



Mapping Amyloid Regions in Gad m 1 with Peptide Arrays

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Abstract

Amyloid formation is basically featured by a protein-protein interaction in which the reacting regions are the segments assembling into cross β -sheets. To identify these segments both theoretical and experimental tools have been developed. Here, we focus on the use of peptide arrays to probe the binding of several amyloid-specific probes such as the OC and A11 anti-amyloid conformation-selective antibodies and of monomers and preformed fibrils. These arrays use libraries containing partly overlapping peptides derived from the sequence of Gad m 1, the major allergen from Atlantic cod, which forms amyloids under gastrointestinal relevant conditions.

Key words Peptide arrays, Amyloids, Anti-amyloid antibodies, Food allergens, Gad m 1

1 Introduction

Proteins forming amyloid assemblies are diverse and exhibit unrelated sequences, native 3D-structures and ligand-binding repertoire [1–5]. Despite this diversity, all amyloid fibrils share a basic architecture, the intermolecular cross- β sheet motif, and a mechanism of formation, a seeded-polymerization [6–8]. The information for cross- β -sheet rearrangement is contained in short sequence stretches of about 5–11 amino acids [9]. These segments fold into β -strands that form β -sheets which pack interdigitating their side-chains [9, 10]. Amyloid-forming regions can be identifying using predictive algorithms such as AMYLPRED [11], FISH AMYLOID [12], and ZIPPERDB [13], among others.

Peptide arrays are useful tool to screen sequence-dependent interactions such as those involved in amyloid formation [14–16]. These arrays are made from libraries containing partly overlapping peptides derived from a protein sequence attached to a solid support or membrane by SPOT-synthesis [17]. The resulting array is incubated with the interacting partner that is further detected. This screening procedure is semi-quantitative but avoids

solubility problems of the work in solution and reduces the required amount of protein compared to other assays.

Despite the sequence dependency amyloids behave as conformational epitopes allowing immunodetection by the A11 and OC antibodies [18, 19]. The A11 antibody recognizes assemblies containing out-of-register anti-parallel β sheet structures which result from fibrillary off-pathways [20, 21]. On the contrary, the OC antibody recognizes amyloid fibrils as well as oligomers along the fibrillization route [18, 19, 22]. Therefore, probing OC and A11 immunoreactivity of peptides arrays provides a complementary view for the amyloid formation propensity of regions in a protein sequence.

In addition to the analysis of the amyloid propensity, peptide arrays can be exploited to study relevant sequences in the assembly process such as those involved in nucleation and elongation events [14, 15, 23].

One of the proteins forming amyloids is the *Atlantic cod* β -parvalbumin Gad m1, model of the major fish allergen [24]. Gad m 1 chain is made from 109 residues, contains two Ca^{2+} -binding sites, and displays a helical fold in its Ca^{2+} -state [25]. Exposure of N-terminal His-tagged recombinant chain of Gad m 1 (rGad m 1) to gastric-like acid pH or chelates triggers amyloid assembly. Importantly, this aggregation correlates with two relevant features of food allergens: the resistance to proteases and the increase binding of patient sera IgE [26, 27]. The current chapter describes the experimental protocols for the design, use, and validation of peptide arrays for identifying amyloid relevant regions in Gad m 1, the major fish allergen from *Atlantic cod*.

2 Materials

Unless otherwise noticed, all reagents are from Sigma-Aldrich, and all solutions are prepared with deionized water (dH_2O).

2.1 Peptide Arrays

1. Template sequence: Gad m 1 sequence (UniprotKB-A5I874).
2. Peptide library design.
3. Custom order service (proteomics facility or company).
4. Membrane incubation boxes for easy handling (Box dimensions of $12 \times 2 \times 3$ cm are recommended for reducing the volume of solutions).
5. Tweezers.
6. Gloves.

