

Assessment of IgE Reactivity of β -Casein by Western Blotting After Digestion with Simulated Gastric Fluid

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Abstract

Cow's milk allergy is defined as an immunologically mediated adverse reaction to cow's milk proteins and it is usually, along with hen's egg allergy, the first food allergy identified in childhood.

One of the main aspects to consider when evaluating the allergenic potential of food proteins is the effect of gastric digestion. It is known that allergens are usually able to survive the harsh acidic environment of the stomach, tolerate the presence of surfactants, and resist digestion by pepsin. They might also be digested into high molecular weight peptide fragments, which retain the same, or sometimes increased, IgE-binding. In this respect, western blotting is a highly sensitive and efficient technique that we have used to detect IgE-binding to the digests of milk and egg proteins. Given the importance of the resistance of food proteins to gastric digestion in their capacity to modulate the immune response, we describe in this chapter the assessment of IgE reactivity of a relevant cow's milk allergen, β -casein, by western blotting after simulated digestion under relevant physiological conditions.

Key words Digestibility, Food allergy, Gastric digestion, Immunoassay, SDS-PAGE, Simulated gastric fluid, Western blotting

1 Introduction

A food allergy reaction arises when the immune system overreacts reproducibly to a food (usually to the proteins of the food), leading to an adverse health effect. Some allergies are cell mediated, but IgE-mediated food allergy is believed to be responsible for the most of the food-induced hypersensitivity reactions [1]. IgE binds to high-affinity receptors present on mast cells and basophils. Crosslinking of receptor-bound IgE by an allergen triggers cell activation and degranulation, resulting in the release of histamine and other mediators of the allergic response.

Studies in Western countries have indicated that cow's milk, followed by hen's egg, are the most prevalent allergens during infancy and early childhood, causing serious problems for the allergic individuals, their families and caregivers, and the public health [2, 3].

Cow's milk contains between 3 and 3.5% proteins and includes approximately 20 different proteins that may be involved in allergic sensitization [4]. Caseins (up to 80% of the total milk protein content) are one of the most critical milk allergens accounting for the highest circulating IgE levels [5]. Among them, β -casein (Bos d8) (approximately 27% of the total milk proteins) represents a severe health risk to patients with cow's milk allergy, since 75% of the sera from patients with allergy to cow's milk have IgE directed against β -casein [6]. Whey proteins account for the remaining 20%, being β -lactoglobulin (Bos d5) and α -lactalbumin (Bos d4) the major allergens of this fraction [7].

In the hen's egg, egg white is the main source of allergens. Four proteins, ovalbumin (55% of the protein content), ovomucoid (11%), ovotransferrin (12%), and lysozyme (3%), named from Gal d1 to Gal d4, have been identified as the major ones [8]. Ovalbumin is considered one of the dominant allergens [9], although ovomucoid has been stressed as an important cause of egg hypersensitivity [10].

Although there are no molecular properties common to all food allergens, resistance to digestion is regarded as a shared feature that, together with factors such as the food matrix and the processing conditions, could contribute to the maintenance of the allergenic structures or reveal formerly hidden sites during their passage through the gastrointestinal tract [11].

We have investigated in previous studies the residual IgE-binding in the digestion products of β -casein [12], β -lactoglobulin [13], ovalbumin [14], ovomucoid [15], and lysozyme [16]. Gastric digestion of main milk and egg allergens was simulated using a relevant digestion model mimicking the physiological process. Pepsin-to-protein ratio was taken into account, as well as the pH and the presence of phospholipids. Immunoreactivity retained after gastric digestion against IgE from sera of allergic patients was evaluated by western blotting. Following the separation of the mixture of intact (if not fully digested) and hydrolyzed protein by PAGE-SDS, proteins and peptides were transferred from the polyacrylamide gel onto a nitrocellulose paper that was incubated with allergic patients' sera containing human IgE. Immunodetection was carried out with anti-human IgE and the biotin-streptavidin amplification system (the mouse anti-human IgE conjugated to biotin interacts with the conjugated streptavidin-horseradish peroxidase). Finally, a chemiluminescent substrate was used to obtain a highly precise detection of proteins or their digests from western blots by an image analyzer.

2 Materials

Prepare all solutions using deionized water and analytical grade reagents. Prepare and store all reagents at room temperature (unless indicated otherwise). Diligently follow all waste disposal regulations when disposing waste materials.

2.1 *Simulated Gastric Digestion*

1. Phosphatidylcholine.
2. Porcine pepsin (EC 3.4.23.1, 3640 U/mg β -casein).
3. Simulated gastric fluid: 35 mM NaCl, pH 2. Weigh 1 g of NaCl. Add 400 mL of water. Mix and adjust pH with 1 N HCl and add water to a final volume of 500 mL. Store at 4 °C.

