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RESEARCH PAPER

Stability Indicating Method for SPf66 Antimalarial Peptide in Solution

A. Santoveña,[1] M. J. Dorta,[1] A. Oliva,[1] M. Llabrés,[1] M. E. Patarroyo,[2] and J. B. Fariña[1,*]

[1]Dpto. Ingeniería Química y Tecnología Farmacéutica, Facultad de Farmacia, Universidad de La Laguna, Tenerife, Spain
[2]Instituto de Inmunología, Hospital San Juan de Dios, Bogotá, Colombia

ABSTRACT

Stability studies on the SPf66 antimalarial peptide with different pH and temperature conditions were carried out. The degradation mechanism was elucidated by the size-exclusion chromatography (SEC) technique and the experimental data obtained at 37°C and different pH were fitted to a kinetic degradation model that could explain the loss of its immunogenic capacity. At 5, 25, 37, and 70°C and pH 2, changes were detected in the areas of the different species, although the values obtained could not be fitted to any known degradation kinetics.

Key Words: Degradation kinetics; Dissolution; Oxidation; SEC; SPf66 synthetic peptide; Stability studies.

INTRODUCTION

Recent developments in recombinant DNA monoclonal antibody technology and in the understanding of the immunological structure of proteins witnessed in recent years have led to a new generation of recombinant subunit and synthetic peptide vaccines. These mimic small regions of microbial proteins, but are unfortunately not in themselves sufficiently immunogenic, due either to their small size or to lack of intrinsic immunostimulatory properties. Due to their complex structure and degradation pathways, protein drugs are often unable to induce appropriate immune responses.

SPf66 is the first synthetic hybrid-polymer containing amino-acid sequences derived from three asexual stage (merozoite) proteins linked by two Asparagine-Alanine-Asparagine-Proline (NANP) amino acid sequences from the sexual stage protein (sporozoite) of

*Correspondence: J. B. Fariña, Dpto. Ingeniería Química y Tecnología Farmacéutica, Facultad de Farmacia, Universidad de La Laguna, 38200 La Laguna, Tenerife, Spain; Fax: +34-922-318-506; E-mail: jbfarina@ull.es.

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Plasmodium falciparum.[1] Several preclinical[2] and clinical trials[3,4] have shown that this molecule is safe, immunogenic, and induces protection. Although the vaccine had been tested in several published trials with varying results, recently Graves, Gelband, and Garner showed the efficacy of SPf66 peptide against clinical malaria.[5]

A quantitative size-exclusion chromatography (SEC) method for SPf66 synthetic peptide was developed and validated by determining its specificity [three peaks with elution volume 7.13, 7.84, and 9.08 mL were detected (Fig. 1), with good resolution and selectivity], linearity [employing eight standard solutions of SPf66 malaria vaccine at concentrations of 5–40 μg/mL and analyzing each sample four times, the relative standard deviation (RSD) was 6.73% (n=32)], accuracy [100.8% with an RSD of 6.0% (n=9)], precision [expressed as repeatability, was <2.2% for the total area of all peaks (n=9)], detection and quantitation limits (5.0 and 15.3 μg/mL respectively), and robustness, according to the International Conference of Harmonization Guidelines.[6] The usefulness of the method to detect differences between different peptide samples was confirmed.[7,8] Due to the complexity of peptides and proteins, no single analytical method can detect all possible chemical, physical, and immunological changes in the protein structure. Thus, relative techniques like SEC, or absolute techniques such as multiple angle laser light scattering (MALLS) and matrix-assisted laser desorption/ionization (MALDI) coupled with time of flight (TOF) mass analysis have been used for characterization of each peak detected in the chromatogram of the SPf66 synthetic peptide. These absolute results correspond to the monomer (4632±32 Da, peak 3), dimer (8642± 76 Da, peak 2), and a mixture of trimer-tetramer species (17,953±81 Da, peak 1), confirming the relative molecular weight obtained with conventional SEC (5094±31, 10,395±32, 17,413±209 Da for peaks 3, 2, and 1 respectively). In conclusion, these data may be especially revealing because the process of autopolymerization could alter the biological activity of the molecule.[9]

