Anti Human papillomavirus antibody detection using rhodamine B labeled L1-HPV-16 derived peptides

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Universidad Industrial de Santander
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Escuela de Química
Bucaramanga
2018

Dedicatoria

A mis padres, Libardo Rodríguez y Luz Stella Mantilla por enseñarme la humildad y a hacer las cosas siempre de la mejor manera.

A mis hermanos Cristian, Angie y Viviana por ser mis compañeros de vida.

A mi sobrina hermosa Allison Nahomi por ser esa luz que llego a alumbrar nuestras vidas

Los amo con todo mi corazón.

Agradecimientos

A Dios por permitirme crecer como persona cada día, por poner personas maravillosas en mi camino, por ser mi guía.

A mi familia por apoyarme en todo lo que emprendo, por alentarme cuando sentía que no podía, a mi tía Eva por brindarme su apoyo incondicional, por abrirme las puertas de su casa y dejarme hacer parte de su vida.

A mi novio, por apoyarme y estar conmigo en los momentos más difíciles dando ánimo.

A mi director el profesor Mauricio por sus concejos, su paciencia, por entenderme y estar pendiente de lo necesitara.

Al grupo de Investigación en Bioquímica y Microbiología por abrirme las puertas para aprender y empezar mi sueño de hacer ciencia, a la profesora Stelia por su apoyo y dirección, a la profesora Claudia por ayudarme a formar como profesional, a todos los compañeros del grupo por cada consejo y cada palabra de afecto que me brindaron.

A todos los profesores y compañeros que de alguna u otra forma hicieron parte mi formación académica y personal siempre llevare conmigo cada enseñanza.

Acknowledgment

I thank the Industrial University of Santander, the school of chemistry and other schools for their contribution in the loan of equipment for the execution of this work.

This work was supported by the Colombian Institute for the Development of Science and Technology Francisco José Caldas (COLCIENCIAS) through the program "It's time to return", 2015-2017 and by the Universidad Industrial de Santander through the project. With internal code 1823

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Resumen

TÍTULO: DETECCIÓN DE ANTICUERPOS ANTI-PAPILLOMAVIRUS HUMANO USANDO PÉPTIDOS DERIVADOS DE LA PROTEÍNA L1-HPV-16 MARCADOS CON RODAMINA B*

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Palabras clave: VPH, péptidos, anticuerpos, polarización de fluorescencia, cáncer cervical

Las infecciones persistentes por virus del papiloma humano de alto riesgo se asocian con el desarrollo de cáncer de cuello uterino. Existe evidencia de que la infección por VPH genera anticuerpos séricos, asociados con el desarrollo natural de la infección por VPH. La detección de anticuerpos contra el VPH está relacionada con el riesgo de desarrollar lesiones cervicales asociadas a la infección y a la generación de respuesta inmune protectora después de la vacunación. Este trabajo se realizó para determinar si es posible o no, detectar diferencias en la respuesta de anticuerpos anti-HPV entre mujeres vacunadas con HPV (PC) y no vacunadas (NC) usando péptidos derivados de L1 marcados con rodamina y técnicas no convencionales. Se encontró que estos péptidos presentaban una reactividad más alta con anticuerpos de los sueros de PC-mujeres que con anticuerpos de los sueros de NC-mujeres con $p<0,0001$. Seis de los once péptidos derivados de tres regiones L1fueron reconocidos específicamente por anticuerpos presentes en sueros de mujeres vacunadas con VPH como se detectó mediante ELISA. El Péptido P-BC en el ensayo de fluorescencia de puntos, mostró la mayor reactividad específica para sueros de PC, lo que sugiere que este péptido podría usarse para detectar cambios en la respuesta de anticuerpos contra el VPH con esta técnica. En el ensayo de polarización de fluorescencia se encontraron más moléculas de unión en sueros de PC que en sueros de CN para los péptidos P-BC y P-H4(406-423), la mayoría de ellos anticuerpos según los resultados de ELISA y probablemente generados por la inmunización con VLPs. Además, había moléculas de unión con mayor afinidad por los péptidos P-BC(50:64) y P-FG(271: 286) en sueros PC que en sueros NC. Estos cuatro péptidos discriminaron entre sueros de PC y sueros NC utilizando las constantes de disociación (kd) y Bmax.

***Trabajo de grado para optar el título de química**
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Abstract

TITLE: ANTI HUMAN PAPILLOMAVIRUS ANTIBODY DETECTION USING RHODAMINE B LABELED L1-HPV-16 DERIVED PEPTIDES*

AUTHOR: YEIMY ANDREA RODRIGUEZ MANTIILA**

KEYWORDS: HPV, Peptides, Antibodies, Fluorescence polarization, Cervical cancer

High-risk human papillomavirus persistent infections are associated to cervical cancer development. There is evidence that HPV-infection generates serum antibodies, associated with the natural development of the HPV-infection. Anti-HPV antibody detection is related to both the risk of having or developing cervical lesions associated to HPV infection during natural HPV-infection and the generation of protective immune response after HPV-vaccination. This work was performed to determine whether or not is possible to detect differences in anti-HPV antibodies response between young women HPV-vaccinated (PC) and non-vaccinated (NC) using rhodamine-b labeled L1-derived peptides and non-conventional techniques. It was found that these designed peptides presented higher reactivity with antibodies from the sera of PC-women than with antibodies from the sera of NC-women with $p < 0.0001$. Six out of eleven peptides derived from three L1-regions were specifically recognized by antibodies present in the sera of HPV-vaccine immunized women as was detected by ELISA. Peptide P-BC in the dot fluorescence assay showed the highest specific reactivity to PC-sera, suggesting that this peptide could be used to detect changes in the antibody response against HPV with this technique. In the fluorescence polarization assay was found more binding molecules in PC-sera than in CN-sera for peptides P-BC and P-H4(406-423), most of them antibodies according to the ELISA results and probably generated by the immunization with VLPs. Moreover, there were binding molecules with higher affinity for peptides P-BC(50:64) and P-FG(271:286) in PC-sera than in NC-sera. These four peptides discriminated between PC-sera and NC-sera by using the dissociation constants (kd) and the Bmax.

***Research Project to achieve Bachelor Degree in Chemistry**
****Science Faculty. Chemistry Department. Director: Mauricio Urquiza Martínez PhD, Co-directora: Stelia Carolina Méndez Sánchez PhD**

Introduction

HPV infection is one of the most common sexually transmitted infections worldwide(WHO - Word Health Organization, 2013). The HPV family has more than 150 types of viruses; divided principally into two groups: 1) low-risk HPVs responsible, among others, for genital warts (Pfister & Ter Schegget, 1997) and 2) high risk HPVs, which are necessary but not sufficient cause for cancer development, especially cervical cancer (CCU)(Burd, 2003) and requires some factors such as persistent infections(Yang, Cheng, & Li, 2017), cigarette addiction(Fonseca-Moutinho, 2011) and number of sexual partners between others.

Persistent infection with High-risk HPV-16 and HPV-18 are associated with the generation of cytological alterations encompassing the group of cervical intraepithelial neoplasia (CIN), graded from 1 to 3 according to the severity of these lesions that subsequently generate about 70% of all CCU cases (Balanda et al., 2016; Dalstein et al., 2003; Ferenczy & Franco, 2002; Ho et al., 1995). The presence of anti-HPV antibodies in patients with premalignant cervical lesions and cervical cancer has been associated with the degree of the cervical lesion, viral load and antecedents of HPV infection(Leon et al., 2009; Nelson et al., 2017; Safaeian et al., 2010; Salazar-Piña et al., 2016; Viscidi et al., 1997). There is evidence that a presence of anti-HPV antibodies could be a marker of previous or current HPV infections(Huang et al., 2017; Salazar-Piña et al., 2016) . The detection of anti-HPV antibodies is usually performed with immunoassay techniques such as the enzyme-linked immunosorbent assay (ELISA), using as an antigen virus-like particles (VLPs) built with viral capsid L1-protein (Kirnbauer et al., 1994; Panicker, Rajbhandari, Gurbaxani, Querec, & Unger, 2015; Sehr, Müller, Höpfl, Widschwendter, & Pawlita, 2002; Viscidi et al., 1997). However, the VLP-production is expensive and requires cold chain to avoid particle

denaturation (Noad & Roy, 2003). For this reason, peptides derived from L1 epitopes have been used in ELISA as alternative antigens to detect anti-HPV antibodies from patients with cervical intraepithelial neoplasia (CIN) or cancer of the neck, uterine with high sensitivity and specificity (Urquiza et al., 2005; Urquiza, Sanchez, et al., 2008; Urquiza, Guevara, Sanchez, Vanegas, & Patarroyo, 2008).

Another technique used for the detection of antibodies is fluorescence polarization (FP), developed in 1926 by Perrin (Perrin, 1926) and used to study of the receptor-hormone binding (X. Zhang et al., 2017), molecule - protein (Rossi & Taylor, 2011) and antigen-antibody (Nasir & Jolley, 1999; Pérez-Bendito, Gómez-Hens, & Gaikwad, 1994; S. Zhang, Wang, Nesterenko, Eremin, & Shen, 2007; X. Zhang et al., 2017). In this technique a fluorescent molecule excited by polarized light rotates emitting light in all directions and the light intensities emitted by the molecule perpendicular and parallel to the light ray will depend on the size of the molecule. A small molecule will rotate faster than a large molecule, generating lower anysotropy than in the large molecule. A small fluorescent molecule will have lower anysotropy when is no bound than when it is bound to large molecule because the speed of rotation of the fluorescent molecule will decrease. The relationship between the perpendicular and parallel fluorescent intensities are directly related to the amount of large protein bound to the fluorescent molecule, including antigen-antibody (Lea & Simeonov, 2011) . Fluorescence polarization is versatile and inexpensive due to the decrease in reagents, incubation times and sample preparation time; in addition, this technique allows both the antibody quantification and the determination of the apparent affinity constants by fluorescence polarization immunization assay (FPIA)(Hall et al., 2016).

Small fluorescent antigens can be used in different platforms for detecting and quantifying antibodies for example the immobilization of the antigen-antibody complex on Nitrocellulose membrane or PDVF and the fluorescence intensity is recorded on electronic imagen analyzer. This technique uses small amount of sera, antigens and reagents, shorter times of analysis and could be used in a commercial test.

Here we presented the results of two low-cost methods: 1) fluorescence polarization and modified immunodot for the detection of anti-HPV antibodies using rhodamine-B labeled peptides from epitopes of the HPV-16 L1 protein as antigens.

1. Objetives

1.1 General objetive

Diseñar y sintetizar péptidos derivados de la proteína L1 del virus del papiloma humano que puedan tener potencial para detectar y generar anticuerpos neutralizantes contra el VPH.

1.2 Specific objetives

- Design peptides based on the three-dimensional structure of the HPV L1 protein16 from regions that are exposed to the solvent and that are recognized by neutralizing antibodies.
- Synthesize the engineered peptides labeled and not labeled with rhodamine B by solid phase synthesis.
- characterize the peptides synthesized, in terms of purity, molecular weight, secondary structure, absorption and emission of light.
- To evaluate the specificity and sensitivity of the interaction of the peptides synthesized with antibodies from people vaccinated or infected with HPV using different physicochemical techniques.

2. Materials and methods

2.1 peptide design

The peptides were designed from virus surface location of the of B cell epitopes recognized by antibodies from HPV-infected or VLP-vaccinated people (Urquiza et al., 2005), on the three-dimensional structure of the L1 protein of HPV-16 reported in RSCB Protein Data Bank (1DZL)(Chen, Garcea, Goldberg, Casini, & Harrison, 2000), determined by using GETAREA software. The structure visualization was made using the PYMOL program. Wincoot program was used to perform the modification of the peptide sequence and to determine the effect on the structure. To decrease the peptide configurational entropy two residues non-exposed in native structure and located less than 5 Å apart were replaced by cysteines trying to induce intracatenary disulfide formation.

