Linear Polymerization of a Synthetic Peptide of the V3 Region from HIV-1 JY1 Isolate Using Acetamidomethyl-protected Thiol Groups of Cysteine Residues

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ABSTRACT

A method to carry out the linear polymerization of a synthetic peptide using acetamidomethyl (Acm)-protected thiol groups of cysteine residues, was developed. This polymer showed to be useful in the improvement of peptide immunogenicity. To achieve the polymerization, two Cys(Acm) residues were incorporated at both ends of the peptide. The model peptide contains B and T cell epitopes placed in tandem. The B cell epitope comprises 15 amino acids of the V3 region from HIV-1 JY1 isolate, and the helper T cell epitope belongs to the region 830–844 of the tetanus toxoid. The polymerization reaction consisted in an instant process of deprotection and oxidation of thiol groups at a high monomer concentration. This reaction proceeded quickly with about 80% of conversion. The monomer and the polymers were analyzed by gel filtration chromatography, reverse phase-high performance liquid chromatography, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and mass spectrometry. The sera obtained from mice immunized with the monomer and the polymer variants were assayed against a conjugated BSA-JY1 peptide in an indirect ELISA. The highest titer values corresponded to the polymer variant ($p < 0.01$). This result emphasizes that this strategy can be used to increase the immunogenicity of synthetic peptides in vaccines or therapeutics.

Keywords: cysteine peptide polymers, solid phase synthesis, vaccine peptide

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RESUMEN

Polimerización lineal de un péptido sintético de la región V3 del aislamiento JY1 del VIH-1, mediante residuos de cisteína protegidos con acetamidometilo en el grupo tiol. Se desarrolló un método para realizar la polimerización lineal de un péptido sintético protegido temporalmente con acetamidometilo (Acm) en los grupos tiol de las cisteínas. El polímero obtenido fue capaz de mejorar la inmunogenicidad de los monómeros del péptido. Para lograr la polimerización, se incorporaron dos residuos de Cys(Acm) en ambos extremos del péptido. El péptido modelo contiene epitopos B y T situados consecutivamente. El epitopo B comprende 15 aminoácidos de la región V3 del aislamiento JY1 del VIH-1 y el epitopo T pertenece a la región 830–844 del toxoide tetánico. La reacción de polimerización consistió en la desprotección y oxidación instantánea de los grupos tiol a altas concentraciones del monómero. Esta reacción procedió aproximadamente con 80% de conversión. El monómero y el polímero fueron analizados por cromatografía de filtración en gel, cromatografía líquida de alta resolución en fase reversa, electroforesis desnaturalizante en geles de poliacrilamida y espectrometría de masas. Los sueros obtenidos de los ratones inmunizados con el monómero y el polímero, fueron ensayados contra un conjugado BSA-péptido JY1 en un ELISA indirecto. Los valores de los títulos más altos correspondieron a la inmunización con el polímero ($p < 0,01$). Estos resultados confirman que la estrategia de polimerización lineal con Cys(Acm), puede ser utilizada para aumentar la inmunogenicidad de los péptidos sintéticos en vacunas o en preparados terapéuticos.

Palabras claves: polímeros de péptidos por cisteínas, síntesis en fase sólida, vacunas de péptidos

*I*ntroduction

An important application of synthetic peptides in biology lies in their use as immunogens to elicit anti-peptide antibodies able to cross-react with the cognate parent protein. Such antibodies are very useful reagents for isolating and characterizing gene products [1, 2], and for analyzing the antigenicity or the biological activity of proteins [3]. Another reason for the current interest in synthetic peptides is their use as synthetic vaccines against viral, bacterial and parasitic diseases [4]. Synthetic-peptide vaccines are regarded as a novel approach in vaccinology because they are safe, cheap, easy to store and handle, and ideally suited to specific targeting, which is not possible with classical vaccines.