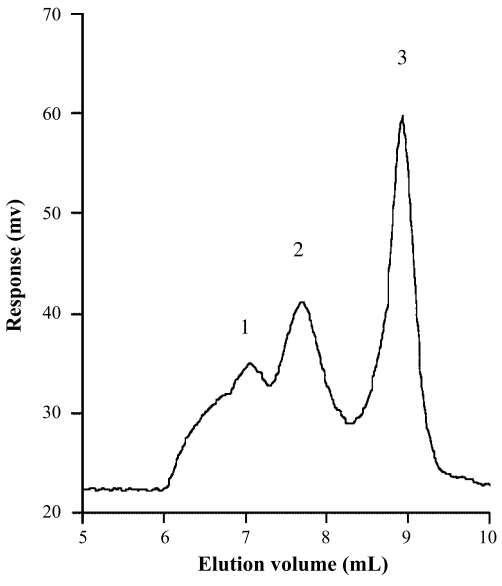

Figure 1. SEC separation of different species of SPf66 antimalarial synthetic peptide with UV-Vis detected at 214 nm.

The principal aim of vaccine technology is the preparation and optimization of a single-administration vaccine to provide complete and long-lasting protection following a single immunization.[10] The controlled-release vaccine approach has shown the most promise for achieving a single-administration vaccine. Several different vehicles have been used to accomplish controlled-release of vaccine; antigen-liposomes, unilamellar vesicles, emulsions, and polymers have all been tested. The main issues to be addressed in future activities are the development of procedures for aseptic manufacture of controlled-release systems and the stability or stabilization of antigens during microencapsulation and release. Single-dose vaccine formulations can only be achieved for antigens whose immunogenicity remains intact over a period of several weeks to a few months under in vivo conditions.[11]

A successful delivery system for a biotechnological product should maintain drug stability during the manufacturing steps, storage, administration, and delivery of the drug to the target site.[12] Owing to the fragile nature of biological macromolecules, it is important to safeguard their structural and chemical stability. Destabilized proteins lose biological efficacy and become aggregated, increasing the possibility of an unwanted immune response.[13] Many manufacturing methods can contribute to this by exposing proteins to potentially damaging conditions, such as aqueous/organic interfaces, elevated temperatures, vigorous agitation, hydrophobic surfaces, detergents, and different buffer components that play an important role due to the pH dependence of protein solubility and stability.[14]

The aim of the present work was to evaluate the capability of an SEC method to study the stability of the SPf66 antimalarial synthetic peptide in different solutions with pH between 2 and 7.4, and different temperatures from 5° to 70°C, in order to gain better knowledge of its behavior under these conditions.

MATERIALS AND METHODS

Materials

SPf66 synthetic peptide (batch 15–7) was synthesized at the Instituto de Inmunología, Hospital San Juan de Dios, Bogotá, Colombia, under solid-phase synthesis conditions described by Merrifield[15] and modified by Houghten,[16] according to the t-Boc technique. The amino acid sequence of the SPf66 peptide is: GDELEAETQNVYAAPNANPYSLFQKEKMVLPNANPPANKKNAG with two cysteine residues at the amino and carboxy-terminal end, which enabled the polymerization of the molecule through an oxidation mechanism, obtaining a final product composed of individual peptides joined by disulfide bridges.

Size-Exclusion Chromatography (SEC) with UV Detection

The chromatographic system used (Waters, Milford, MA) consisted of a pump, a Model 600E Multisolvent delivery system, a 700 Wisp Sample Processor, a 490 programmable multiwavelength detector, and a Protein Pak 125 column (300×7.8 mm I.D., Waters) packed with 10-μm particles of 125 Å pore size as stationary phase. The data acquisition software used was Maxima 820. The mobile phase was an acetonitrile/water (30/70) v/v mixture with 0.05% trifluoroacetic acid at a flow rate of 1.0 mL/min at room temperature, and UV detection at 214 nm was used. Deionized water used to prepare mobile phase was purified by a Millipore Milli-Q system; all other chemicals and reagents were high-performance liquid chromatography (HPLC) grade. All solvents were filtered with 0.45 μm pore-size filters (Millipore). The mobile phase was filtered and degassed.

Stability

pH Studies

The SPf66 peptide was dissolved in different buffers with 0.2 of ionic strength: Glycin I Sörensen at pH 2, Phthalate II Clark and Lubs at pH 4, and Phosphate Sörensen at pH 7.4,[17] with the commonly used pH conditions for in vitro release assays in order to obtain a final concentration of 400 μg/mL, stored at 37°C. Samples were taken after different times, depending on the storage conditions, and were dissolved in the mobile phase to obtain a concentration over a range of 5–40 μg/mL. All the samples were analyzed in triplicate.