2.2 Synthesis of peptides

Peptides were synthesized in solid phase by the synthesis of multiple peptides in tea bags (Houghten, 1985), using the F-MOC strategy. In brief 100 mg of 4HMBC resin (100-200 mesh, substitution 0.58 mmol/g) (Cruz et al., 2014) per bag; once the peptide sequence on the resin was complete this peptide resin was divide in two parts: 1) used to carry out the labeling of the peptides with rhodamine B, following the coupling protocol for amino acids by increasing the coupling time to 24 hours. 2) The other part of the resin was acetylated using anhydrous acetic acid in dichloromethane (DCM) for 1 hour. The cleavage of the peptides was performed with

trifluoroacetic acid (TFA) / trisisopropylsilane (TIS) / ethanedithiol / H2O (92.5 / 2.5 / 2.5: 2.5) for 2 hours (Cruz et al., 2014).

These peptides were characterized by Reverse Phase High Efficiency Liquid Chromatography (RP-HPLC) using a ZORBAX Eclipse XDB-C18 column (4.6 × 150 mm, 5 µm) mobile phase (A) H2O with 0.1% TFA and (B)) acetonitrile (ACN) with 0.1% TFA; the molar mass was determined by MALDI-TOF mass spectrometry (desorption / ionization of matrix-assisted laser with a time of flight) the matrix used was CHCA (a-cyano-4-hydroxycinnamicacid) of sigma Aldrich, (prepared at 1mg / ml in acetonitrile: water and 0.01% TFA), the method of sample disposition in the target was the sandwich method (Gobom et al., 2001); the secondary structure of the peptides was determined by circular dichroism (CD) in a wavelength range of 190 to 250 nm and a bandwidth of 0.5 nm with a speed of 50 nm / min in a CD spectrometer (J-815 Jasco Corporation, Japan) In addition, rhodamine-labeled peptides were characterized by visible ultraviolet (UV-vis) spectroscopy and fluorescence.

2.3 Human serum obtantion

Sera from eight VLP vaccinated women(Gardasil, Merck, USA), having received three doses of immunization, (positive controls PC) and sera from eight girls under 14 years of age, having had no the first intercourse (negative controls NC), were obtained by means venipuncture at the faculty of health of the Industrial University of Santander and at the university hospital with the parents or legal guardian authorization. The protocol had the approval of the ethical committee. Each women and parents signed the informed consent. These sera were used for the ELISA assays and fluorescence assays using the rhodamine B-labeled synthesized peptides as antigen.

2.4 Enzyme-linked immunosorbent assay (ELISA)

100 µL of peptide (10 µg/mL) diluted in PBS was added to high-binding 96 well plates, incubated overnight at 4 ° C, then these plates were washed 4 times with PBS containing 0,05% Twen 20 (PBS-T). Non-specific binding sites of these wells were blocked with 2% skimmed milk in PBS-T during 2 hours at 37 °C. The wells were 4 times washed again PBS-T. These blocked wells were incubated with 100 µL of serum diluted 1:20 in blocking buffer 1 h at 37 ° C, followed by 4 washings as before. These wells containing bound antibody were incubated with 100 µL of diluted peroxidase conjugated anti human IgG antibody during 1 hour, followed by 4 washings as performed before. TMB peroxidase substrate diluted in buffer was added to each well, incubated for 10 minutes and the color reaction was stopped by adding 0.1M H_2SO_4 before the absorbance was read at 450 nm in an ELISA plate reader (Urquiza et al., 2005).

2.5 Antigen-antibody complex formation on nitrocellulose membrane

Equal volumes of peptide (20 µM, 10 µM 5 µM and 2 µM) and PC or NC serum dilutions (1:10, 1:20, 1:40, 1:80) were mixed and incubated for 15 minutes at room temperature and 1.2 µL of this mix were dropped on a nitrocellulose membrane previously activated in PBS at pH 7.4, (137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 2 mM KH2PO4), containing 0.2% Tween 20, incubated for 10 minutes in humidified chamber to prevent the nitrocellulose membrane drying; subsequently the membrane was washed 3 times with PBS and the fluorescence of the peptide on the nitrocellulose membrane was recorded on a -documentation system Chemidoc (Biorad, Hercules, USA), adjusting the filter to rhodamine (605BP50) with a radiation exposure time of 0.5 seconds, the image analysis was done with the software Image Lab. The experiment the antigen-antibody

complex formation was carried out in triplicate and two independent experiments for each PC- and NC- serum

2.6 Study of antigen-antibody complex formation by Fluorescence Polarization (FPIA)

The fluorescence polarization assays were performed on 96-well black plates (slp life science 30296); in each well a mix of 60 µL of serum and 10 µL of peptide to reach a final volume of 70 µL, at different peptide concentrations from 20 µM to 25 nM and serum dilutions from 1:10 to 1: 320 in phosphate buffered saline PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 2 mM KH2PO4) with 0.1% Tween 20 at pH 7.4. This mix was incubated during 2 hours at 4 ° C, the polarization readings were made in the microplate reader Synergy H1 (Hybrid Multi-Mode Reader) with the Gen5 software, using a cube of filters for polarization with excitation wavelength of 525 nm and emission at 585 nm.

2.7 Statistical analyses

The statistical analysis were done with the program by Prism 5 (GraphPad) software, the comparison of two groups were performed by the Student's T test, and were considered different if $P < 0.005$

3. Results

Based on GETAREA analysis of the three-dimensional structure of the L1 protein pentamer (Chen et al., 2000) and the structure of the complex of these pentamers with neutralizing antibodies(Guan et al., 2015), three regions were selected from L1-HPV protein, exposed on the viral capsid surface; two of them were L1-regions that bind to neutralizing antibodies. According to the L1 structure these regions were the loops BC and FG and the helix h4 from the C-terminal region of the L1 protein (Figure 1).

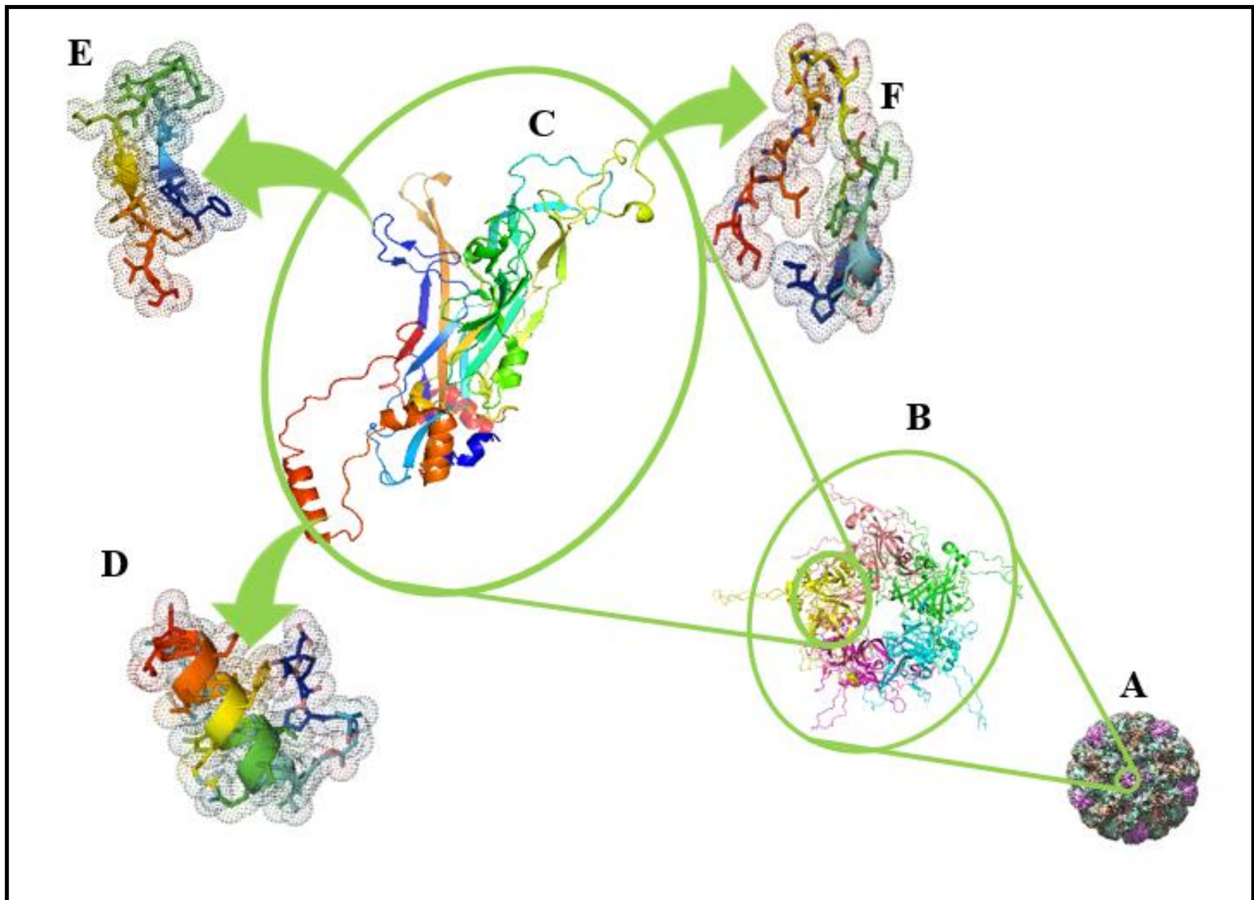

Figure 1. Structure of the human papilloma virus showing L1, the main capsid protein. (A) icosahedral capsid with 72 capsomeres (B) capsomere (pentamer) consisting of five L1 proteins (C) L1 protein used for the design of the peptides. (D) Amino terminal region of the L1 protein where P-H4 and its analogs P-H4 (406: 423), P-H4 (423: 426), P-H4 (417: 420), P-H4 were obtained (420: 424) (E) BC region, peptide P-BC and its analogues P-BC (50:64) and P45 (51:63). (F) Region F-G, P-FG and its analogs P-FG (276: 287) and P-FG (271: 286). (taken from Protein Data Bank (Chen et al., 2000; Guan et al., 2015), edited by the authors)

A peptide derived from the loop BC, having 35% of its sequence exposed on the L1-tridimensional structure, presents high binding activity to serum antibodies from patients with cervical lesion and low binding activity to serum antibodies from normal cytology women (Urquiza et al., 2005); also two key amino acids in the interaction with different neutralizing monoclonal antibodies have been

identified in this loop (Guan et al., 2015). We selected L1 residues from 50 to 64 because this region contains 50% of its sequence exposed in the L1 protein and the amino acids identified in the interaction with monoclonal antibodies. We also selected residues from 271 to 287 belonging to the loop FG, which is the binding site different monoclonal antibodies described by Chen et al. and contains 5, 8 and 10 residues that interact with these antibodies(Guan et al., 2015); moreover, 65% of this sequence is exposed on the L1 protein structure. The sequences of loop BC and loop FG were modified by changing by Cysteines two of the amino acids that were mostly no exposed on the surface and that are less than 5Å apart in the L1 protein tridimensional structure; these two Cysteines were added to the sequence to try to form an intracatenary disulfide bond, reducing the number of structures that these peptides can acquire. From loop BC were designed the analogues P-BC (50:64) and P-BC (51:63) and from the loop FG were designed the analogues P-FG (276: 287) and P-FG (271: 286).

The other selected region was the h4 helix located at the C-terminus of the L1-protein; the sequence of this region is conserved among the different high-risk HPVs, it is exposed on the pentamer surface and a peptide from this region binds to serum antibodies showing different reactivity amongst patients with cervical cancer, infected patients and women with normal cytology (Urquiza, Guevara, et al., 2008). Peptide P-H4, LQ**PPP**GGTLEDTYRFVTSQAIA, was generated from this region; 59% of its sequence is exposed on the surface of the pentamer and this sequence is mostly conserved even in HPV-18, (HPV18 VPPPPTTSLVDTYRFVQSVAIT); using the same approach P-H4 was modified changing different amino acids by cysteines obtaining the analogues P-H4 (406: 423), P-H4 (423: 426), P-H4 (417: 420) and P-H4 (420: 424).