It is generally accepted that in the aim of raising antibodies, small peptides should be coupled to a high molecular weight carrier. This methodology has limitations when used for vaccination purposes [5]. The cross-linking of the peptide with a carrier may modify antigenic determinants, generating structures that inappropriately represent the native antigen. Also, the cross-linking process may result in unquantifiable structures. Undesirable immune responses such as hypersensitivity and epitopic suppression, may result from immunization with some carriers [6]. These problems are being solved at present by developing new strategies using synthetic peptides as subunit vaccine,

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such as multiple antigen peptide systems (MAPs) [7], peptide dendrimers [8], lipopeptides [9], and linear polymers [10, 11].

The linear polymerization strategy using disulfide bonds in order to cross-link monomers has been used by Patarroyo and co-workers for vaccination purposes [12]. Due to the use in their methodology of cysteine residues protected with MeOBzl group, the monomer units are obtained with free thiol groups. This fact has a direct influence on the undesirable uncontrollable polymerization. Therefore, it is necessary to reduce the crude material previous to the oxidation step. On the contrary, this step can be avoided when the thiol group of the cysteine present in the monomer peptide is protected with the acetamidomethyl (Acm) group, which is stable to hydrofluoric acid (HF) deprotection conditions. The Acm group is widely used as a protecting group for the thiol of cysteines during the solid-phase peptide synthesis [13]. Until now, Boc-Cys(Acm)-OH has been used to make peptides cyclic and to selectively from disulfide bonds in combination with other protective groups [14].

This report describes the use of cysteine residues with the Acm-protected thiol groups for the linear polymerization of synthetic peptides through disulfide bond formation. The temporal Acm protection permits an easy manipulation, characterization and conservation of the monomers. The polymerization reaction is carried out at high peptide concentrations by simultaneous deprotection/oxidation with iodine, proceeding quickly to generate high molecular weight polymers. Besides, it was examined whether this polymer could induce a high level immune response against HIV-1.

Materials and Methods

Materials

The amino acids protected with the Boc group were purchased from Bachem (Switzerland). Solvents for synthesis (dichloromethane [DCM], 2-propanol, *N*,*N'*-dimethylformamide [DMF]) were obtained from Merck (Germany). TAB9 was obtained from CIGB (Havana, Cuba). TAB9 is a recombinant multiepitope polypeptide including the V3 region from the divergent HIV-1 isolates LR150, JY1, RF, MN, BRVA, and IIIB, in this order; fused to the amino terminal 47 amino acids of P64K protein from *Neisseria meningitidis* [15].

Peptide design

The design of the peptide used consists of a Cys(Acm) amino acid at both carboxyl and amino terminus of the sequence. The model peptide contains B and helper T cell (Th) epitopes placed in tandem. The B cell epitope comprises the central 15 amino acids (RQSTPIGLGQALYTT) from the V3 loop of the gp 120 protein of JY1 HIV isolate [16]. The Th epitope belongs to region 830–844 (QYIKA NSKFIGITEL) of tetanus toxoid [17]. B and Th epitopes were spaced by a cathepsin processing site [11]. The whole sequence has the following amino acids: CGRQST PIGLGQALYTTKKQYIKAN SKFIGITELGC.

Peptide synthesis

The peptide was synthesized using the classical "tea bag" method [18]. The 4-methylbenzhydrylamine resin (MBHA) (100–200 mesh, 1–1.2 mmol/g, Fluka, Switzerland) was used. The coupling reaction of the protected amino acids was achieved by activation with 1,3-diisopropylcarbodiimide. The completion of the reactions was verified by Kaiser's test [19]. The peptide was cleaved from the resin by the Low-High procedure using anhydrous HF with the corresponding mixtures of nucleophilic agents (dimethylsulfide, ethanoditiol, *p*-cresol and *p*-thiocresol) [20]. Subsequently, the resin was washed with diethylether and dried out under vacuum. The crude product was extracted from the resin with 30% acetic acid. The final extract was diluted with water and lyophilized.

