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A simple and sensitive immunoassay method for the detection of low-molecular-weight proteins and neuropeptides

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Abstract:

BACKGROUND: Low-molecular-weight proteins and peptides perform numerous regulatory roles in biological systems and could be useful as biomarkers. Hence, qualitative and quantitative measurement of peptide levels has become increasingly important.

AIM: This present study was aimed at developing a simple and sensitive dot immunoblot assay (DIA) by modifying existing protocols.

MATERIALS AND METHODS: Using polyclonal antibody generated against FMRFamide peptide, at a dilution ratio of 1:10,000 in buffer, as a primary antibody, alkaline phosphatase-conjugated goat anti-rabbit antibody as secondary antibody and nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate development system, peptides were dotted on 0.2µm nitrocellulose membrane in volumes of 1µl, 0.5µl and 0.2µl.

RESULTS: Results show immunoreactivity of FMRFamide antibody to small quantities of dotted neuropeptide as low as 0.2ng and 33.4pM.

CONCLUSION: This indicates that this protocol is sensitive with the additional advantage of simplicity, speed, small sample volume, low cost, and production of nonhazardous waste. This DIA protocol could be useful in resource-poor settings and laboratories with low budgets.

Keywords:

Dot immunoblot assay, low-molecular-weight proteins, neuropeptides, peptides

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Introduction

Low-molecular-weight proteins and peptides are small polypeptides, normally ranging from 3 to 100 amino acid residues (DeWied, 1969; Kastin *et al*., 1979; Strand, 1999). They perform numerous regulatory roles in biological systems and could be useful as biomarkers (Schulte *et al*., 2005). Hence, qualitative and quantitative measurements of low-molecular-weight proteins and peptide levels have become increasingly important (Rubakhin and Sweedler, 2008; Gillette and Carr, 2013).

One of the major problems associated with the assessment of low-molecular-weight proteins and peptides is their small molecular mass and often low quantity which makes them difficult to detect in samples. For example, FMRFamide neuropeptide, which was first discovered in the mollusc (Price and Greenberg, 1977), is a peptide with very small molecular weight. Attempt at isolating FMRFamide-related peptides (FaRPs) in tissue samples of the sea cucumber, *Holothuria scabra*, has led to the development of a sensitive detection technique for this peptide. In this paper, we present a simple and sensitive immunoassay method for the detection of

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low-molecular-weight proteins and peptides, as applied in the study of FaRPs.

Materials and Methods

Synthetic peptide

Synthetic FMRFamide peptide (>98% pure) was purchased from GenScript (USA).

Antisera

Polyclonal antibody, raised against the tetrapeptide FMRFamide, was used in the experiment. Commercially available rabbit polyclonal antibody against FMRFamide was procured from ImmunoStar Inc. (USA). The antibody was raised against FMRFamide–thyroglobulin conjugate and preabsorbed with 100 µg thyroglobulin, according to the manufacturer.

Immunoassay

An 8 cm × 6 cm size membrane cut from Amersham 0.2 µm nitrocellulose membrane (GE Healthcare Life Sciences) was divided into "wells" using a pencil and ruler. Serial dilutions of 1 µg/µl of FMRFamide neuropeptide were spotted on the membrane in volumes of 1 µl, 0.5 µl, and 0.2 µl. The membrane was air-dried at room temperature for 30 min and baked at 100°C for 30 min to fix the peptides to the membrane. After cooling to room temperature, the membrane was washed for 10 min in a small volume of 0.1M Tris buffer (pH 7.4) with 0.05% Tween 20 (v/v; buffer 1), and then incubated for 60 min in a blocking solution containing 3% bovine serum albumin in 0.1M Tris buffer (pH 7.4). The membrane was then incubated overnight in anti-FMRFamide polyclonal antibody (1:10,000 in buffer 1) and washed three times for 5 min each in buffer 1 before incubation in alkaline phosphatase-conjugated goat anti-rabbit secondary antibody (1:500 in buffer 1) for 1 h. The membrane was washed again three times for 5 min each in buffer 1 and developed with nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate substrate (1:100 in development buffer). After about 15 min, the membrane was washed in distilled water, air-dried, and photographed. The number of mole of neuropeptide in each well was calculated using the following formula:

$$\text{Mole} = \frac{\text{Concentration of dotted neuropeptide}}{\text{Molar mass of neuropeptide (598.76g)}}$$

Results

The concentrations of dotted peptide on the nitrocellulose membrane are provided in Tables 1 and 2. The results, as presented in Figure 1, show immunoreactivity of FMRFamide antibody to different concentrations of FMRFamide neuropeptide at 1 µl, 0.5 µl, and 0.2 µl with the least detectable neuropeptide concentration at about 0.0002 µg or 0.2 ng which corresponds to 3.34×10^{-13} M or 33.4 pM of FMRFamide.