The peptide characterization of six (3 native sequences and 3 analogs) out of the 11 synthesized peptides, selected according to ELISA results are shown in Table 1. The mass variations between the expected and the experimental mass did not correspond to the loss of an amino acid because the mass differences range from 1 to 16 Da; probably were due to secondary reactions occurring during the cleavage of the peptides.

Table 1 ***Peptide Characterization***

	Expected Mass (g/mol)	Experimental mass (m/z)	Retentio n time (min)	PI	Net Charge at pH 7	Secondary structure
P-BC	2347.8	2364.07	7.89	14	4	No defined
P-BC(50:64)	2278.74	2278.6	797	10,38	4	α-helix
P-FG	2306.48	2322.8	8,32	3,71	-1	No defined
P-FG(271:286)	2300.48	2314.7	8,95	3,71	-1	No defined
P-H4	2773.06	2769.9	7,55	3,93	-1	α-helix
P-H4(406:423)	2738.06	2730.1	7,8	3,93	-1	α-helix

Initially an ELISA screening assay was performed to determine the binding activity and the specificity of these 11 peptides to serum antibodies from VLP-vaccinated women. In the ELISA assay performed, all the peptides showed OD higher than 1.2 with serum antibodies from VLP-vaccinated women and OD lower than 0.6 with serum antibodies from negative control women, being the reactivity of PC-sera significant higher than the NC-sera ($p < 0.0001$) (Figure 2). Particularly, the OD of P-BC and its derivatives P-BC (50:64), P-BC (51:63) with PC-sera were 6.6, 5.1 and 3.8 times higher than the OD with NC-sera (Figure 2A), respectively. Defining the specific reactivity as the ratio between OD of PC-sera over OD of the NC-sera, amongst the BC-loop peptides P-BC presented the highest specific reactivity followed by P-BC (50:64), both were selected to perform additional experiments. Amongst the FG-loop peptides P-FG and its derivatives P-FG (276: 287) and P-FG (271: 286) showed the specific reactivity of 4.6, 5.5 and

5.9, respectively (Figure 2B). P-FG and P-FG (271: 286) were chosen for the next experiments. The specific reactivity of the P-H4 and its derivatives P-H4 (406: 423), P-H4 (423: 426), P-H4 (417: 420), P-H4 (420: 424), were 5.4, 6.4, 5.1, 3.8, 3.1, respectively. P-H4 and P-H4 (406: 423) were used for additional experiments.

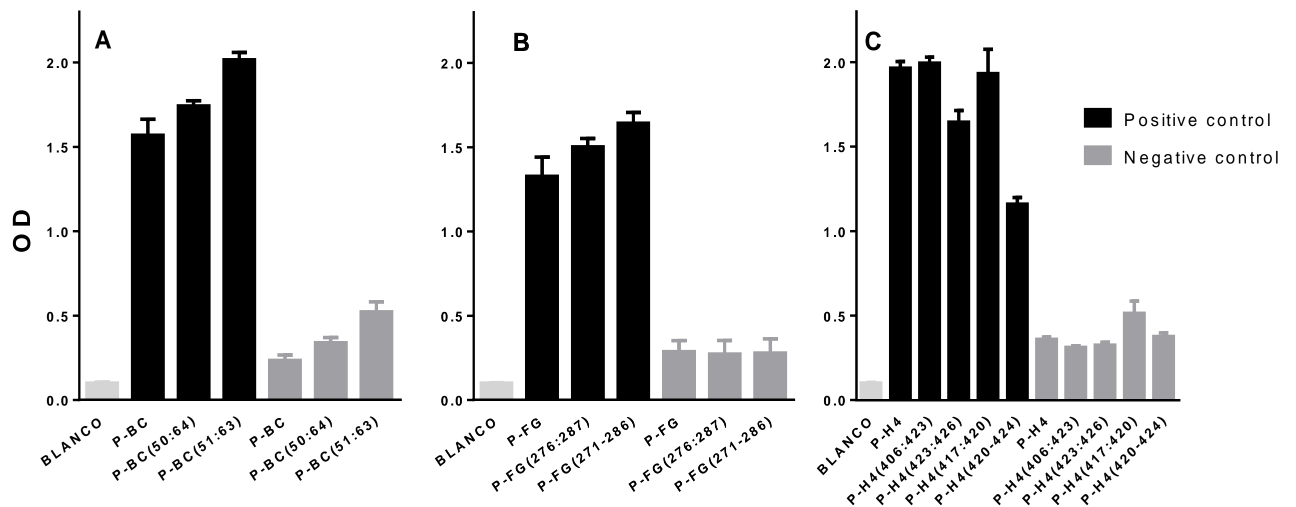

Figure 2 ELISA assay using as antigens the 11 designed peptides

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A. Peptide P-BC and its derivatives showing optical densities with the positive control sera between (1,568 ± 0,054 and 2,015 ± 0,025) and in negative control sera between (0,2353 ± 0,017 and 0.5220 ± 0.03) B. Peptide P-FG and derivatives presenting optical densities with positive control sera between (1,327 ± 0,11 and 1,642 ± 0,065) and negative control sera between (0,2783 ± 0,084 and 0,2870 ± 0,065) C. Peptide H-4 and derivatives having optical densities with positive control sera between (1,159 ± 0,04008 and 1,964 ± 0,03967) and with negative control sera between (0,3117 ± 0,009597 and 0,5133 ± 0,07327). According to the T-student test the difference in reactivity between PC-sera and NC-sera was significant ($p<0.0001$)

In preliminary experiments (data not shown) we have determined that in presence of serum these rhodamines labelled peptides did not directly bind to nitrocellulose membrane, but they

form complexes with serum proteins, including antibodies, able to bind to this nitrocellulose membrane. We prepared different solutions mixing rhodamine labelled peptides at different concentrations and different serum dilutions; 1.2 μL of this mix poured on nitrocellulose membrane, extensively washed the rhodamine fluorescence intensity was recorded. The six peptides selected on the ELISA assay, were assayed using the twelve sera (6 PC-sera and 6-NC sera). Initially, two independent experiments, each one having three replicates were performed with peptide at 20, 10, 5 and 2.5 μM and serum dilutions of 1:10, 1:20, 1:40 and 1:80 and the peptide fluorescence was recorded. The dots of the mix peptides and PC-sera showed significant higher rhodamine fluorescence intensity than the dots of the mix peptides and NC-sera ($p<0.0001$) in most of the conditions that were assayed (Figure 3). The P-BC at 20 μM with sera at 1:10 and at 5 μM with sera at 1:40 showed significant higher fluorescence intensity with PC-sera than with NC-sera ($p <0.0001$) (Figure 3A) but no at 10 μM with sera 1:20 and at 2.5 μM-with sera 1:80. The fluorescence of the dot of P-BC (50:64) at 20 μM with sera 1:10 and at 10 μM with sera 1:20, was significant higher with PC-sera than with NC-sera ($p <0.0001$) (Figure 3B). The fluorescence intensity of the dots of P-FG was significant higher for PC-sera than for NC-sera only at 5 μM with sera 1:40 (Figure 3C) and for P-FG (271: 286) at 20 μM with sera 1:10 and at 10 μM with sera 1:20 (Figure 3D). The fluorescence intensity of the dots of P-H4 (Figure 3E) and P-H4 (406: 423) (Figure 3F) was significant higher for PC-sera than for NC-sera at 20 μM with sera 1:10, at 10 μM with sera 1:20 and at 5 μM with sera 1:40 ($p <0.0001$). Based on these results and looking for an increase in the specificity of this assay additional experiments were performed but at 5 μM of peptide and a serum dilution of 1:10 (Figure 4). Under these conditions the specific reactivity (defined as the ratio of fluorescence intensity of PC/NC) increased for these peptides, being the difference in fluorescence intensity between PC-sera and

NC-sera significant ($p < 0.005$); specially for P-BC that fluorescence intensity was twice for PC-sera than for NC-sera. Under this condition P-FG did not show any significant difference in the fluorescence intensity between PC-sera and NC-sera. Additionally, the proteins of these sera were separated in a non-denaturant gel, transferred to nitrocellulose membrane and incubated with rhodamine labelled peptides to determine the pattern of protein binding to these peptides. The intensity was very low in most of the cases but for example it was found that P-FG (271: 286) interacted with different proteins present in the serum and the fluorescence intensity of some of these peptide binding proteins was higher in PC-serum than in NC-serum (Apendice W)

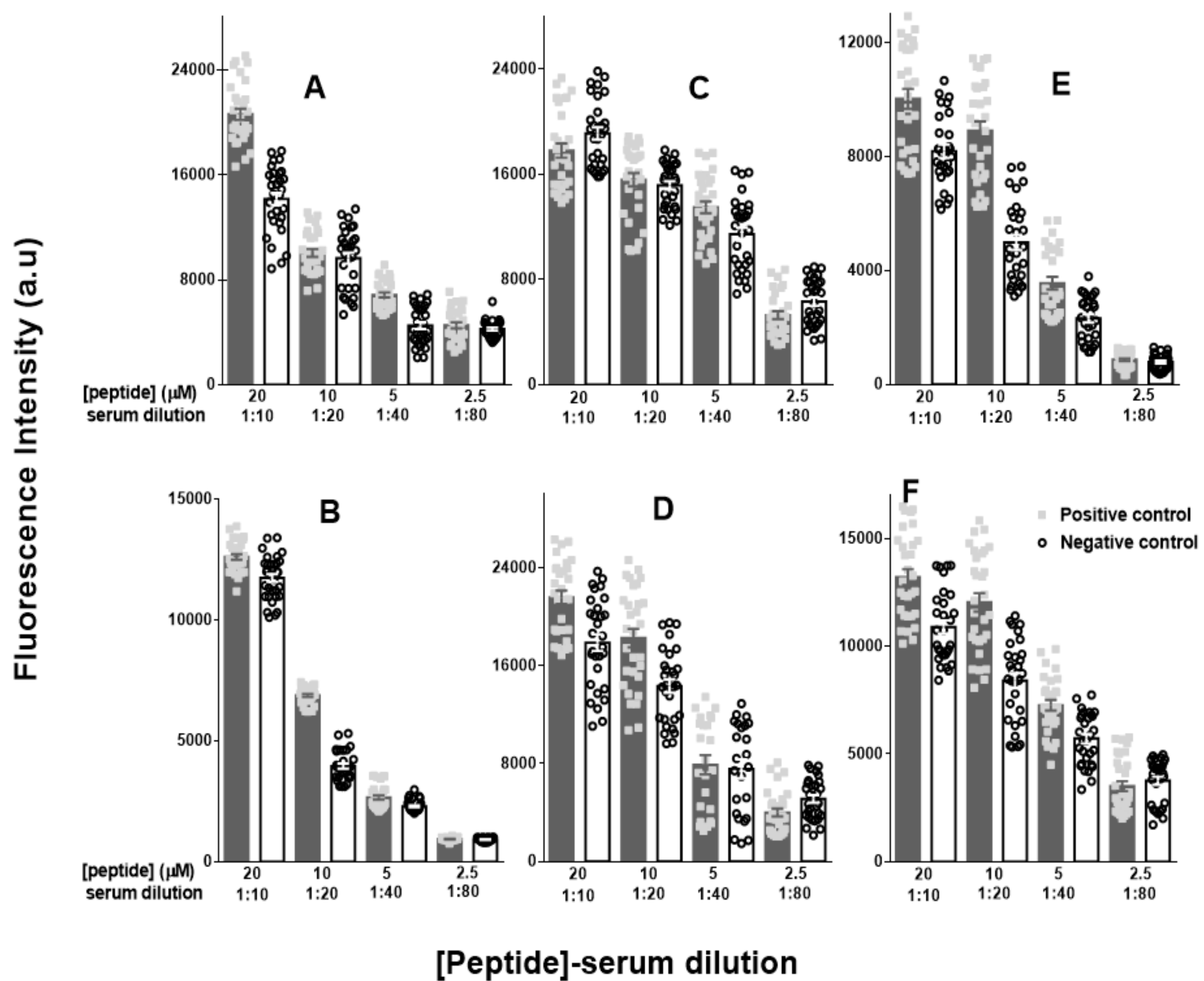

Figure 3 Fluorescence intensity analysis of dots containing molecular complex of serum molecules and fluorescent peptides on nitrocellulose membrane.

