International Journal of Biomedicine 14(3) (2024) 516-519
http://dx.doi.org/10.21103/Article14(3)_OA21

ORIGINAL ARTICLE

Diagnostic Immunology

INTERNATIONAL JOURNAL OF BIOMEDICINE

Development of Standard and Competitive ELISA for Detection and Quantification of PRP-1 in Biological Fluids by Using anti-PRP-1 Polyclonal Antiserum

N. V. Tumasyan[1], I. K. Sahakyan[1*], N. V. Kocharyan[1], A. A. Khachatryan[2], T. K. Davtyan[3], K. A. Galoyan[4], S. S. Abrahamyan[1]

[1]*Buniatian Institute of Biochemistry of NAS RA, Yerevan, Republic of Armenia*
[2]*National Instruments AM LLC, Yerevan, Republic of Armenia*
[3]*Rhea Pharmaceutical Armenia, Yerevan, Republic of Armenia*
[4]*University of Miami, Miller School of Medicine, Miami, FL, USA*

Abstract

This study aimed to establish a sensitive method for the detection of proline-rich polypeptide-1 (PRP-1) in biological fluids. PRP-1, also known as galarmin, is a fragment of neurophysin-vasopressin-associated glycoprotein synthesized by brain neurosecretory cells and consisting of 15 amino acid residues. An enzyme-linked immunosorbent assay (ELISA) was used for PRP-1 quantification. An ELISA system has been developed using polyclonal antibodies we raised against the synthetic PRP-1. According to the analysis, the concentration PRP-1 of 25 ng/mL was accepted as the main appropriate coating concentration for further experiments in 1:100 and 1:500 antibody dilutions. Then, a competitive ELISA was developed to quantify PRP-1 in the fluids. Based on the results, an appropriate condition was chosen to be the best condition for PRP-1 detection: the appropriate quantity of the immobilized PRP-1 (25 ng/mL); anti-rabbit primary antibodies against PRP-1 (1:500); anti-rabbit secondary antibodies conjugated to peroxidase (1:1000), and extravidin (1:1000); as a result, the minimum detectable amount of PRP-1 in the fluid was 1.5 ng/mL. Thus, this method provides a good detection limit and sensitivity and is easy to use. In addition, a large number of rats and human serum and plasma samples can be analyzed rapidly and simultaneously, which is what we intend to realize in the future. (**International Journal of Biomedicine. 2024;14(3):516-519.)**

Keywords: proline-rich polypeptide-1 • galarmin • ELISA

For citation: Tumasyan NV, Sahakyan IK, Kocharyan NV, Khachatryan AA, Davtyan TK, Galoyan KA, Abrahamyan SS. Development of Standard and Competitive ELISA for Detection and Quantification of PRP-1 in Biological Fluids by Using anti-PRP-1 Polyclonal Antiserum. International Journal of Biomedicine. 2024;14(3):516-519. doi:10.21103/Article14(3)_OA21

Abbreviations

ELISA, enzyme-linked immunosorbent assay; **OD**, optical density; **PRP-1**, proline-rich peptide-1.

Introduction

Proline-rich polypeptide-1 (PRP-1), also known as galarmin, is a fragment of neurophysin-vasopressin-associated glycoprotein synthesized by brain neurosecretory cells and consisting of 15 amino acid residues.[1] In electrophysiological, histochemical, and immunohistochemical studies, the neuroprotective and immunomodulatory action of PRP-1 has been established in rats with non-specific and specific neuronal injury.[2,3] The immunohistochemical assay demonstrated that treatment with PRP-1 resulted in the recovery and growth of the nerve fibers, glia proliferation, and motoneuron survival.[4] In addition, the PRP-1 involvement in the neuroprotective action of the venom of the Central Asian cobra *Naja oxiana* has been found in trauma-injured rats.[4]

It is important and necessary to develop highly sensitive and specific analytical methods for determining proline-rich neuroprotective peptides, especially PRP-1. The high level

of specificity of immunoanalytical tests is achieved due to specific and high-affinity reversible binding of antigens to antibodies. For this purpose, we used the ELISA method. This study describes a standard and competitive ELISA with a specific design for detecting and quantifying galarmin using an anti-rabbit primary antibody to galarmin and a secondary anti-rabbit peroxidase-conjugated antibody for sandwich ELISA.