Table 1: Volume and concentration of dotted peptide

Dotted volume	Concentration of dotted neuropeptide				
	Lane 1	Lane 2	Lane 3	Lane 4	Lane 5
1 µl	1 µg	0.1 µg	0.01 µg	0.001 µg	0.0001 µg
0.5 µl	0.5 µg	0.05 µg	0.005 µg	0.0005 µg	0.00005 µg
0.2 µl	0.2 µg	0.02 µg	0.002 µg	0.0002 µg	0.00002 µg

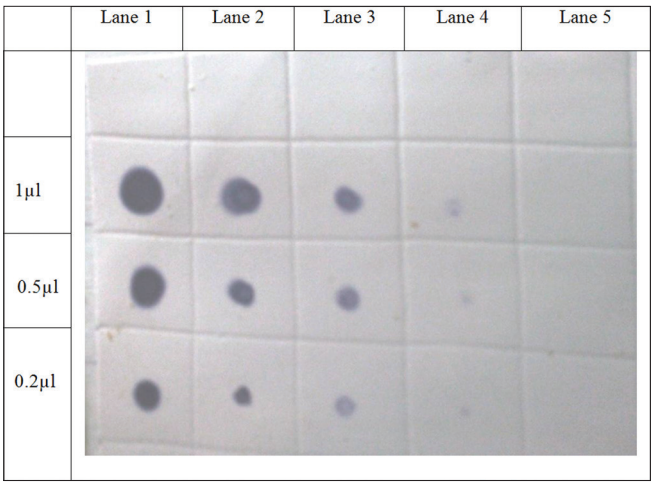

Figure 1: Dot immunoblot assay of FMRFamide peptide

Discussion

In the present study, dot immunoblot assay (DIA) was capable of detecting FMRFamide neuropeptide at concentrations as low as 0.2 ng and 33.4 pM but not beyond 0.02 ng or 0.334x fM. The lowest sample volume of 0.2 µl was also observed to have the highest sensitivity. Three reasons could be responsible for the high degree of sensitivity shown by this technique. First is the low sample volume dotted on the membrane and second is the dilution factor with which the primary antibody 1:10,000 in the buffer. Another possible contributing factor is the development system used. Our preliminary study with under the same conditions has shown that alkaline phosphatase-conjugated antibody is more sensitive with lower background than 3,3′,5,5′-tetramethylbenzidine. This DIA can be used in the isolation of neuropeptides from tissues (Ajayi and Withyachumnarnkul, 2013). In addition, when compared to similar protocols such as that of Salzet *et al.*, with sensitivity of 1.32 pmole (Salzet *et al.*, 1993; Loi *et al.*, 1997), we find this protocol to be equally sensitive. It has the additional advantage of simplicity, speed, small sample volume, low cost, and production of nonhazardous waste. This DIA protocol is recommended for laboratories with low budgets and might be useful in resource-poor settings.

Table 2: Molar mass of dotted peptide

	Lane 1	Lane 2	Lane 3	Lane 4	Lane 5
1 µl	1.67×10^{-9}M	1.67×10^{-10}M	1.67×10^{-11}M	1.67×10^{-12}M	1.67×10^{-13}M
0.5 µl	8.35×10^{-10}M	8.35×10^{-11}M	8.35×10^{-12}M	8.35×10^{-13}M	8.35×10^{-14}M
0.2 µl	3.34×10^{-10}M	3.34×10^{-11}M	3.34×10^{-12}M	3.34×10^{-13}M	3.34×10^{-14}M

Conclusion

This sensitive protocol is simple, is fast, requires small sample volume, is cost-effective, and does not produce any hazardous waste. Hence, this DIA protocol is recommended for laboratories with low budgets, and this might be useful in resource-poor settings.

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Conflicts of interest
There are no conflicts of interest.

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