Mix of fluorescent peptides at 20, 10, 5 and 2.5 μM with 1:10, 1:20, 1:40, 1:80 dilutions of PC- and NC-sera. (A) fluorescence intensity of P-BC interacting with PC- and NC-sera. (B) P-BC (50:64) (C) P-FG (D) P-FG(271:286)(E) P-H4 (F) P-H4(406:423)

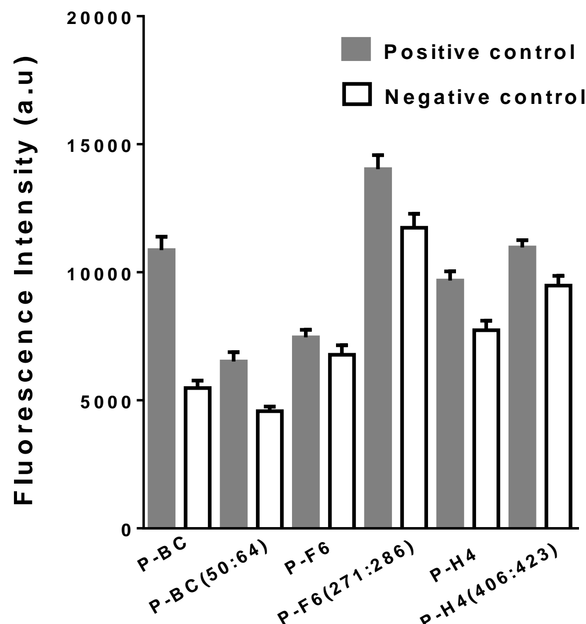

Figure 3 Fluorescence intensity of the dot assay on nitrocellulose membrane using rhodamine labeled peptide at 5µM and dilution of serum 1:10.

Taking into account that there is an interaction between the peptide and serum molecules, we were interested in to study this interaction in solution using fluorescence polarization. The initial conditions of this experiment were fluorescent peptide concentration from 20 µM to 25 nM and serum dilutions from 1:10 to 1:320 to perform a screening to determine the conditions in which there is significant difference in the anisotropy between the interaction with PC-sera and NC-sera. At high peptide concentrations there was observed no changes in anisotropy neither with PC- nor NC-sera at different serum dilutions and is very similar to peptide in buffer and at low peptide concentration the anisotropy was very low and similar to the value obtained with serum in buffer (about 400 mP). The binding assay using one peptide concentration: P-BC (Figure 4A), P-BC (50: 64) (Figure 4B), P-H4 (Figure 4C) and P-H4 (406: 423) (Figure 4D) at 5 µM; P-FG (Figure 4E) and P-FG (271: 286) at 200 nM; in several serum dilution from 1:10 to 1:180 showed

the saturable binding of these peptides to serum molecules; this binding can be fitting by one binding site curve obtaining the Kd and Bmax values and was significant different with PC-sera than with NC-sera (Figure 4 and Table 2). The Bmax and Kd values of peptide binding to PC-sera were significant different to the peptide binding to NC-sera ($p < 0.001$), except that P-H4 binding was similar with PC-sera than with NC-sera.

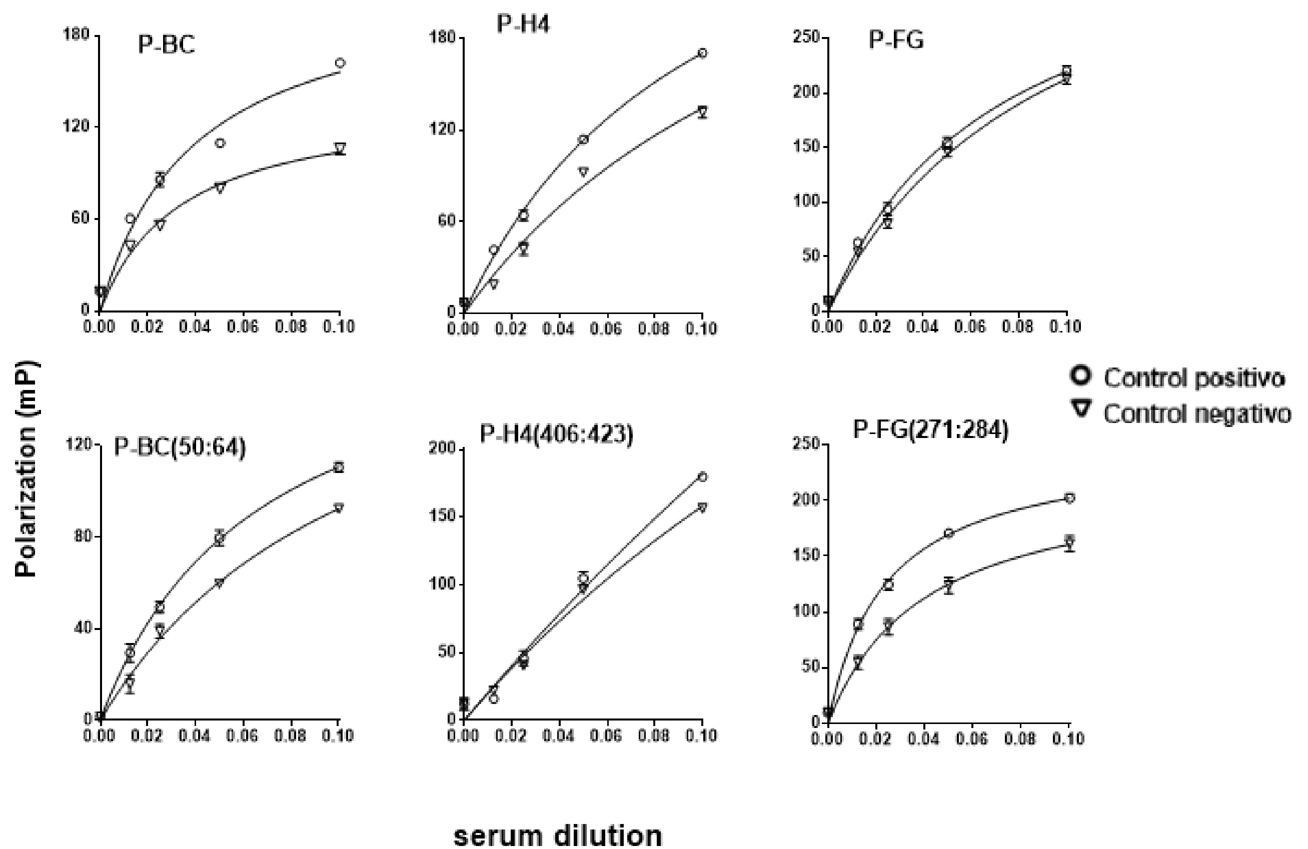

Figure 4 Fluorescence polarization values vs. serum dilution showing saturation curves for the peptides P-BC (A) P-BC (50:64) (B), P-H4 (C), P-H4 (406: 423) (D) P-FG (E) and P-FG (271: 286)

The Bmax values of P-BC, P-H4 (406: 423) and P-FG (271: 286) were higher when these peptides bind to proteins from PC-sera than from NC-sera, but the dissociation constant was

higher for PC-sera than from NC-sera, except for P-FG peptide (271: 286). The Kd and the Bmax for P-BC (50:64), P-H4 and P-FG were lower for PC-sera than from NC-sera

Table 2 *Bmax and Kd values for the interaction of fluorescent peptides and PC- or NC-sera determined by fluorescence polarization assay*

Peptide	Bmax		Kd		*P-value	
	PC	NC	PC	NC	Bmax	kd
P-BC	217 ±11	139 ±8	0.0039±0.004	0.034±0.005	<0.0001	<0.0001
P-BC(50:64)	184±13	196±23	0.006±0.009	0.112±0.021	0.003	<0.0001
P-H4	344 ±21	347±48	0.102±0.010	0.158±0.032	0.5966	<0.0001
P-H4(406-423)	1415±847	690±179	0.680±0.046	0.336±0.011	0.4311	<0.0001
P-FG	373 ±22	411±29	0.070±0.008	0.930±0.011	0.213	<0.0001
P-FG(271:286)	250 ±8	225 ±8	0.024±0.002	0.040±0.003	0.0028	<0.0001

*P values obtained when comparing the different controls

4. Discussion

In general the rhodamine moiety did not affect the binding activity of these peptides; in fact P-H4 (406-423), and P-FG (271: 286) presented higher reactivity than the native peptide, except for P-BC (50:64) that showed lower serum reactivity that could be due to steric effect of the rhodamine that prevents the peptide serum protein interaction.

Amongst the three different assays performed to determine the interaction between these L1-derived peptides and anti-HPV antibodies, ELISA showed the highest specific reactivity. This could be due to the fact that ELISA is very specific to determine antibody interaction, avoiding the detection of the interaction of other serum molecules with these peptides. Despite the lower specific reactivity of the two fluorescent peptide assays these are simpler to perform and cheaper

than the ELISA; moreover, fluorescence polarization assay is in solution and give us Kd and Bmax of this interaction.

In the dot fluorescence assay at the appropriate peptide and serum concentrations, significant differences in the peptide reactivity were found between PC-sera and NC-sera, despite the high fluorescence of dots from the mix NC-sera and peptides. The high non-specific fluorescence was probably due to peptide interactions with other serum molecules that could involve charge interaction because most of these peptides were charged at pH 7.4 (Table 1). In fact, P-FG (271: 286) at high concentrations interacted with many serum proteins and few of them showed higher intensity with PC-sera than with NC-sera as it was determined with this fluorescent peptide incubated with serum proteins separated in non-denaturant gel, transfer to nitrocellulose membranes. The highest specific reactivity to PC-sera in the dot fluorescence assay was obtained with P-BC and suggest that this peptide could be used to detect changes in the antibody response against HPV.

On the other hand, in the fluorescence polarization assay was found that there are more P-BC and P-H4(406-423) binding molecules in PC-sera than in CN-sera, most of them antibody that bind to these peptides according to the ELISA and probably generated by the immunization with VLPs taking into account the higher reactivity of PC-sera than NC-sera. On the other hand, there are P-BC(50:64) and P-FG(271:286) binding molecules with higher affinity for these peptides in PC-sera than in NC-sera. In summary these four peptides in the fluorescence polarization assay discriminated between PC-sera and NC-sera by using the dissociation constants (kd) and the Bmax. This assay is promissory because is quantitative, obtaining Bmax and Kd which are related to the amount of antibodies and the affinity of these antibodies against these peptides; moreover

can be performed multiple tests in a short time. This assay can be used to study the antibody response to HPV during VLP vaccination at during the HPV-infection. The high reactivity of these peptides with NC-sera in the two assays using fluorescent peptides, probably due to binding to other serum proteins, can be overcome using simple protocol of antibody isolation.

5. Conclusion

Fluorescent peptides from the L1 protein can be used to detect of antibodies induced by vaccination using different techniques, the labeling of these peptides with fluorochromes allows us to study peptide- antibody interactions with unconventional techniques potentially usable in new large-scale screening techniques, not only for detecting and probably quantification of HPV induced antibodies but also antibodies induced for various diseases.