2.2 Probing Gad m 1 Peptide Arrays with Anti-amyloid Antibodies

1. Methanol and ethanol.
2. Tris-buffered saline (TBS): 25 mM Tris-Cl, pH 7.4, 137 mM NaCl, 2.7 mM KCl.
3. Tris-buffered saline, 0.05% Tween 20 (TBST).
4. Blocking solution: TBST, 1% (w/v) BSA. Since Gad m 1 is a Ca^{2+} -binding protein, milk-based blocking agents should be avoided.
5. Antibody solution: TBST, 1% (w/v) BSA.
6. Binding buffer: 50 mM Tris-HCl pH 7.5, 100 mM NaCl, 5 mM EDTA.
7. OC: Anti-Amyloid Fibrils OC Rabbit polyclonal IgG (Millipore #AB2286) in antibody solution at a 1:2000 dilution.
8. A11:Anti-Oligomer A11 Rabbit polyclonal IgG. (Millipore #AB9234) in antibody solution at a 1:2000 dilution.
9. GAR-HRP: horseradish peroxidase (HRP)-conjugated goat anti rabbit IgG antibody (Millipore # AP307P) in antibody solution at a 1:5000 dilution.
10. ECL (electrochemiluminescence) kit (HRP substrate and solutions) and materials (filter paper, plastic wrap, etc.).
11. Sodium azide.
12. Plastic disposable tubes of 1.5, 15 and 50 mL.
13. Image station with chemiluminescent detection and analysis setting (we use the XRS+ ChemiDoc™ on chemiluminescent settings and Image Lab software).
14. Orbital shaker.

2.3 Use of Peptide Arrays for the Determination of Gad m 1 Relevant Regions for Its Amyloid Assembly

1. Methanol and ethanol.
2. Tris-buffered saline (TBS): 25 mM Tris-Cl pH 7.4, 137 mM NaCl, 2.7 mM KCl.
3. Tris-buffered saline, 0.05% Tween 20 (TBST).
4. Blocking solution: TBST, 2% (w/v) BSA. Since Gad m 1 is a Ca^{2+} -binding protein, milk-based blocking agents should be avoided.
5. Antibody solution: TBST, 2% (w/v) BSA.
6. Binding buffer: 50 mM Tris-HCl pH 7.5, 100 mM NaCl, 5 mM EDTA.
7. rGad m 1 wt monomer solution: 2 mg/mL protein concentration in dH_2O (*see Note 1*).
8. rGad m 1 I12V,T13K,A14T,A17E mutant: 2 mg/mL protein concentration in dH_2O (*see Note 2*).
9. rGad m1 wt fibrils: 2 mg/mL protein concentration in binding buffer (*see Note 3*).

10. 6×His Epitope Tag HRP-conjugated antibody (ThermoFisher #MA1-21315-HRP) in antibody solution at 1:4000 dilution.
11. ECL kit (HRP substrate and solutions) and materials (filter paper, plastic wrap, etc.).
12. Plastic disposable tubes of 1.5, 15, and 50 mL.
13. Bath sonicator.
14. Image station with chemiluminescent detection and analysis settings.
15. Orbital shaker.

2.4 Membrane Re-probing

1. Stripping solution: TBS, 8 M urea, 1% (w/v) SDS, 0.5% (v/v) β -mercaptoethanol.
2. Tris-buffered saline (TBS): 25 mM Tris-Cl pH 7.4, 137 mM NaCl, 2.7 mM KCl.
3. Methanol/ethanol.
4. Shaking incubator.

3 Methods

3.1 Gad m 1 Peptide Arrays

Before approaching the use of a peptide array to map and characterize an amyloid-related process, evidences for the occurrence of the assembly event and of the conditions needed for it must be available. The SPOT technology is very well commercialized, making the ordering of a peptide array a quick and cheap process. Therefore, the major task before ordering is the peptide array design including the following items:

1. Choose the template sequence. Gad m 1 sequence (UniprotKB-A5I874). For proteins undergoing posttranslational modification such as signal sequence processing, the mature chain should be the sequence of choice.
2. Design the peptide library. To this effect the following parameters must be determined:
 - (a) Peptide length (12 amino acids) (*see Note 4*).
 - (b) Sequential shift between two consecutive peptides (two amino acids steps among them) which dictates the common core between consecutive peptides (Fig. 1a).
 - (c) The immobilization support dictates the immobilization density and solvent compatibility. The Amino-PEG500-UC540 sheet provides an immobilization density of ≈ 10 nmol per spot ≈ 400 nmol/cm²) and conditions methanol/ethanol solvents for wetting of the dry array.
 - (d) The number of required membranes.

