3 and Figure 2. It was observed that all three peptides exhibited complete resistance against enzymatic degradation under the conditions studied. No difference was found between the stability profiles of the cyclopeptides, studied at these pH values (Table 3). In contrast, predominant decomposition was observed with the linear peptide. It is interesting to note that in this case the rate of the enzymatic hydrolysis was pH dependent. Under more acidic conditions (at pH 3.5), the amount of intact peptide was significantly less (8%) than that at pH 5.0 (33%) during the 3 h incubation period with lysosome preparations (Table 3 and Figure 2).

CONCLUSIONS

This study reports the synthesis and proteolysis of one linear (H-LLEDPVGTVA-NH_2) and three cyclic derivatives, with peptide bond, disulfide bridge and thioether link, of the ^{278}LLEDPVGTVA^{287} sequence of HSV gD-1. The stability of these peptides was compared in human serum and in rat lysosomal preparations. The data obtained in 10% and 50% human sera show that all types of cyclization enhanced the stability of the peptides compared with the linear peptide but at different levels. Complete stability was only achieved by the introduction of a thioether link, while the presence of a disulfide bridge or peptide bond resulted in improved, but partial resistance against hydrolytic decomposition. The results also show that in lysosomal preparations the presence of a cyclic primary structure could provide full protection against enzymatic hydrolysis.

Taken together, the results indicate that the presence of a cyclic structure in the epitope peptide selected for the study could provide a high level of protection against proteolytic degradation in the lysosomal compartment of rat liver cells. In addition, by the introduction of a thioether link into the cyclopeptide full resistance could be achieved against proteolytic degradation in diluted human serum. These findings indicate that by appropriate structural modification it is feasible to construct a synthetic antigen with high stability against enzymatic degradation in complex biological fluids. Further studies are in progress to identify the enzymes responsible for degradation in diluted human sera as well as in the lysosomal preparations and to gain more detailed information on the mechanism of action.

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