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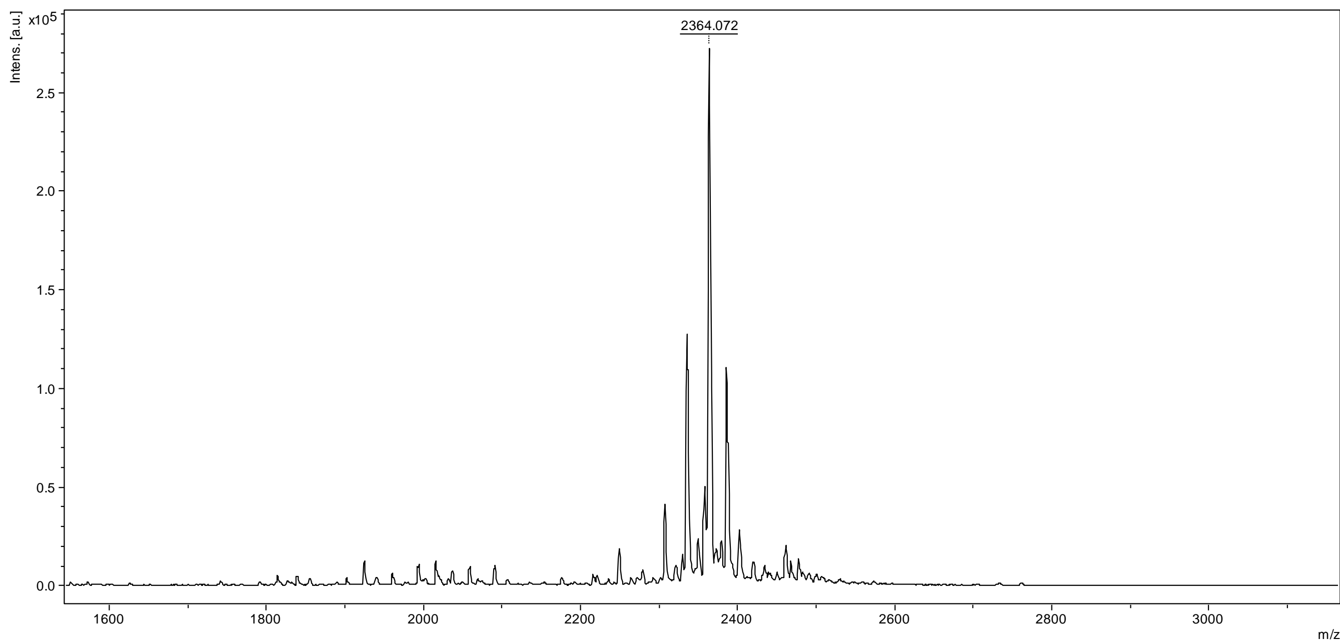
Apéndices

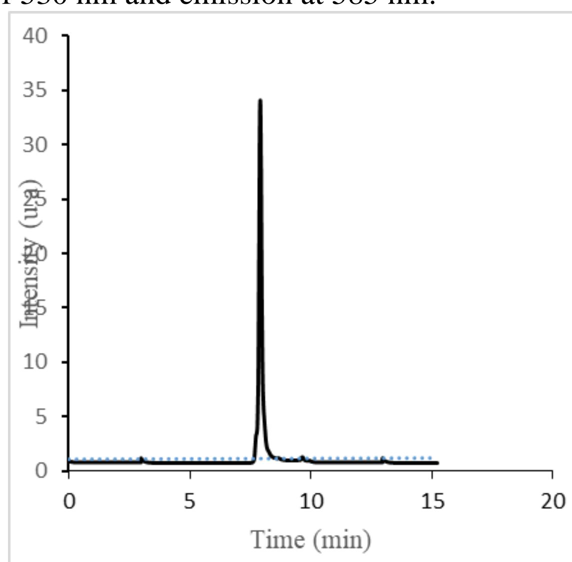
Apéndice A Mass spectrum P-BC

molecular ion peak at 2364, 072 m / z. the theoretical value of the mass for this peptide is 2347.8 there is a mass difference of +16 which can be due to the hydroxylation of one of the prolines of the peptide P-BC.



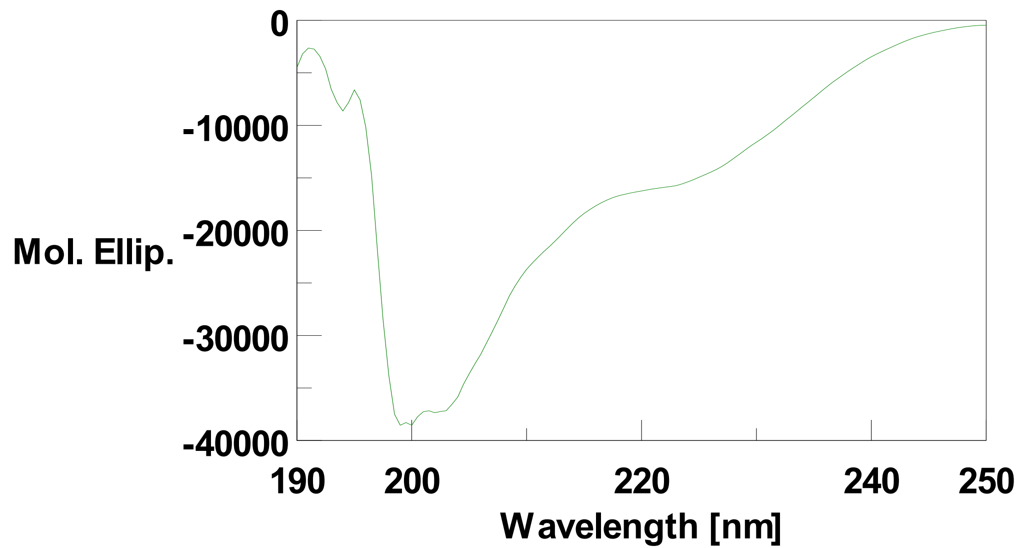
Apéndice B Chromatogram P-BC.

With a retention time of 7.89 min, a gradient of 5-100% acetonitrile was used, with detection by fluorescence, wavelength of 530 nm and emission at 585 nm.



Apéndice C Circular Dichroism of P-BC

According to circular dichroism, the peptide has no defined secondary structure.



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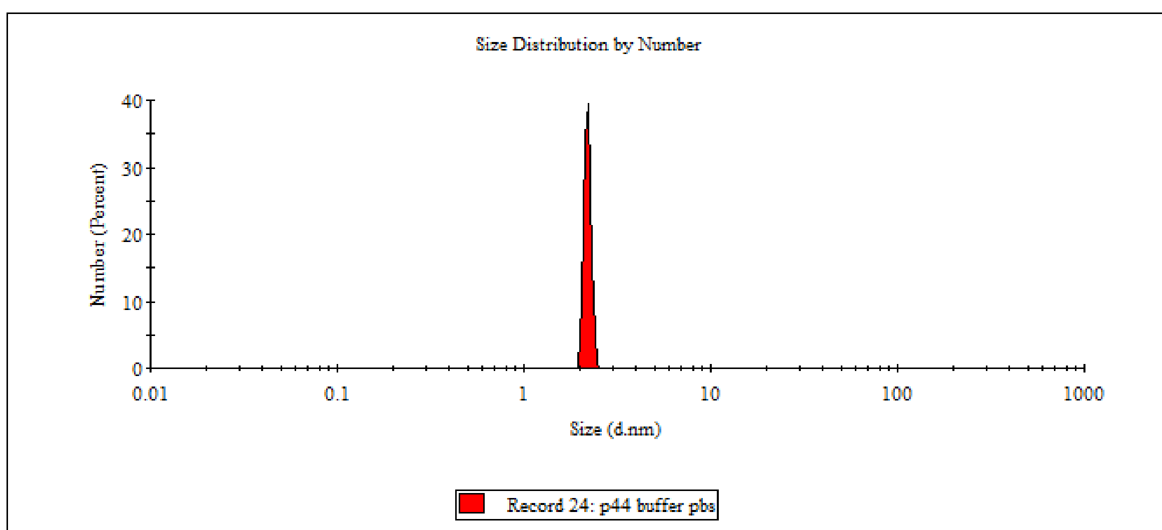
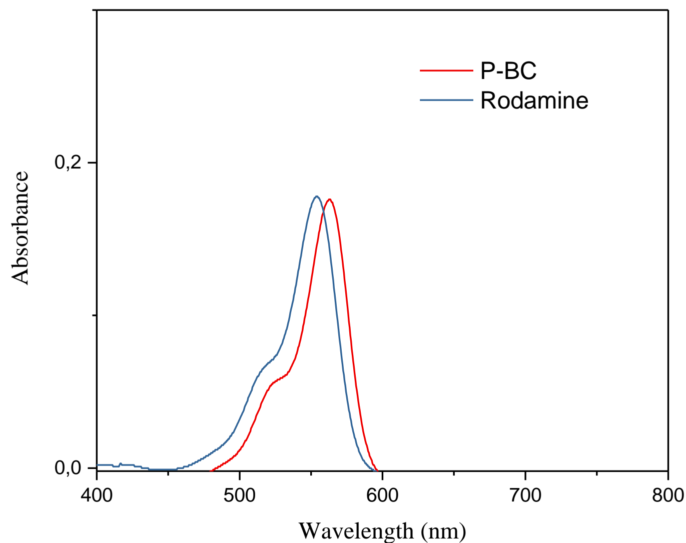
Apéndice D. Average diameter of P-BC

measured with the equipment dynamic light scattering (DLS) (4.7 nm)

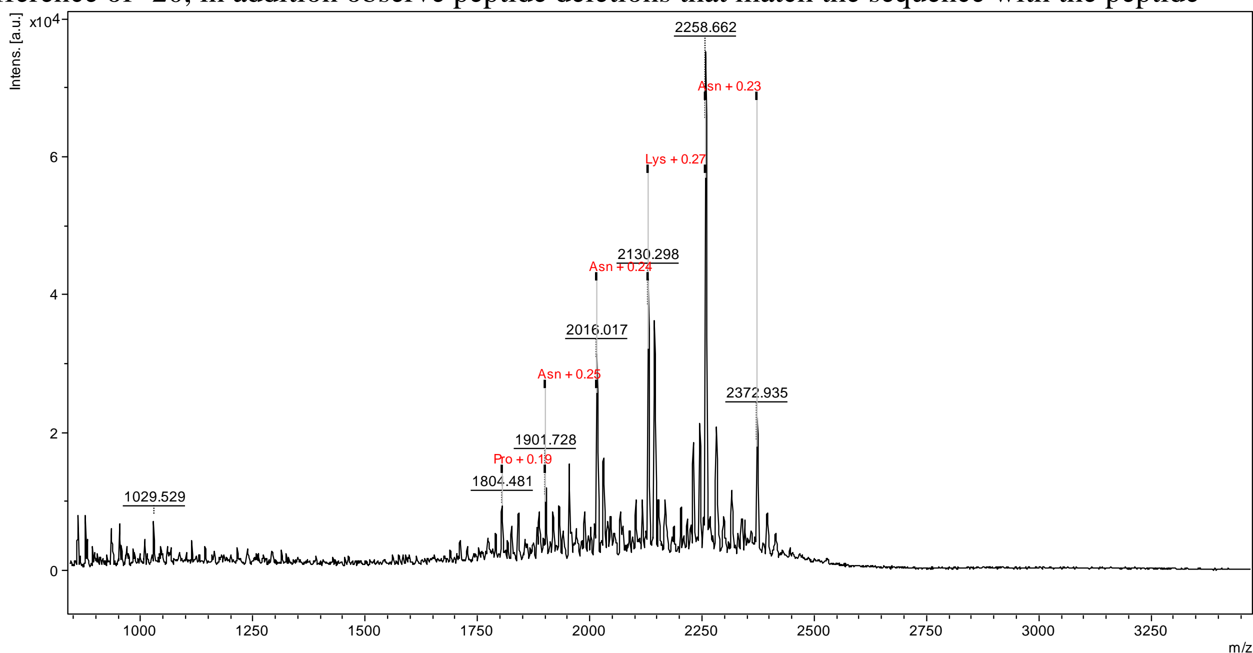


Apéndice E Uv-vis spectrum by comparing the wavelength of absorption of rhodamine and peptide P-BC labeled with rhodamine.
A shift towards a shorter wavelength in the labeled peptide is observed



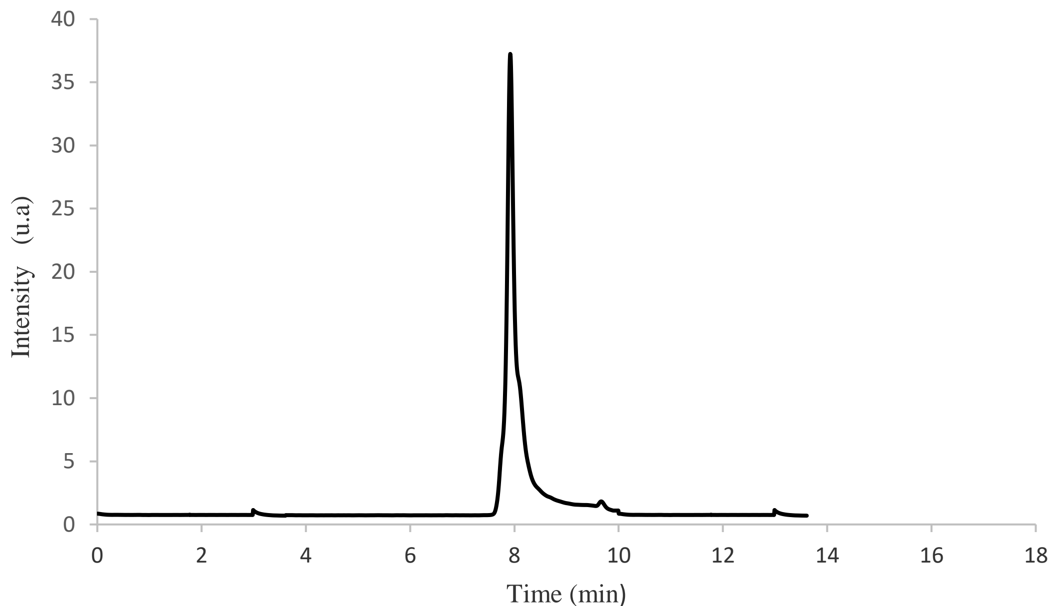
Apéndice F. Mass spectrum for P-BC (50:64).