Materials and Methods

Production of PRP-1 Antiserum

For immunization, 1 to 1.5-year-old wild male (3-4) rabbits [Oryctolagus cuniculus (Linnaeus, 1758)] of the Californian breed, weighing 2 kg, were used. Animals were anesthetized using pentobarbital (Nembutal; serial no. 71308321 and registration no. 0285003) at a dose of 30-35 mg/kg. The Institutional Animal Ethics Committee of the Buniatian Institute of Biochemistry of the NAS (IRB 0001621; IORG0009782) approved the study protocol. The rabbits were housed in cages (cage model RBB-S-01) comprised of 2 sections and maintained at a room temperature of 18-22°C

The antiserum to the synthetic galarmin was obtained by rabbit immunization with PRP-1-BSA conjugate mixed with Freund's complete adjuvant. We prepared a 1.5 ml suspension, which included PRP-1 (2 mg), BSA (3 mg), PBS pH 7.4 (1.5 mL), and glutaraldehyde (15 µL). 0.3 mL of this suspension was mixed with 0.3 mL of Freund's complete adjuvant (product no. F5881; Sigma-Aldrich; Merck KGaA) and injected in equal portions in the region of both popliteal lymph nodes. 1.2 mL suspension was stored at -20°C for reimmunization. Immunization was repeated one month later by injecting suspension in the following locations: into the region of the left popliteal lymph node (0.45 mL), into the auricular vein (0.3 mL), and intramuscularly on the right side (0.45 mL). Whole blood samples were collected from individual animals on days 7, 9, and 11, mixed, and kept in the refrigerator (at +4°C). Then, 10 mL of this mixture was lyophilized, and the antiserum specificity was tested by immunodiffusion and ELISA.

Purification of PRP-1 Antiserum by Affinity Chromatography

Purification of PRP-1 antiserum by affinity chromatography was performed using AminoLink™ Plus Immobilization kit (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions.

Standard ELISA for the PRP-1 Detection

To prepare an ELISA plate for determining PRP-1 concentration, Immulon-2 ELISA plates were coated overnight with PRP-1 (0.5-500 ng/mL) or unrelated antigen trypsinogen in 100 µL coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$). Plates were washed 3 times with wash buffer (0.01 M PBS [pH 7.4] with 0.1% Tween-20), then incubated one hour with 200 µL per well of blocking buffer (1% BSA in wash buffer) to prevent non-specific binding. Plates were again washed 5 times and incubated with 1:50-1:1500 diluted rabbit primary antiserum in 100 µL dilution buffer (PBS with 0.1% BSA, 0.2% Tween-20) for 90 min in quadruplicate or duplicate wells and then washed 5 times. Then, plates were incubated for 90 min with 100 µL per well of the biotinylated goat anti-rabbit IgG diluted in dilution buffer (1:100 or 1:3000). Plates were again washed 5 times and incubated for 60 min at room temperature with 100 µL per well of horseradish peroxidase (HRP)-conjugated extravidin diluted in PBS (1:100 or 1:3000). After washing 5 times 100 µL HRP substrate buffer (8 mg orthophenylene diamine, 12 mL citrate acid phosphate, with 5 µL 30% H_2O_2 added immediately before use) was added to each well. The reaction was stopped with 1N H_2SO_4 (50 µL per well) after 30 min in the dark. The OD was reported on an ELISA reader at 450 nm of wavelength, with background PRP-1 binding to the standard subtracted. A competitive ELISA was further developed to quantitatively assess minimal PRP-1 counts in rat and human biological fluids (serum, plasma, and cerebrospinal fluid).