The purity of the crude peptide was verified by high performance liquid chromatography (HPLC) using an RP C18 Vydac column (10 x 250 mm) (Amersham Pharmacia Biotech, Sweden) with a 5%–60% gradient of acetonitrile (0.05% trifluoroacetic acid) in water (0.1% trifluoroacetic acid) for 40 min. The flow rate was a 0.8 mL/min and UV detection at 226 nm.

Polymerization reactions for deprotection/oxidation of S-Acm with iodine

The crude peptide presenting the cysteine residues protected with Acm was dissolved at 25 mmol/L in AcOH/H_2O (3:2) and 1.5 Eq of HCl 80 mM. Afterwards, 10 Eq of iodine in DMF was added. Finally, the polymerization reaction was stopped adding L-ascorbic acid after 30 min. The reaction mixture was separated by chromatography on a column PD10 (1,5 x 10 cm) containing sephadex G-25 equilibrated in 1% AcOH to remove reagents and to prevent precipitation of polymers. Extensive dialysis against 1% aqueous acetic acid was undertaken followed by lyophilization. The scheme for the synthesis of polymers is shown in Figure 1.

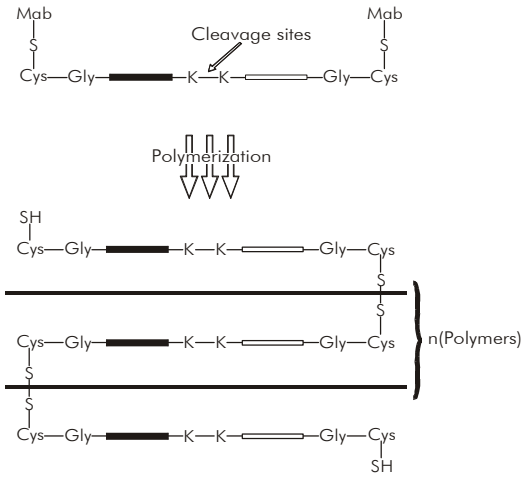

Figure 1. Reaction scheme for the formation of a polymer. Between B and T cell epitopes a cathepsin cleavage site (-KK-) was inserted.

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Polymer characterization

The polymer was analyzed by reverse phase-high performance liquid chromatography (RP-HPLC), using the same conditions as described above for the crude peptide. Based on the numbers of bands and the comparasion with a molecular weight standard, the polymerization degree of the product was evaluated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli [21].

The polymer was also characterized via HPLC gel filtration chromatography using a TSK G 2000SW (8.0 x 300 mm) equilibrated and eluted with 10% of acetonitrile (0.05% trifluoroacetic acid) in water (0.1% trifluoroacetic acid). Lysozyme, chymotrypsinogen A, bovine albumin were used as molecular weight markers.

Biological Assays

Immunization. Female Balb/c mice (10 animals per group) between 6 and 8 weeks old were immunized subcutaneously using incomplete/complete Freund's adjuvant on days 0, 14 and 28 with 40 µg of antigens in a final volume of 100 µL. They were bled 10 days after the last dosis.

ELISA. A conventional indirect ELISA was performed. Briefly, the plaques were coated with 4 µg/mL TAB9 protein at 37 ºC for 3 h. After three washes with 0.05% Tween 20 in distilled water, they were blocked with 1% milk in phosphate-buffered saline (PBS, blocking solution) for 1 h at 37 ºC. Then, the plaques were washed again, the samples were diluted in blocking solution containing 0.05% Tween 20 and 5% sheep serum, and incubated for 1 h with an anti-mouse IgG-peroxidase conjugate. Finally, the reaction was developed for 10 min using *o*-phenylenediamine (0.05%) substrate and H_2O_2 (0.05%), and the optical density (OD) values were determined at 492 nm (Sensident Scan, Merck, Germany). The sample sera were diluted 1:100, 1:1000 and 1:10,000, and their OD values were interpolated in a curve using a previous standard serum.