Molecular ion peak at 2258 z / m, the theoretical value for this peptide is 2278.7, there is a mass difference of -20, in addition observe peptide deletions that match the sequence with the peptide



Apéndice G. ***Chromatogram P-BC (50:64)***

with retention time of 7.97 min, a gradient of 5-100% acetonitrile was used, with detection by fluorescence excitation wavelength 530 nm and emission at 585 nm



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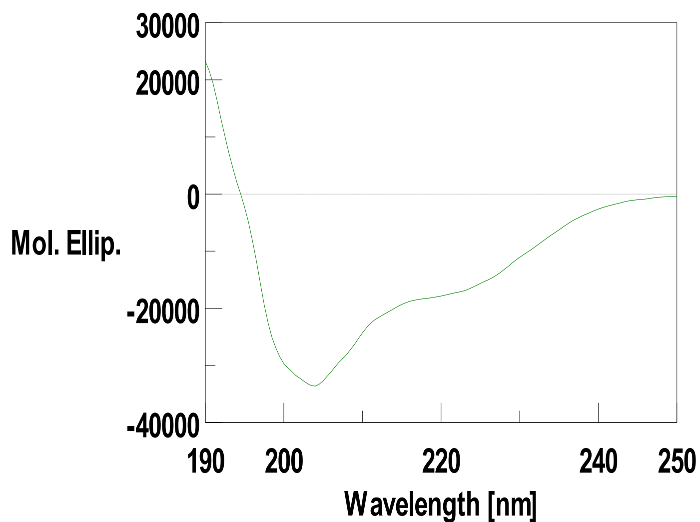
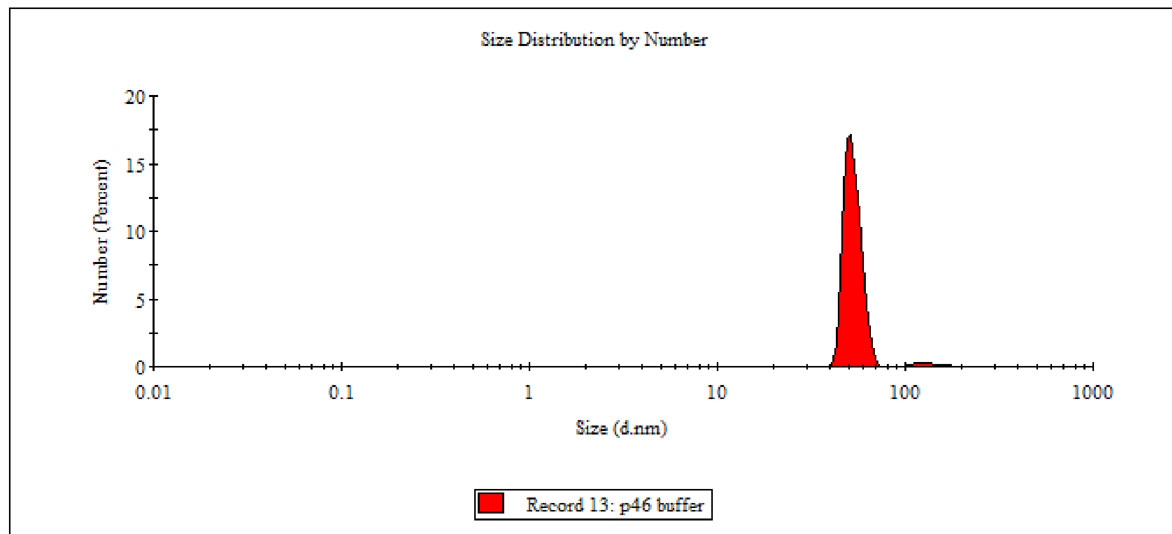
Apéndice H. Circular dichroism for the P-BC analog, P-BC (50:64)

a change in the secondary structure is observed due to the influence of the two amino acids substituted by cysteines, it is observed that the peptide tends to form an Alpha helix



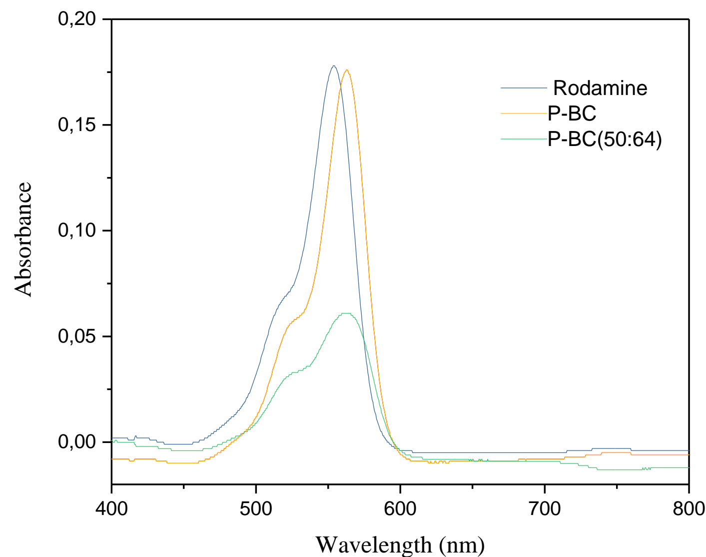
Apéndice I Average diameter of P-BC (50-64)

measured with the equipment dynamic light scattering (DLS) (159.2 nm)



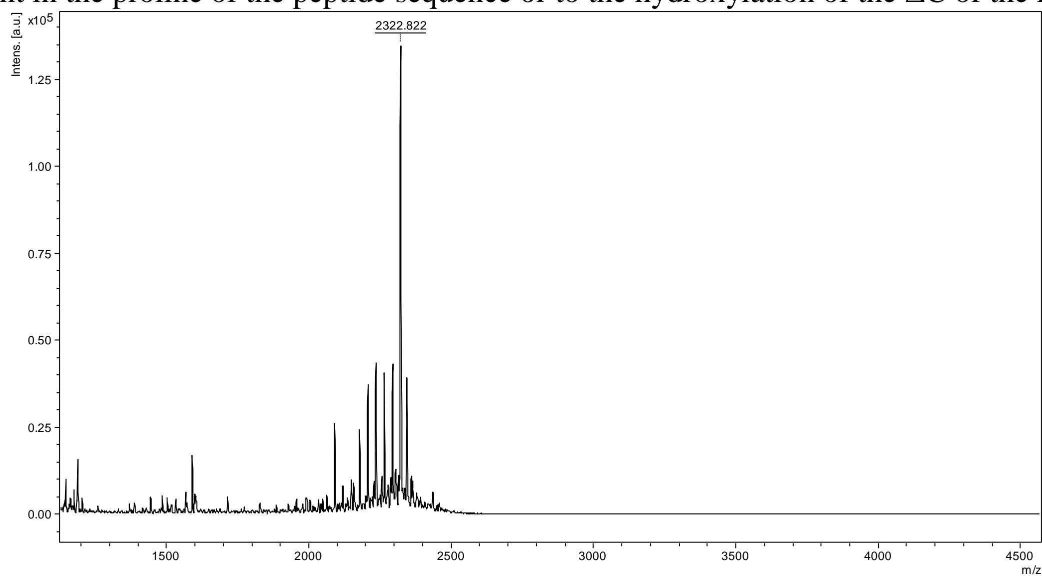
Apéndice J Uv-vis spectrum by comparing the rhodamine absorption wavelength to the P-BC and P-BC peptides (50-64)

Labeled with rhodamine, there is no change in the displacement of the maximum absorption peak between the two peptides



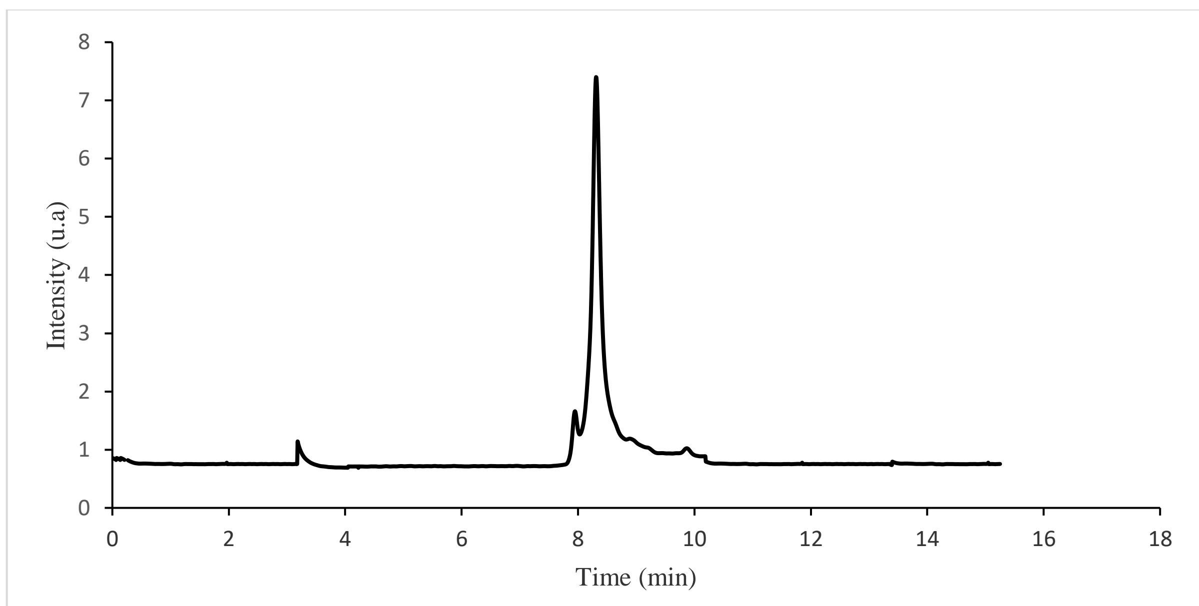
Apéndice K Mass spectrum peptide P-FG

molecular ion peak at 2322.822 m / z. the theoretical value of the mass for the peptide P-FG is 2306.48, there is a mass difference of +16 which may be due to the hydroxylation of the C4 present in the proline of the peptide sequence or to the hydroxylation of the ΔC of the lysine.