Competitive ELISA for Quantification of PRP-1

Immulon-2 ELISA plates (VWR Scientific, Mississauga ON) were coated overnight with 100 µL per well of 25 ng/mL PRP-1 diluted in coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$). Plates were washed 3 times with washing buffer (PBS with 0.1% Tween-20), then incubated for one hour with 200 µL per well of blocking buffer (1% BSA in wash buffer) to prevent non-specific binding. Plates were washed 5 times, and further incubation was performed as follows: 50 µL serially two-fold tittered PRP-1 standard solution (at an initial concentration of 25 ng/mL in total 100 µL per well) mixed with 50 µL 1:25 or 1:750 antiserum (for obtaining the final dilution 1:50 or 1:1500) was added to each well. To determine the maximum binding (Bmax), the antiserum mentioned above without PRP-1 was added to another column, and the plates were incubated for 90 min at room temperature. After washing 5 times, the plates were incubated for 90 min at room temperature with 100 µL per well of biotinylated goat anti-rabbit IgG secondary antibody diluted 1:100 or 1:3000. Plates were again washed 5 times, and 100 µL per well of horseradish peroxidase (HRP)-conjugated extravidin diluted in PBS in radio 1:200 or 1:3000 for 90 min at room temperature were added. Plates were washed five times, and 100 µL per well of substrate buffer (8 mg orthophenylene diamine, 12 mL citrate acid phosphate, with 5 µL 30% H_2O_2 added immediately before use) for HRP was added. The reaction was stopped with 1N H_2SO_4 (50 µL per well) after 30 min in the dark. The OD was reported on an ELISA reader at 450 nm of wavelength, with background PRP-1 binding to the standard subtracted.

All data obtained were analyzed using the statistical package SPSS11. The ODs from each dual well were separated and classified as OD1 and OD2 for each concentration. The character of the curves was determined, and the most appropriate curve matching the rule of the ELISA method was chosen. Using the Kruskal-Wallis test for multiple comparisons, the comparative analysis was done to choose the right coating concentration PRP-1 for further competitive ELISA development. In competitive ELISA development experiments, the means for OD1 and OD2 were calculated. The dose-dependent curves were drawn for each experimental set of the OD mean to get the most appropriate curve with minimum disorders and perfect shape. The minimum detectible concentrations were identified.

Results and Discussion

To obtain the best results in the quantitative determination of galarmin in blood serum samples of experimental animals, we conducted exploratory studies using the following conditions: primary antiserum in dilutions 1:50 – 1:1500 and 0.5-500 ng/mL of galarmin (Figure 1).

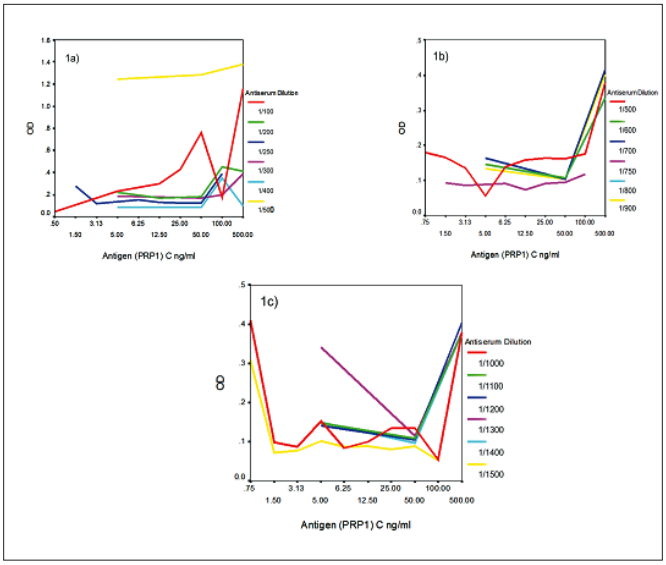

Fig 1. *The dose-dependent curve for different primary antibody dilutions (1:50 – 1:1500).*

The Kruskal-Wallis test showed insignificant differences between the observed concentrations both when diluting the primary antiserum (Figure 1a) 1:100 with 0.5-50 ng/mL (P=0.273) and (Figure 1b) 1:500 with 5-50 ng/mL (P=0.158) of immobilized antigen. However, the rank means for the PRP-1 concentration of 25 ng/mL (1:100, 1:500 of primary antiserum dilution) were the highest insignificantly in the respective dilution groups. This concentration was accepted as the main coating concentration for further experiments in respective antibody dilutions.

The competitive ELISA was developed with the mentioned parameters. For competitive ELISA, the higher the sample antigen concentration, the weaker the eventual signal. This is the rule to identify the detection limits. The results were analyzed using the curve estimation in regression for each set to detect the best curve matching the statement above.

1. Coated PRP-1 in the concentration of 25 ng/mL with tittered PRP-1 in the range of 25-0 ng/mL mixed with anti-PRP-1 in the final dilution 1:100 and the secondary antibodies and extravidin in dilution 1:200 (Figure 2).