Statistical analysis

Statistical analysis was performed using the F test to assess variance homogeneity, and Student's *t* test for the comparison of means. All OD values were transformed to ln to homogenize variance among groups.

Results and Discussion

Peptide preparation

The synthesis of the peptide with Acm protection of Cys residues was achieved using the solid phase synthesis. The Acm group was stable to HF deprotection conditions during the cleavage of the peptide. The chromatogram of the peptide with Cys(Acm) groups is shown in Figure 2A. As it can be seen, the crude material is not presented in any polymeric species.

Polymerization reaction by deprotection/ oxidation of S-Acm with iodine

A simple protocol similar to that used in the preparation of cyclic peptides, was adapted to assemble the linear polymer. The polymerization reaction was developed in AcOH/H_2O (3:2), in which the substrates are highly solubles. The reaction proceeds quickly and the evaluation by RP-HPLC of the conversion level of monomers to the polymer was estimated in more than 80% (Figure 2B). About 10% of peptide cyclization took place as a side reaction. This undesirable product was verified by FAB mass spectrometry (MW calculated, 3887.5; MW found, 3887.6). As it was expected, the retention time for the polymeric species was higher than that for the monomer and the cyclic peptide.

It is well known that peptide concentration, reaction time and solubility are important factors in the polymerization reaction. In addition, we have found that when the polymerization reaction is developed at high temperatures, intramolecular cyclization is favored (data not shown).

The polymerization degree can be followed by gel filtration and/or SDS-PAGE. However, the resolution by gel filtration did not allow to separate each particular unit (Figure 3). SDS-PAGE of the polymer formed at 25 mM is shown in Figure 4. The polymerized peptide appears as a smear spanning the entire lane.

Biological properties

Balb/c mice were immunized with the peptide and the polymer. The humoral response was evaluated by an indirect ELISA. After four doses, the antibody response generated in the group immunized with the polymer was significatively higher than the response for the peptide ($p < 0.01$) (Figure 5). This result agrees with previous reports where an enhancement of the immunogenicity has been achieved by linear polymerization of a peptide [11].

In this paper, the polymerization of a peptide that contains B and T cell epitopes sequentially combined through cysteine residues with the thiol group protected with Acm, was described. The polymerized pep-

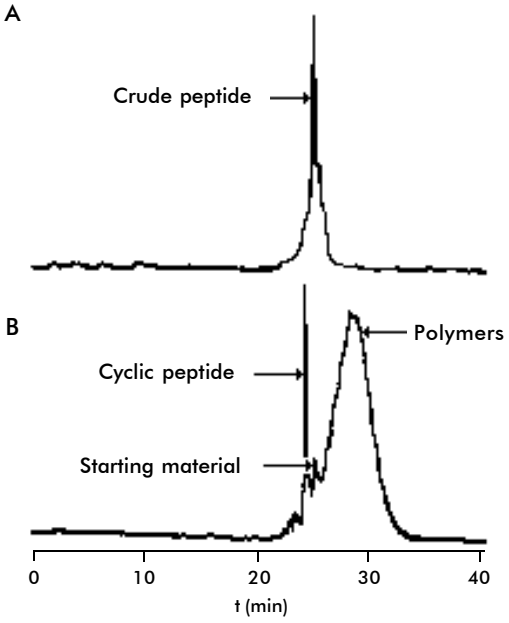

Figure 2. A) RP-HPLC using a Vydac C18 column of crude peptide C(Acm)GRQSTPIGLGQALYTTKKQYIKANSKFIGITEL GC(Acm). B) HPLC of the crude peptide after stirring at room temperature for 30 min in AcOH/H_2O (3:2) with 10 Eq of iodine. The arrows indicate cyclic peptide and polymer peaks.