Temperature Studies

The peptide was prepared at a concentration of 40 μg/mL in mobile phase (pH 2), stored at 5, 25, 37, and 70°C. These conditions are generally used during conservation of the peptide, in the release assays, and to accelerate the degradation process observed. Samples were taken at different times and directly analyzed in duplicate.

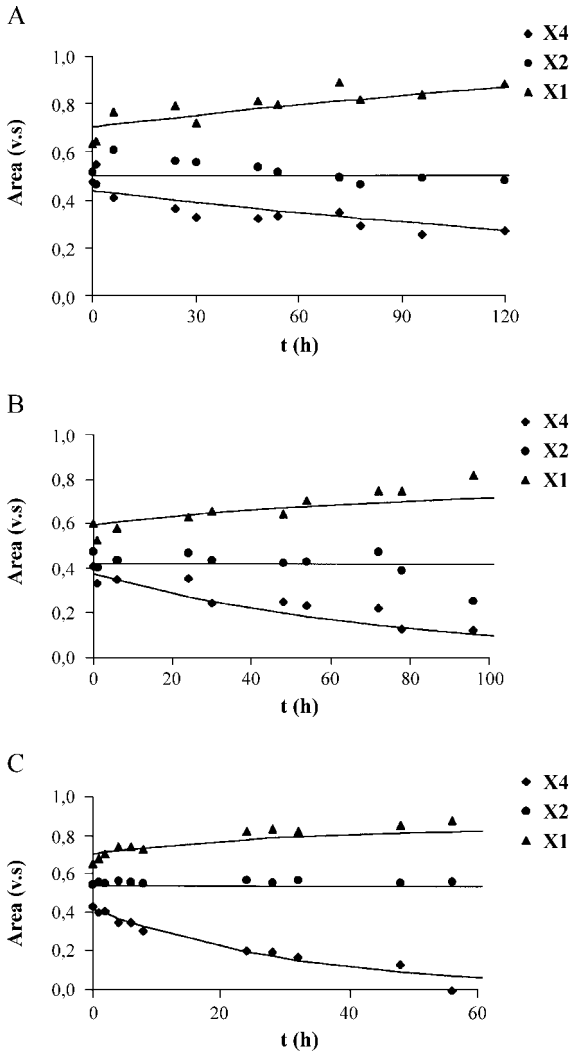

Figure 2. Degradation kinetics of SPf66 peptide corresponding to each species detected at pH 2 (A), pH 4 (B), and pH 7.4 (C).

Table 1. Rate constants and initial areas obtained for each peak of SPf66 peptide at different pH.

	pH 2	pH 4	pH 7.4
k_{40}	0	0	0.02[a] (0.005;0.036)
k_{41}	0.004[a] (0.003;0.005)	0.010[a] (0.0084;0.0135)	0.011[a] (0.002;0.019)
k_{42}	0	0	0
X_{40}	437,490[a] (402,709;472,271)	375,993[a] (302,359;449,628)	420,399[a] (350,731;490,066)
X_{10}	702,851[a] (667,498;738,203)	593,631[a] (523,863;663,399)	703,723[a] (641,328;766,118)
X_{20}	499,295[a] (475,154;523,437)	420,952[a] (375,860;466,043)	539,730[a] (500,380;579,081)

[a]Confidence intervals.

Fit of Stability Results

The experimental results were fitted using the NonlinearFit function from Mathematica 4.0 program (Wolfram Research, Redwood City, CA), which allowed the different parameters of the model to be obtained as in the next subroutine:

```
In [1]:=
fun[z_, t_, X10_, X20_, X40_, k40_, k41_, k42_]:
= Which[z == 1.,
X10 + X40(1 − e^−(k40+k41+k42)t)k41 / (k40 + k41 + k42),
z == 2.,
X20 + X40(1 − e^−(k40+k41+k42)t)k42 / (k40 + k41 + k42),
z == 4.,
X40e^−(k40+k41+k42)t]

In [2]:=
Y1p = Map[{1., #[[1]], #[[2]]} &, X1p]
Y2p = Map[{2., #[[1]], #[[2]]} &, X2p]
Y4p = Map[{4., #[[1]], #[[2]]} &, X4p]
Yp = Join[Y1p, Y2p, Y4p];

In [3]:=
soluno = NonlinearRegress[Yp, fun
[z, t, X10, X20, X40, k40, k41, k42],
{z, t},
{{X10, 714., 614., 814.},
{X20, 605., 505., 705.},
{X40, 206., 106., 306.},
{k40, 0.034., 0.0., 0.068.},
{k41, 0.016, 0.0, 0.032},
{k42, 0.015, 0.0, 0.02}}]
```

the values in the third step of the subroutine being in this case those for the pH 7 degradation assay.