The tendency to reduce absorbance values was observed starting from the concentration of 0.19 ng/mL (97%), with some disorders in the checked points of concentration 1.5 ng/mL and 6.25 ng/mL (Table 1).

2. Coated PRP-1 in the concentration of 25 ng/mL with tittered PRP-1 in the range of 25-0 ng/mL mixed with anti-PRP-1 in the final dilution 1:500 and the secondary antibodies and extravidin in dilution 1:1000 (Figure 3).

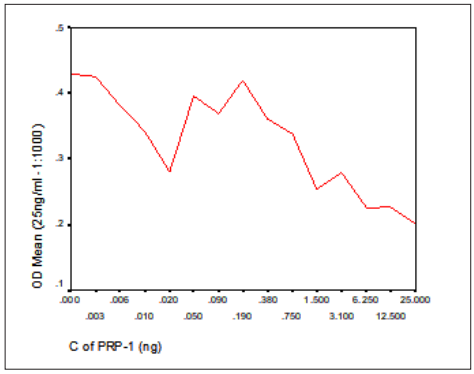

Fig. 2. *OD means of coated 25 ng/mL PRP-1 with anti-PRP-1 in a 1:100 dilution at the titration PRP-1 range of 25-0 ng/mL.*

Table 1.

The ratio between the absorbance at 450 nm in the presence (OD_0) and absence (OD_{max}) of 25 ng/mL of coated PRP-1 against a concentration of tittered PRP-1.

PRP-1(ng/mL)	OD	%OD
0	0.429	100
0.003	0.426	99.3007
0.006	0.381	88.81119
0.01	0.342	79.72028
0.02	0.28	65.26807
0.05	0.396	92.30769
0.09	0.369	86.01399
0.19	0.42	97.9021
0.38	0.361	84.14918
0.75	0.339	79.02098
1.5	0.254	59.20746
3.1	0.279	65.03497
6.25	0.226	52.68065
12.5	0.227	52.91375
25	0.202	47.08625

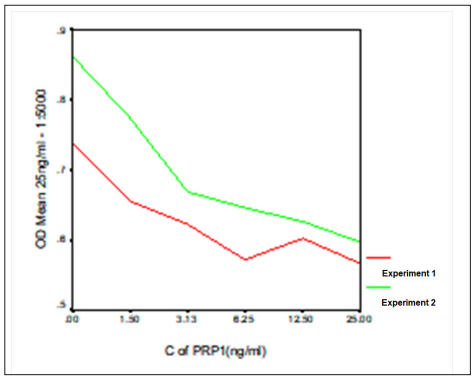

Fig. 3. *OD means of coated 25ng/ml PRP-1 with anti-PRP-1 in a 1:500 dilution at the titration PRP-1 range of 25-0 ng/mL.*

The minimum detectible amount of the peptide is 1.5 ng/mL. In Experiment 1, an insignificant disorder at 6.25 ng/mL occurs (Table 2).

Table 2.

The ratio between the absorbance at 450 nm in the presence (OD_0) and absence (OD_{max}) of 25 ng/ml of coated PRP-1 against a concentration of tittered PRP-1 in two experiments.

Experiment 1		
PRP-1 (ng/mL)	OD	%OD
0	0.7365	100
1.5	0.655	88.93415
3.125	0.6215	84.38561
6.25	0.571	77.52885
12.5	0.6015	81.67006
25	0.566	76.84997
Experiment 2		
PRP-1 (ng/mL)	OD	%OD
0	0.862	100
1.5	0.7745	89.84919
3.125	0.6685	77.5522
6.25	0.646	74.942
12.5	0.626	72.62181
25	0.5965	69.19954

Three experiments were compared to identify the lowest detectible concentration of PRP-1 in the biological fluids at the minimum concentration of the coated PRP-1 and the maximum dilution of anti-PRP-1. All three experiments have almost the same pattern of OD reduction. However, Experiment 3 has a weaker OD than the other 2 due to the lowly diluted secondary antibody (Figure 4).