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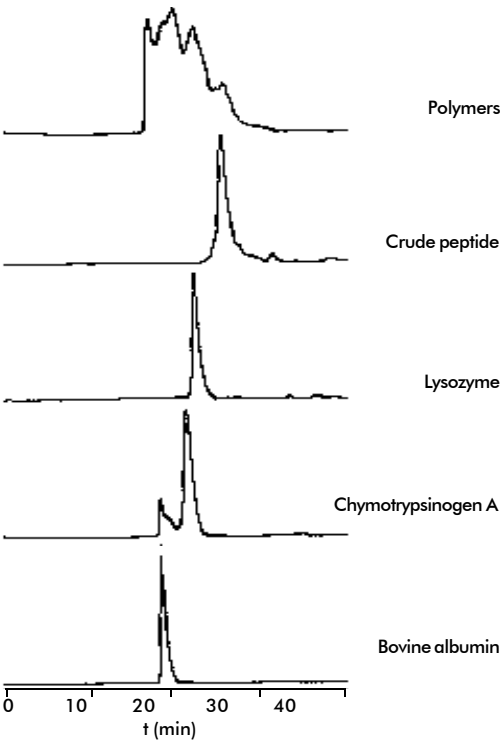

Figure 3. Gel filtration chromatography of peptide polymerization shown in Figure 2. The molecular weight standards shown are lysozyme, MW 14,000; chymotrypsinogen A, MW 25,000; and bovine albumin, MW 67,000. TSK G 2000SW column, equilibrated and eluted with 10% of acetonitrile, was used.

tides have been effectively used to induce antibody-mediated responses to the native epitopes of malaria [12], HIV-1 [22], and hepatitis B [23]. The findings of these studies and others indicate that these synthetic polymers are highly immunogenic. These data provide an additional support to the hypothesis that peptide polymers are good candidates for the development of subunit vaccines against microbial diseases in which circulating antibodies play a protective role.

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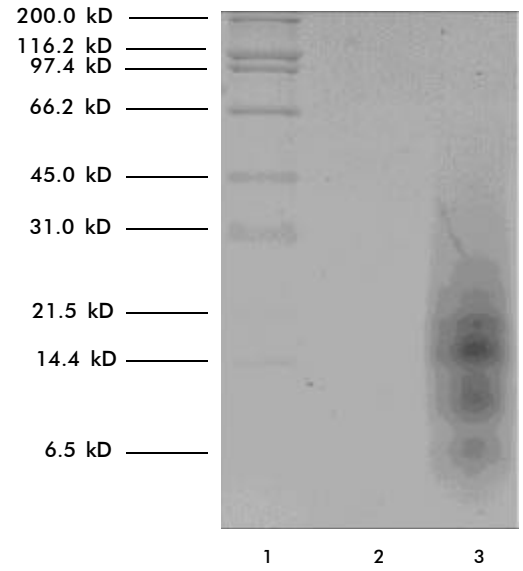

Figure 4. SDS-PAGE of the polymer under non-reducing conditions. The sample polymer was loaded onto the gel after extensive dialysis in a membrane of 6000–8000 molecular weight cut-off. Visualization was achieved by Coomassie Blue staining. Lane 1, molecular weight standards; lane 2, monomer peptide; lane 3, polymers. For unknown reasons, the band of the monomer peptide was not detected under standard conditions.

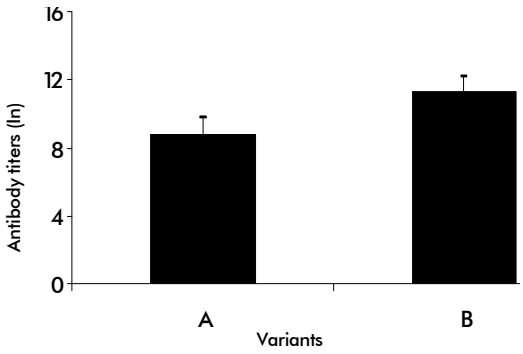

Figure 5. Antibody titers of Balb/c mice immunized with 40 μg of peptide (A) and 40 μg of the polymer variants (B). Antigens were emulsified in Freund's adjuvant and were administered subcutaneously. Titers are expressed as the geometric mean ± standard deviation.

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