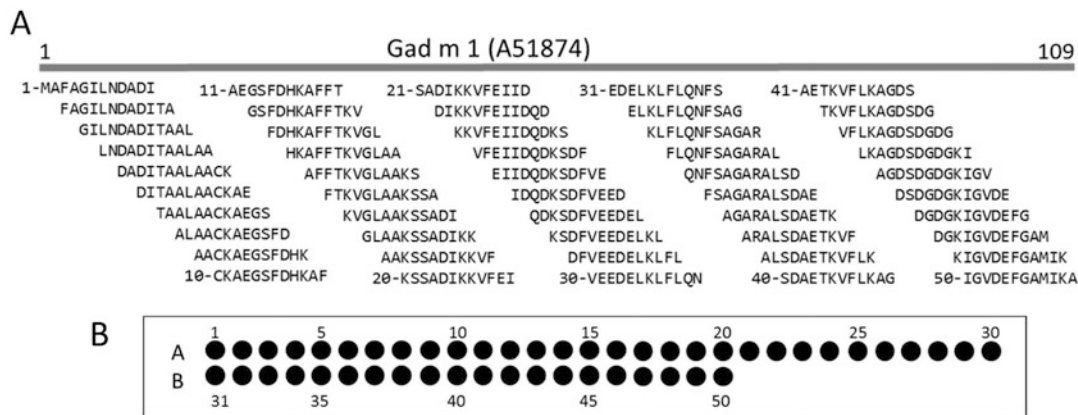


Fig. 1 Design of Gad m 1 peptide array. **(a)** Template Gad m 1 chain (UniprotKB-A51874). The 109 amino acid residues of the chain (gray line) were divided into 50 peptides, each of which was made by 12 amino acids with a sequence shift of 2 amino acids between consecutive peptides and 8 residues overlapping. **(b)** Peptide array scheme. SPOT-membranes were supplied as two-row (A and B), each of which contained peptides 1–30 (row A) and 31–50 (row B)

3. Place a custom order either at a proteomics facility or company. Membranes are provided with a real size image of their Pon-
ceau red staining to allow cutting and easy spot identification (Fig. 1b). Importantly, membrane should be always manipu-
lated with gloves and tweezers.

3.2 Probing Gad m 1 Peptide Arrays with Anti-amyloid Antibodies

1. Wet the dry peptide array by soaking the membranes for 10 min in methanol, rinsed once with ethanol for 1 min, and then rinsed three more times with TBS 10 min each.
2. Add 20 mL of binding buffer and incubate for 12 h at room temperature.
3. Remove the previous solution and add 20 mL of the blocking solution and incubate for 1–2 h at room temperature with gentle shaking.
4. Remove the blocking solution and add anti-amyloid (either OC or A11) antibody in antibody solution at 1:2000 dilution (5 µL of antibody in 10 mL of antibody buffer) and incubate with gentle shaking for 2 h at room temperature.
5. Remove antibody solutions. For reuse purposes (maximum five times), add 0.1% (w/v) sodium azide for preservation and store the solutions at 4 °C.
6. Add 20 mL of TBST, incubate for 10 min and remove the solution. Repeat the process three times.
7. Add 10 mL of secondary antibody solution and incubate for 1 h at room temperature (see Note 5).

8. Remove the secondary antibody solution. This solution can be reused within the same day considering possible reductions in efficiency.
9. Add 25 mL of TBST, incubate for 10 min, and remove the solution. Repeat the process three times. A last wash with TBS is recommended.
10. Develop using an ECL kit, following the manufacturer's indications.
11. Acquire chemiluminescent signal with an imaging station using various developing times (0.5, 1, and 5 min) (Fig. 2a).
12. Rapidly, wash the membrane with three times with TBST, one with TBS and rinse with ethanol to avoid irreversible membrane damage (overreaction causes visible brownish of spots that cannot be washed away with any described stripping