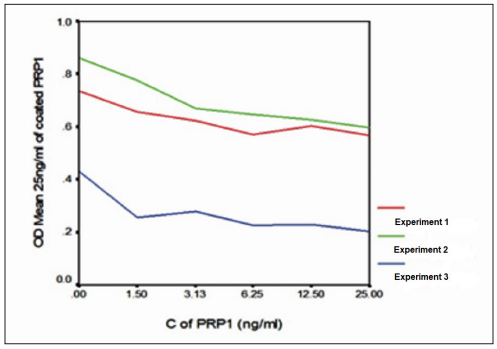

Fig. 4. *Competitive ELISA at 25ng/ml coated PRP-1 with primary antiserum in a 1:100 dilution, secondary antibody and extravidin at a 1:200 dilution (Experiment 3), and primary antibody at a 1:500 dilution, secondary antibody and extravidin at a 1:1000 dilution (Experiments 1, 2).*

Conclusion

During the development of the standard ELISA, to prepare an ELISA plate for determining PRP-1 concentration, PRP-1 was first immobilized in each well at 0.5 ng/mL-500 ng/mL concentration. Then, the polyclonal antiserum against PRP-1 in 1:50-1:1500 dilution was used to detect the latter's specificity. According to the analysis, 25 ng/mL concentration was accepted as the main appropriate coating concentration for further experiments in 1:100 and 1:500 antibody dilutions.

As for the development of the competitive ELISA needed for the PRP-1 quantification in the fluids, the following 2 conditions were identified:

•The minimum detectable amount of PRP-1 in the fluid was 3.1 ng/mL at coated PRP-1 in the concentration of 25 ng/mL, at a 1:100 dilution of anti-PRP-1, and secondary antibody and extravidin at a 1:200 dilution

•The minimum detectable amount of PRP-1 in the fluid was 1.5 ng/mL at coated PRP-1 in the concentration of 25 ng/mL, at a 1:500 dilution of anti-PRP-1, and secondary antibody and extravidin at a 1:1000 dilution

At the same time, the disordering specificity of PRP-1 was defined as a rule at a concentration of 6.25 ng/mL of tittered PRP-1. As it is seen from the results mentioned above, the second one was chosen to be the best condition for PRP-1 detection and quantification in the biological fluids [the appropriate quantity of the immobilized PRP-1 (25 ng/mL); anti-rabbit primary antibodies against PRP-1 (1:500); anti-rabbit secondary antibodies conjugated to peroxidase (1:1000), and extravidin (1:1000)].

Thus, using polyclonal antibodies raised against the synthetic PRP-1, an ELISA system has been developed that has demonstrated the method's sensitivity and specificity. This method provides a good detection limit and sensitivity and is easy to use. In addition, a large number of rats and human serum and plasma samples can be analyzed rapidly and simultaneously, which is what we intend to realize in the future.

References

1. Galoyan AA. Biochemistry of Novel Cardioactive Hormones and Immunomodulators of the Functional System Neurosecretory Hypothalamus – Endocrine Heart. Nauka, Moscow; 1997.
2. Galoyan AA, Sarkissian JS, Kipriyan TK, Sarkissian EJ, Chavushyan EA, Sulkhanyan RM, et al. Protective effect of a new hypothalamic peptide against cobra venom and trauma-induced neuronal injury. Neurochem Res. 2001 Sep;26(8-9):1023-38. doi: 10.1023/a:1012353005489.
3. Galoyan AA, Sarkissian JHS, Chavushyan VA, et al. Study of the newhypothalamic Proline-rich peptide (PRP-1) protective effect on morpho-functional changes in rat hippocampus using a model of Alzheimer's Disease induced by intracerebroventricular injection of Beta-Amyloid peptide Aβ (25-35). J. Neurochemistry. 2004;21(4):265-288.
4. Abrahamyan SS, Sarkissian JS, Meliksetyan IB, Galoyan AA. Survival of trauma-injured neurons in rat brain by treatment with proline-rich peptide (PRP-1): an immunohistochemical study. Neurochem Res. 2004 Apr;29(4):695-708. doi: 10.1023/b:nere.0000018840.19073.0b. PMID: 15098931.
5. Abrahamyan SS, Meliksetyan IB, Chavushyan VA, Aloyan ML, Sarkissian JS. Protective action of snake venom Naja naja oxiana at spinal cord hemisection. Ideggyogy Sz. 2007 Mar 30;60(3-4):148-53. PMID: 17451057.

***Corresponding author**: Inesa Sahakyan, PhD. Buniatian Institute of Biochemistry of NAS RA, Yerevan, Republic of Armenia. E-mail: inesasahakyan5@gmail.com*