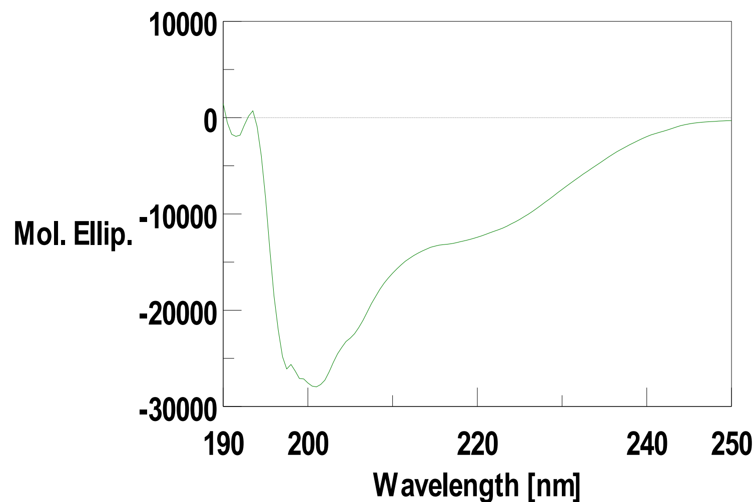


Apéndice L Chromatogram of the peptide P-FG

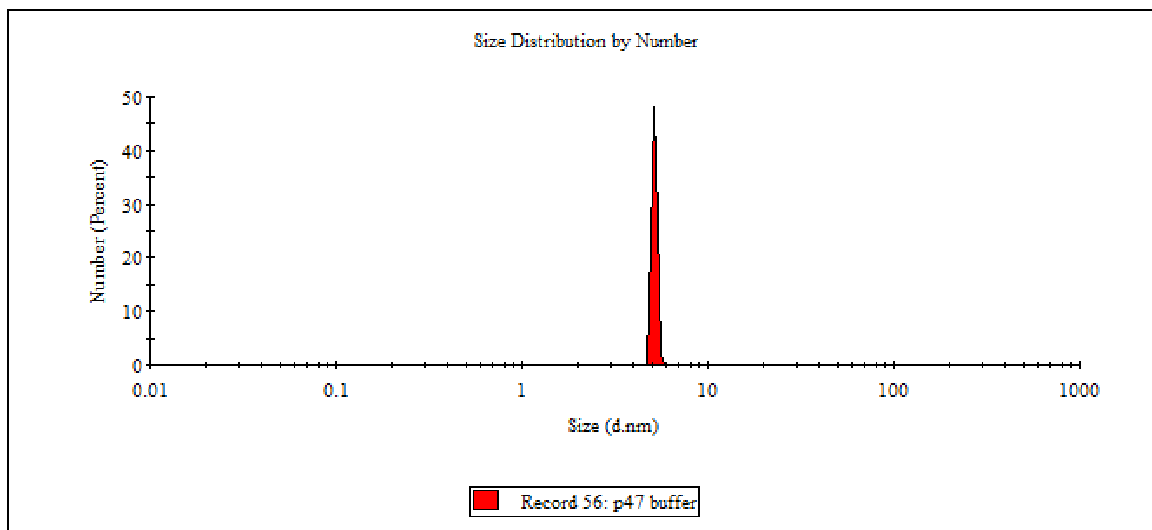
with retention time of 8.32 min, a gradient of 5-100% acetonitrile was used, with detection by fluorescence excitation wavelength 530 nm and emission at 585 nm



Apéndice M Circular Dichroism of P-FG taken at the Catholic University of Valparaíso-Chile. According to circular dichroism, the peptide has no defined secondary structure

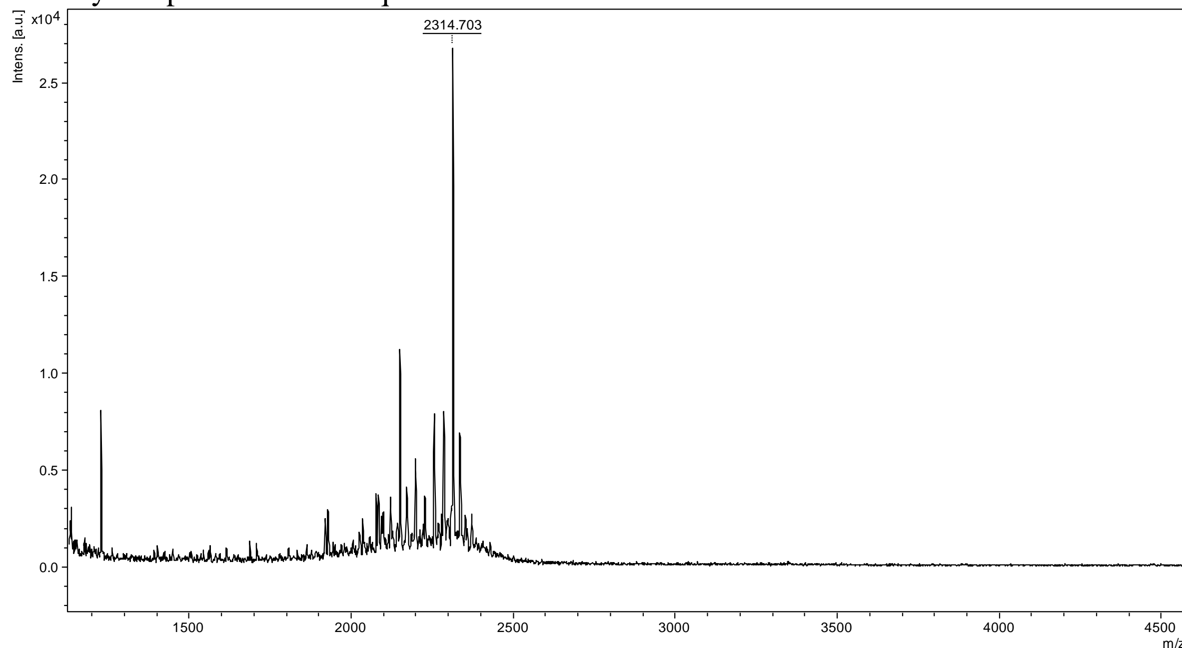


Apéndice N . Average diameter of the peptide P-FG
measured with the equipment dynamic light scattering (DLS) (5.2 nm)

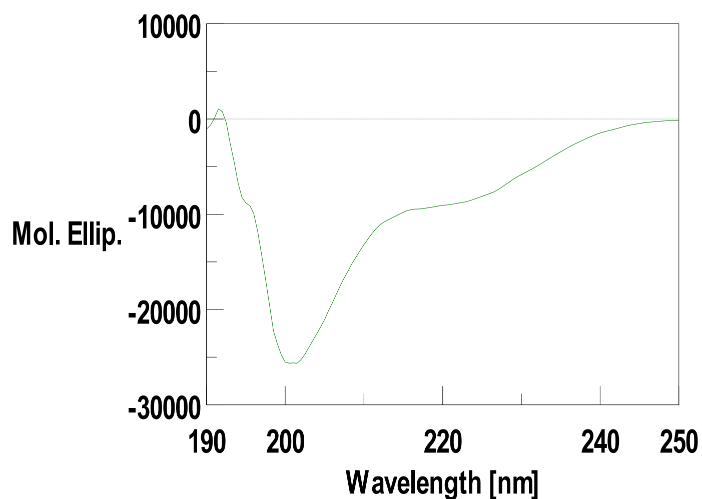


Apéndice O Mass spectrum peptide P-FG(271:286)

Molecular ion peak at 2314.7 m / z. the theoretical value of the mass for the peptide P-FG(271:286) is 2300.48, there is a mass difference of +14 which can be due to methylation of the εN of the lysine present in the sequence

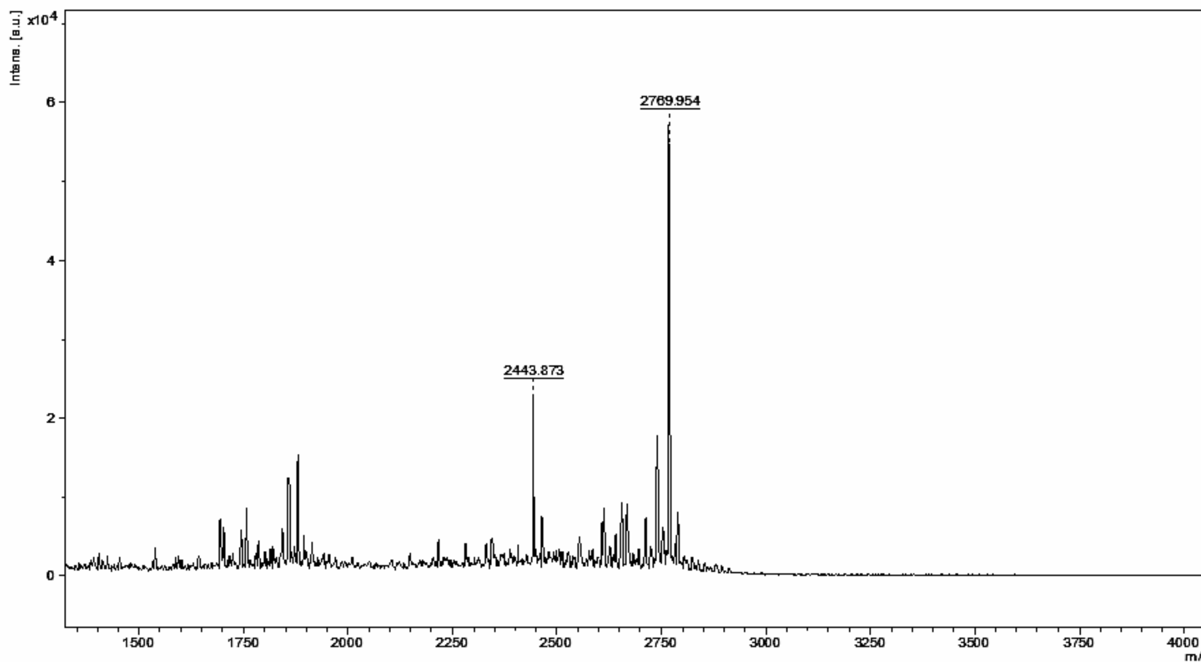


Apéndice P Circular Dichroism of P-FG (271: 286).
According to circular dichroism, the peptide has no defined secondary structure



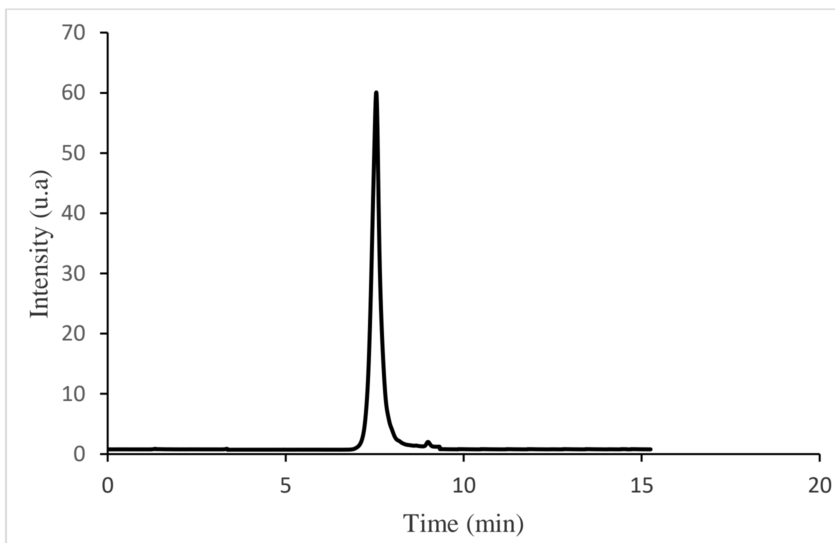
Apéndice Q. Mass spectrum the peptide P-H4.

molecular ion peak at 2769. m / z. the theoretical value of the mass for the peptide P-H4 is 2773 there is a mass difference of -3