RESULTS AND DISCUSSION

In the case of the stability studies carried out with the SPf66 peptide at 37°C and with different buffers that maintain their pH during the assay, the degradation process observed followed the same pattern: a decrease in the area of the peak corresponding to the trimer-tetramer species, then an increase in the dimer and monomer area, and finally a decrease in the areas of all peptide species. This pattern occurred faster at neutral than acid pH.

The data obtained in these stability studies were first fitted to a mechanism consisting of the transformation of a trimer-tetramer species to a monomer via a dimer, but the experimental data did not fit to it. Consequently, the data were fitted to the following kinetic degradation model, using the Mathematica 4.0 program (Wolfram Research) with the subroutine mentioned in the Material and Methods section:

$$X_1 \overset{k_{41}}{\leftarrow} X_4 \overset{k_{42}}{\rightarrow} X_2 \qquad (1)$$
$$\downarrow k_{40}$$

where X_1, X_2, and X_4—the areas of the peaks corresponding to the monomer, dimer, and trimer-tetramer mixture respectively; k_{41}, k_{42}, and k_{40}—the degradation rate constants for each reaction.

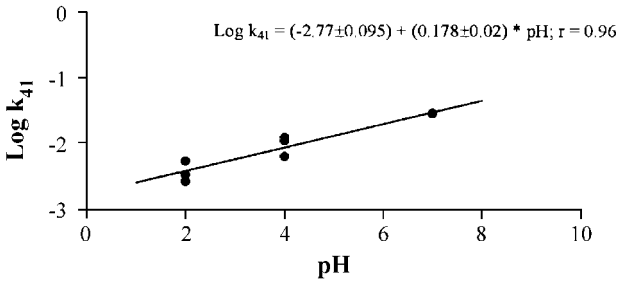

Figure 3. Plot K_{41} rate constant vs. different pH solutions of SPf66 at 37°C.

This model shows the transformation of trimer-tetramer to dimer and monomer species or to new species unidentified. The transformation of dimer to monomer species is not probable because the dimer peak area remains nearly constant while the monomer peak area increases (see Fig. 2). The new, unidentified species formed by hydrolysis of trimer-tetramer with lower molecular weight than the monomer are not detected by SEC.

The equations for each peak detected are:

$$X_4 = X_{40}e^{-(k_{40}+k_{41}+k_{42})t} \quad (2)$$

$$X_2 = X_{20} + \frac{X_{40}(1 - e^{-(k_{40}+k_{41}+k_{42})t})k_{42}}{k_{40} + k_{41} + k_{42}} \quad (3)$$

$$X_1 = X_{10} + \frac{X_{40}(1 - e^{-(k_{40}+k_{41}+k_{42})t})k_{41}}{k_{40} + k_{41} + k_{42}} \quad (4)$$

where X_{40}, X_{20}, and X_{10}—the initial area of trimer-tetramer, dimer, and monomer species respectively.

Table 1 shows the different rate constants and the initial areas obtained for each peak at the different pH studied. The increase in each rate constant occurred with the increase in pH of the sample solution (faster at neutral pH).

Figure 2 shows the fit of the values for each peak (dots) to the kinetic degradation model (continuous line), at the different pH values studied.