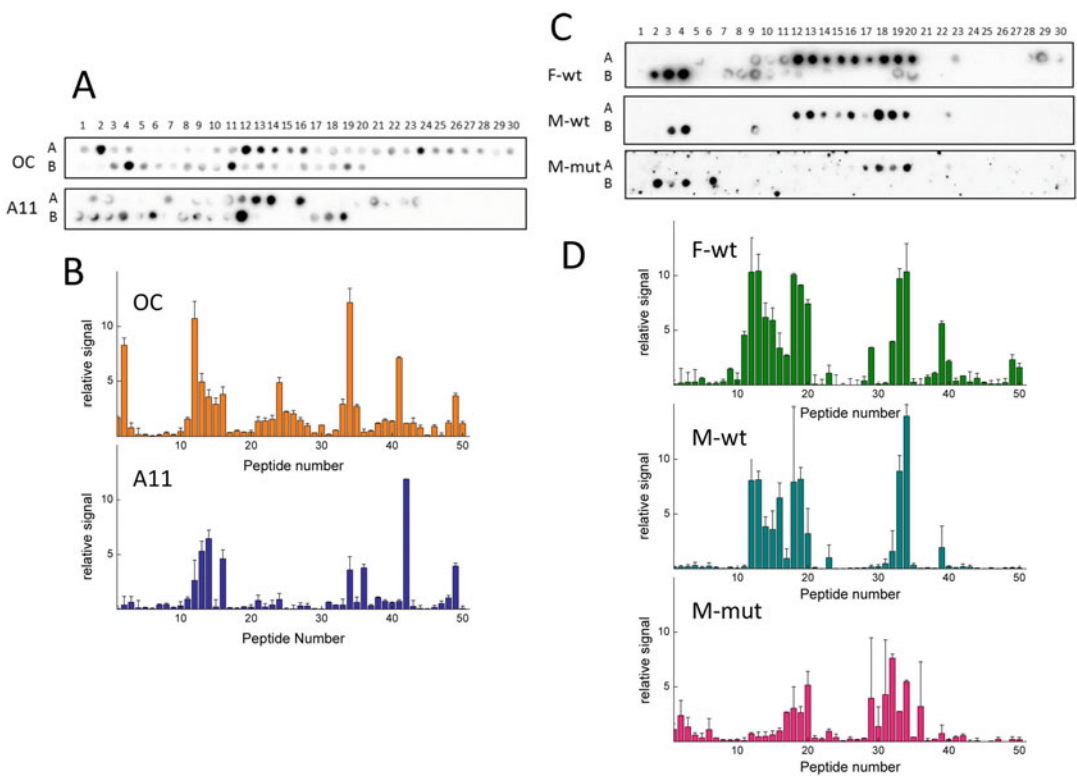


Fig. 2 Use of Gad m 1 peptide arrays to probe amyloid forming regions and recognition sites with relevance in amyloid formation (a) OC and A11 binding to Gad m 1 peptide arrays. (b) Relative signal of peptides binding the anti-amyloid OC and A11 antibodies. Data are displayed as the average of two independent duplicates. (c) Binding of rGad m 1 wt fibrils (F-wt), rGad m 1 wt monomers (M-wt) and rGad m 1 mutant monomers (M-mut) to peptide arrays. Development was performed using an HRP-conjugated antibody against the N-terminal His6 tag present in all the chains. (d) Relative signals of the binding of the distinct Gad m 1 states displayed as the average and standard deviations of two independent experiments

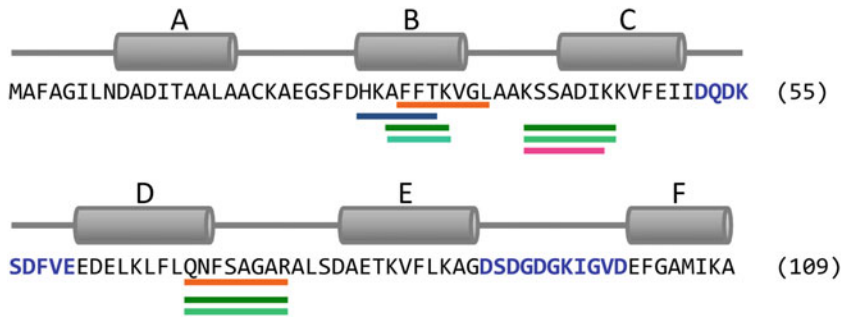


Fig. 3 Hot spots of Gad m 1 sequence. Native Ca^{2+} -bound Gad m 1 displays a globular fold constituted by six helical segments A–F (grey cylinders). The Ca^{2+} -binding sites contained between helices C–D and E–F (sequence in blue). Hot spots for amyloid formation are indicated by the following lines: orange, OC binding; blue, A11 binding; olive, fibril binding; green, monomer binding; magenta, amyloid-prone monomer binding

procedure). Reuse of membranes requires a stripping step (*see* Subheading 3.4).

13. Quantify and process the signals. Image stations are provided with software for quantification of spots and background. For membrane comparison, substrate background from each spot signal and express them as percentage of the total peptide array signal (*see* **Note 6**). Plot the relative signal as a function of peptide number (Fig. 2b).
14. Consider a signal threshold and impose a specificity criterion such as binding to at least two consecutive peptides. To obtain the anti-amyloid antibody binding sequences, identify peptides with positive signal and determine the sequence common cores (Fig. 3).

3.3 Use of Peptide Arrays for the Determination of Gad m 1 Relevant Regions for Its Amyloid Assembly

Amyloid formation is a complex process featured mechanistically by a seeded-polymerization and a hierarchically assembly. Amyloid precursor (usually refer to as monomers) must form a nuclei. This seed grows by monomer incorporation yielding protofibrils that further assemble into fibril, protofibers, and fibers. This simplistic view underlines the relevance of recognition sites for nucleation, protofibril growth, and assembly.