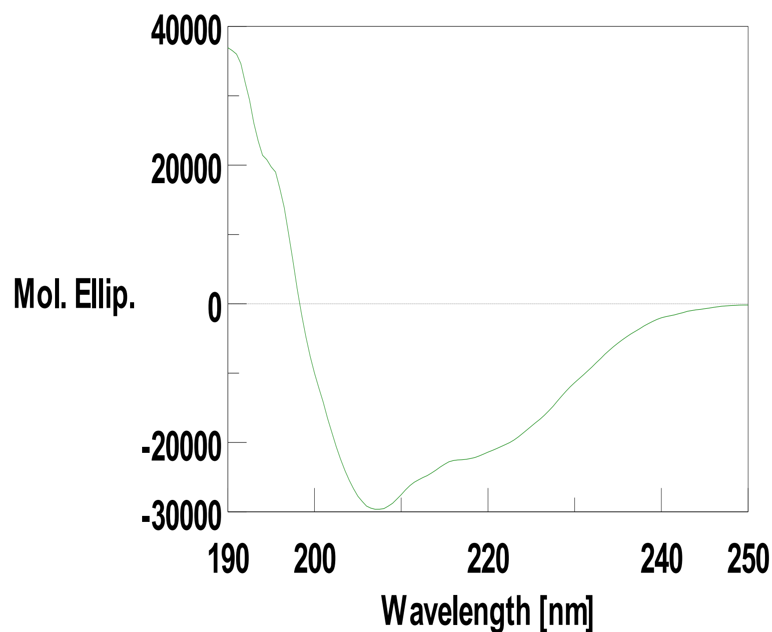


Apéndice R Chromatogram P-H4

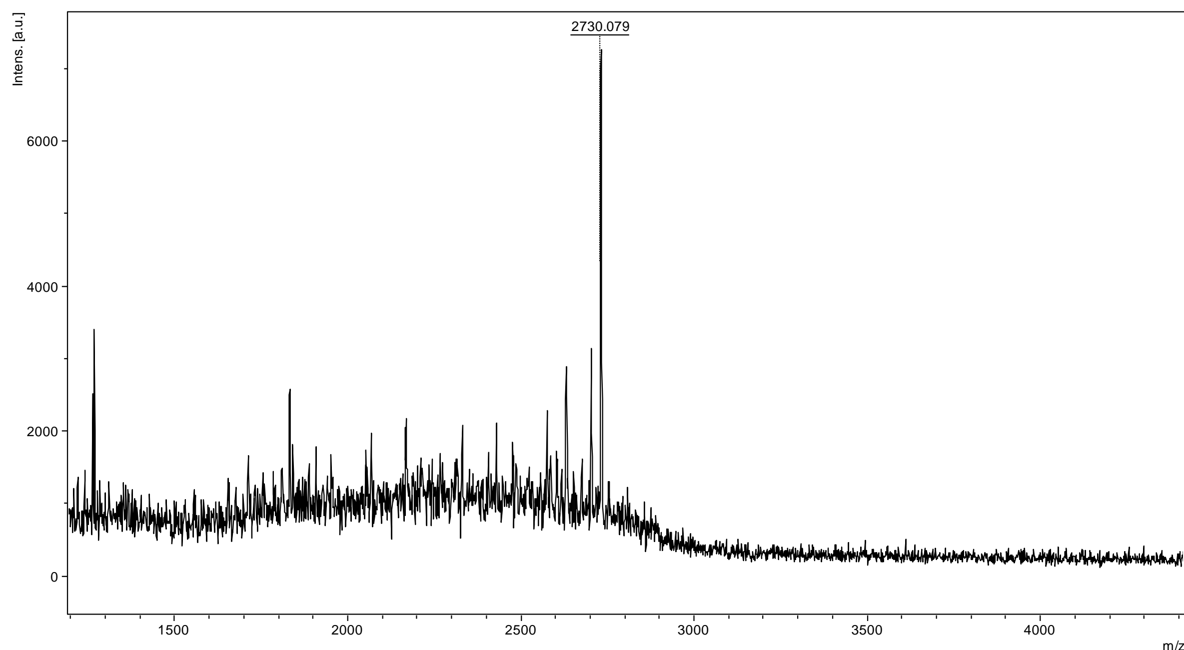
with retention time of 7.55 min, a gradient of 5-100% acetonitrile was used, with detection by fluorescence excitation wavelength 530 nm and emission at 585 nm



Apéndice S Circular Dichroism P-H4
taken at the Catholic University of Valparaíso-Chile. According to the circular dichroism the peptide has a secondary structure alpha helix.

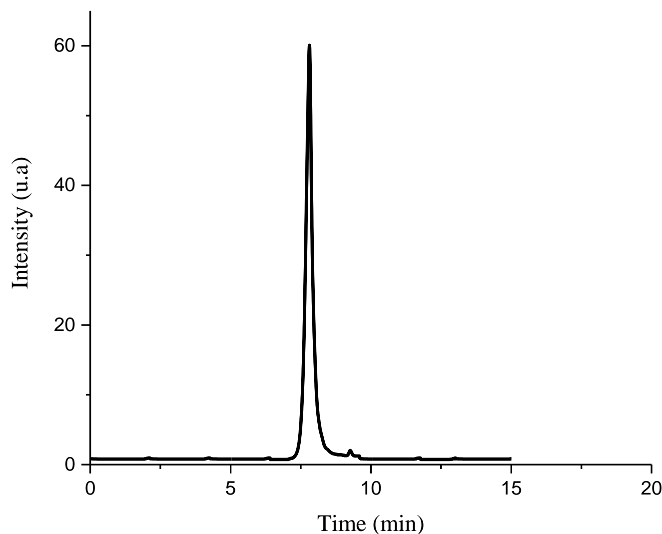


Apéndice T Mass spectrum peptide P-H4 (406: 423)
, molecular ion peak at 2730.079. m / z. the theoretical value of the mass for the peptide P-H4 (406: 423) is 2738.06 there is a mass difference of +8



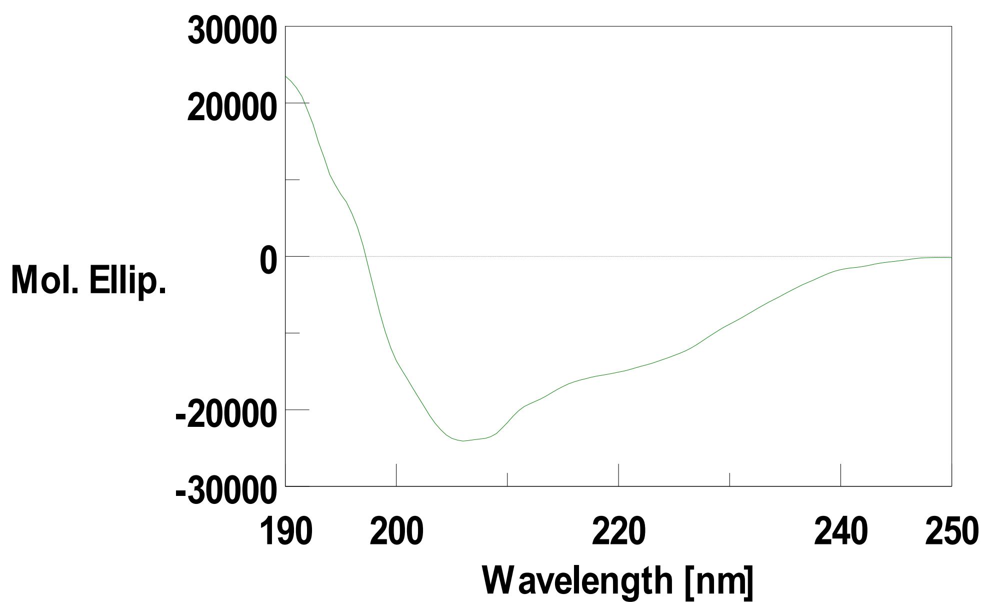
Apéndice U Chromatogram P-H4 (403: 426)

with retention time of 7.8 min, a gradient of 5-100% acetonitrile was used, with detection by fluorescence excitation wavelength 530 nm and emission at 585 nm



Apéndice V Circular Dichroism P-H4 (406: 423)

taken at the Catholic University of Valparaíso-Chile. According to the circular dichroism the peptide has a secondary structure alpha helix.



Apéndice W Data separation of serum proteins in a non-denaturing gel and peptide P-BC(271:28*6)*

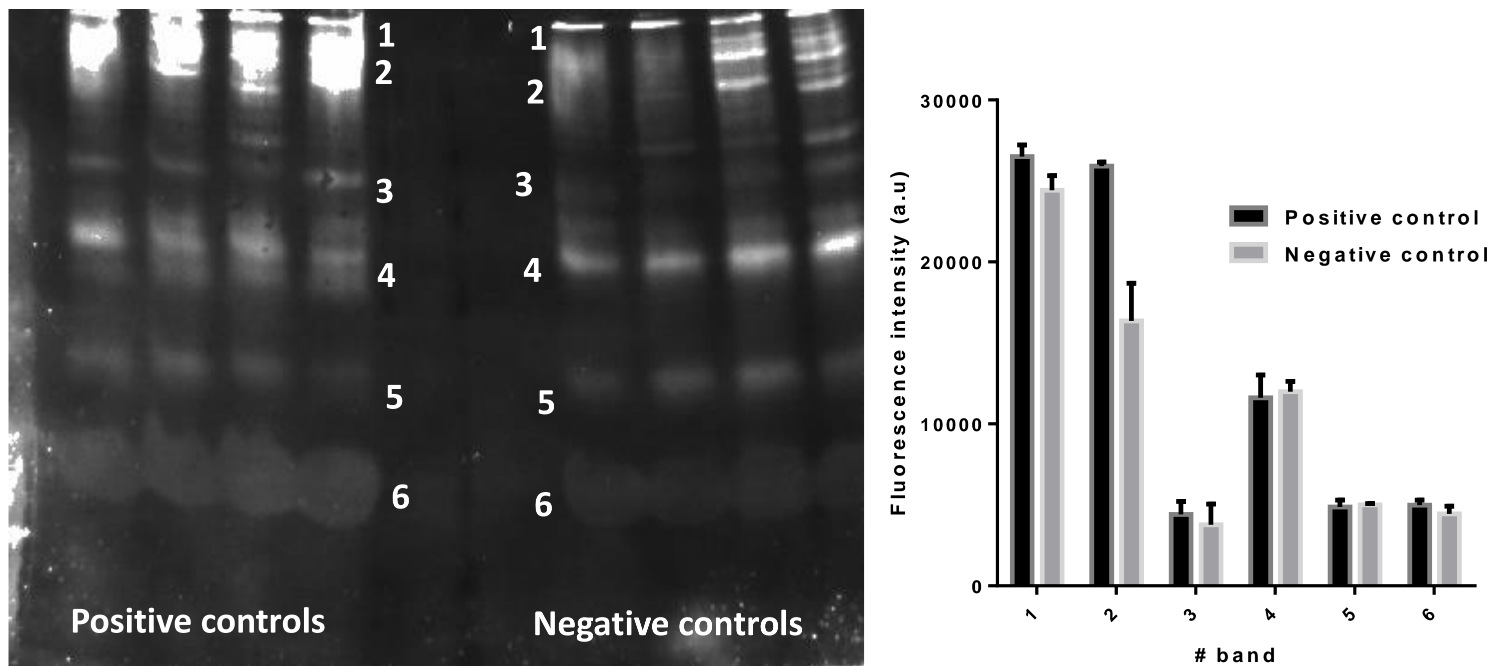


Figure 1 separation of serum proteins in a non-denaturing gel, transferred to nitrocellulose membrane and incubated with rhodamine-labeled peptide P-FG(271-286) to determine the pattern of protein binding to this peptide, the intensity of the peptide in the membrane is represented in numbered bands of 1-6; the band with greater intensity in the positive controls with P <0.05 is the band number 2

Table 1. Fluorescence intensity of each band together with the P values comparing the different controls

# Band	P value	Mean CP	Mean CN
1	0,1238	26501 ± 731	24426 ± 899
2	0,0064	25913 ± 242	16362 ± 2323
3	0,6834	4416 ± 799	3773 ± 1271
4	0,8148	11606 ± 1413	11986 ± 640
5	0,5807	4875 ± 417	4493 ± 504
6	0,9578	4978 ± 311	4950 ± 390