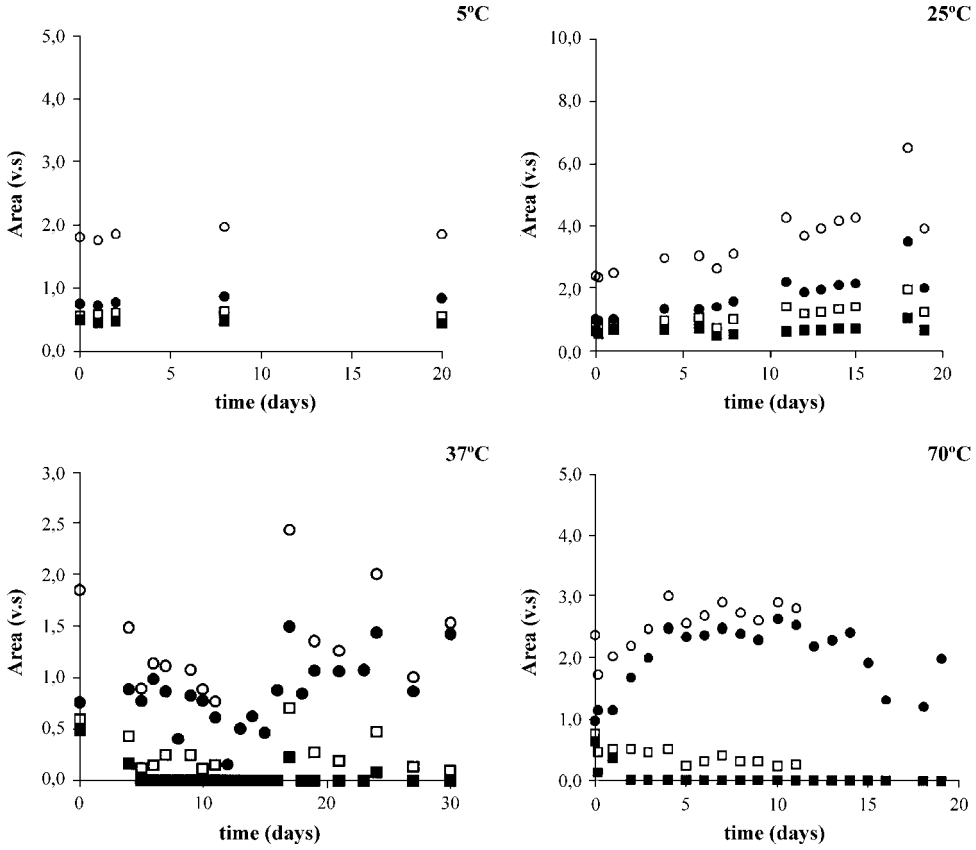

Figure 4. Evolution of the areas of different species of synthetic peptide at different temperatures, with mobile phase (pH 2). ○, total area; ●, peak 1; □, peak 2; ■, peak 3.

The plot of the k_{41} rate constant vs. the different pH solutions (see Fig. 3) shows a straight line (r=0.96) associated with a hydrolytic degradation kinetics. Therefore, this mechanism could be associated with the degradation of the higher molecular weight species (trimer and tetramer).

The influence of temperature on stability shows at 5°C a small decrease in the area of the peak corresponding to trimer-tetramer species (96.7±20), and an increase in the areas of the other species (111±5.96 and 131±39.7% for dimer and monomer respectively) and the total area (116±23.4%) after 22 days of the study (Fig. 4). At 25°C, the values of the total area of the peaks and the areas of the monomer, dimer, and trimer-tetramer species increase (166±2.27, 203±3.65, 165±0.2, and 107±13% at 19 days, respectively). This probably happens because the species formed by the hydrolysis could be present in the chromatogram at the same wavelength as the other species, thus increasing the area values. In contrast, the peaks corresponding to trimer-tetramer species at 37°C disappeared on day 4, and at 70°C on day 1 of the study, thus the area of the dimer peak at 37°C decreases (18.4±26%) and totally disappears at 70°C, followed by the increase in the monomer species (188±13.2 and 204±64.7% at 37° and 70°C) and the decrease in the total area of the peaks (83.5±4.22 and 84.7±26.8% at 37° and 70°C). The data obtained by these stability studies did not follow any known degradation kinetics, probably due to the highly variable values obtained and because the use of the SEC method does not permit the degradation to be monitored. In any case, degradation was obviously faster at higher storage temperatures than at lower ones.

CONCLUSIONS

The results obtained in the present study by SEC show the reliability of the analytical method in indicating that this synthetic peptide became degraded at all pH conditions. These results follow the kinetic degradation model we propose. At 5, 25, 37, and 70°C and pH 2, the results did not fit any known degradation model. In the case of this antimalarial peptide, this information may be especially important because the degradation process could alter its induced humoral immune response and be responsible for the variability in protection obtained in several of the trials carried out.[5,9] Further research is necessary to know if the presence of these different species could modify its immunogenic capacity, as it may depend on their proportion.

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