1. Wet the dry peptide array by soaking the membranes for 10 min in methanol, rinsed once with ethanol for 1 min, and then rinsed three more times with TBS 10 min each.
2. Add 20 mL of the blocking solution and incubate for 1 h at room temperature.
3. Remove the blocking solution.
4. Add 10 mL of the rGad m 1 solutions at 0.2 mg/mL in binding buffer, and perform different trials incubating several times (2–12 h) at room temperature. A brief sonication (15 s)

in a bath sonication of the concentrated stock is recommended for reproducible pipetting.

5. Remove the fibril solution.
6. Add 25 mL of TBST, incubate for 10 min, and remove the solution. Repeat the cycle three times.
7. Add 10 mL of antibody solution containing 6×His Epitope Tag HRP conjugated antibody at a 1:4000 dilution and incubate for a minimum of 2 h at room temperature.
8. Remove the antibody solution.
9. Add 25 mL of TBST, incubate for 10 min, and remove the solution. Repeat the process three times. A last wash with TBS is recommended.
10. Develop using the chemiluminescent HRP substrate and solutions and acquire the signal with an imaging system.
11. Wash rapidly the membrane with three times with TBST, one with TBS and rinse with ethanol to avoid irreversible membrane damage.
12. For quantification, processing and identification of signals proceed as described in Subheadings 3.2, steps 13 and 14 (Fig. 2c, d).
13. Assignment of the role played by the recognition sites in amyloid formation. To this task knowledge of nature of the entity binding (monomer/fibril) and of the recognized peptides (OC/A11 binding) is necessary (*see* Notes 7–9, Fig. 3).

3.4 Membrane Re-probing

For Gad m 1-templated peptide arrays the number of synthesized SPOT membranes was 7, dictating the need of re-probing for experiment duplicates.

1. Place the used membrane in a box with excess (30 mL) of stripping solution.
2. Incubate the membrane at 55 °C for 30 min with moderate shaking (any benchtop incubator shaker with the required temperature range).
3. Remove the stripping solution and wash with excess of TBS for 10 min three times.
4. Rinse with ethanol and allow air-drying.

4 Notes

1. Gad m 1 can be either purchased or produced recombinant (rGad m 1 wt) as an N-terminal 6 × His tagged chain from a pET15b construct containing its synthetic gene inserted between the NdeI and BamHI targets. Expression and

purification procedures have been previously described [26, 27]. rGad m 1 wt behaves as a non-aggregating monomer in its Ca^{2+} -bound state (dH_2O or TBS containing 2 mM CaCl_2). But Ca^{2+} chelation such as that in binding buffer triggers its fibrillation.

2. The rGad m 1 I12V,T13K,A14T,A17E mutant (also named rGad m 1 A) behaves as monomer even in the apo-state [26, 27].
3. Amyloid fibrils are generated incubating rGad m 1 wt solutions at ≥ 2 mg/mL protein concentration in binding buffer at 37°C under quiescent conditions for 72 h [26, 27].
4. The optimal length of the peptides should not exceed 15 residues, for purity reason. A length of 12 residues ensures that amyloid forming stretches of ≤ 8 residues will be conserved in two consecutive peptides.
5. Pre-analysis of artifacts arising from nonspecific secondary antibody binding or undesired ECL reactions must be performed to ensure the specificity of the global signals. In those cases, dilutions and reaction times will need modification.
6. Use of replicates is recommended to ensure specificity of detected signal.
7. Incubating rGad m 1 wt fibrils with the peptide arrays leads to a strong accumulation of signal over peptides: 11–15, 18–20 and 32–34 (Fig. 2c, d). These interactions are highly reproducible and are detected from 2 to 12 h of incubation. Of the recognized regions, peptides 11–15 and 32–34 overlap OC immunoreactivity whereas peptides 18–20 are also covered by the amyloid-prone monomer (*see Note 9*). Therefore, peptides 11–15 and 32–34 represent growing surfaces, fibril contact regions, or both.
8. Incubating rGad m 1 wt monomers under fibrillation conditions for 2–12 h with the peptide arrays leads to labeling over peptides: 11–15, 18–20, and 32–34 (Fig. 2c, d). Under these conditions, fibrillation lag phase is about 50 h indicating that the binding entity to the peptides is the monomer and suggesting that recognized peptides are fibril growing surfaces.
9. Use of incubations with amyloid-prone rGad m 1 mutant monomer leads to signal over peptides 17–20 (Fig. 2c, d), suggesting this sequence as a nonproductive interaction motif involving rare conformations in the soluble monomer.

Acknowledgments

This work was supported by grants from Spanish AEI/EU-FEDER SAF2014-52661-C3 and BFU2015-72271-EXP. M.C. was supported by a Juan de la Cierva Postdoctoral contract.

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