2.2 *SDS Polyacrylamide Gel*

1. Sample buffer: 0.5 M Tris-HCl, pH 6.8, containing 1.6% SDS, 8% glycerol, 4% 2- β -mercaptoethanol, and 0.002% bromophenol blue. Prepare by mixing 5 mL of 1 M Tris-HCl pH 6.8, 160 mg of SDS, 0.8 mL of 100% glycerol, 0.4 mL of 100% β -mercaptoethanol, and 0.2 mL of 0.1% bromophenol blue. Adjust the final volume to 10 mL with water.
2. 4–12% Criterion™ XT Bis-Tris Gel, 12 + 2 well (Bio-Rad).
3. Running buffer: XT-MES running buffer (Bio-Rad). Prepare a 1 $\times$ running buffer by diluting 100 mL of 20 $\times$ stock buffer in 1.9 L of water (*see Note 1*). Store at 4 °C.
4. Criterion cell tank (Bio-Rad).
5. Gel-loading tips.
6. Precision plus protein standards, molecular mass marker ranging in molecular mass from 10 to 250 kDa (Bio-Rad). Store at –20 °C.
7. Fixation solution: 40% ethanol (v/v), 10% acetic acid (v/v). Prepare by mixing 400 mL of ethanol, 100 mL of acetic acid, and 500 mL of water.

2.3 *Immunoblotting*

1. Nitrocellulose membranes, precut, 0.45 μ m, 15 $\times$ 15 cm (Bio-Rad).
2. Extra thick blot filter paper, precut, 14 $\times$ 16 cm (Bio-Rad).
3. Western blot transfer buffer: 0.048 M Tris, 0.039 M glycine, 20% methanol. Dissolve 5.8 g of Trizma (Sigma-Aldrich) and 2.9 g of glycine in 200 mL of water. Add 200 mL of methanol and water to a final volume of 1 L (*see Note 2*). Store at 4 °C.
4. Tris buffered saline (TBS, 10 $\times$): 1.5 M NaCl, 0.5 M Tris, pH 7.6. Dissolve 60.5 g of Trizma and 88 g of NaCl in 500 mL of water. Mix and adjust pH with 1 N HCl. Add water to a final volume of 1 L.
5. TBS containing 0.05% Tween-20 (TBST). Dilute 100 mL of TBS 10 $\times$ in 900 mL of water. Add 0.5 mL of Tween 20 (*see Note 3*).

6. Blocking solution: 1% bovine serum albumin (BSA) in TBST. Dissolve 1 g of bovine serum albumin in 100 mL of TBST. Store at 4 °C.
7. Diluent solution: 0.1% BSA in TBST. Dilute 10 mL of blocking solution in 90 mL of water. Store at 4 °C.
8. Trans-blot® SD semidry transfer cell (Bio-Rad).
9. Plastic container.
10. Ponceau S solution: 0.1% Ponceau S bioReagent in 5% acetic acid. Dissolve 0.2 g of Ponceau S bioReagent in 190 mL of water. Add 10 mL of acetic acid and mix. Store at 4 °C and protected from light to prevent degradation.
11. Membrane washed-out solution: 0.1 M NaOH.
12. Imaging system: Molecular Imager VersaDoc™ MP 4000 System (Bio-Rad).
13. Image acquisition software: Quantity One® 1-D analysis software (Bio-Rad).

2.4 Proteins, Antibodies and Conjugates

1. β -casein from bovine milk.
2. Human sera from milk allergic patients.
3. Mouse anti-human IgE Fc-biotin antibody, clone HP6029.
4. Streptavidin-horseradish peroxidase conjugate.
5. Amersham ECL prime western blotting detection reagent (GE Healthcare).

3 Methods

3.1 In Vitro Digestion Model (See Fig. 1)

3.1.1 Preparation of the Phospholipid Vesicles Solution

1. Dissolve 0.1 g of phosphatidylcholine in 10.4 mL of simulated gastric fluid to obtain a final concentration of 9.58 mg/mL.
2. Sonicate the solution until clear to the eye (for a total of 5 min) (*see Note 4*).
3. After sonication, filter the sample through a 0.22 μ m nylon filter to remove any titanium particle deposited by the sonication.

3.1.2 Simulated Gastric Digestion

1. Dissolve 43 mg of β -casein in 3 mL of simulated gastric fluid and incubate for 10 min at 37 °C.
2. Adjust to pH 2 with 1 N HCl.
3. Complete with simulated gastric fluid until a final volume of 3.416 mL.
4. Take an aliquot of 316 μ L (4 mg of β -casein) in a tube label as CP (control protein) containing 78 μ L of 1 M NaHCO₃ and add 406 μ L of water to obtain a final sample volume of 4 mg/mL.

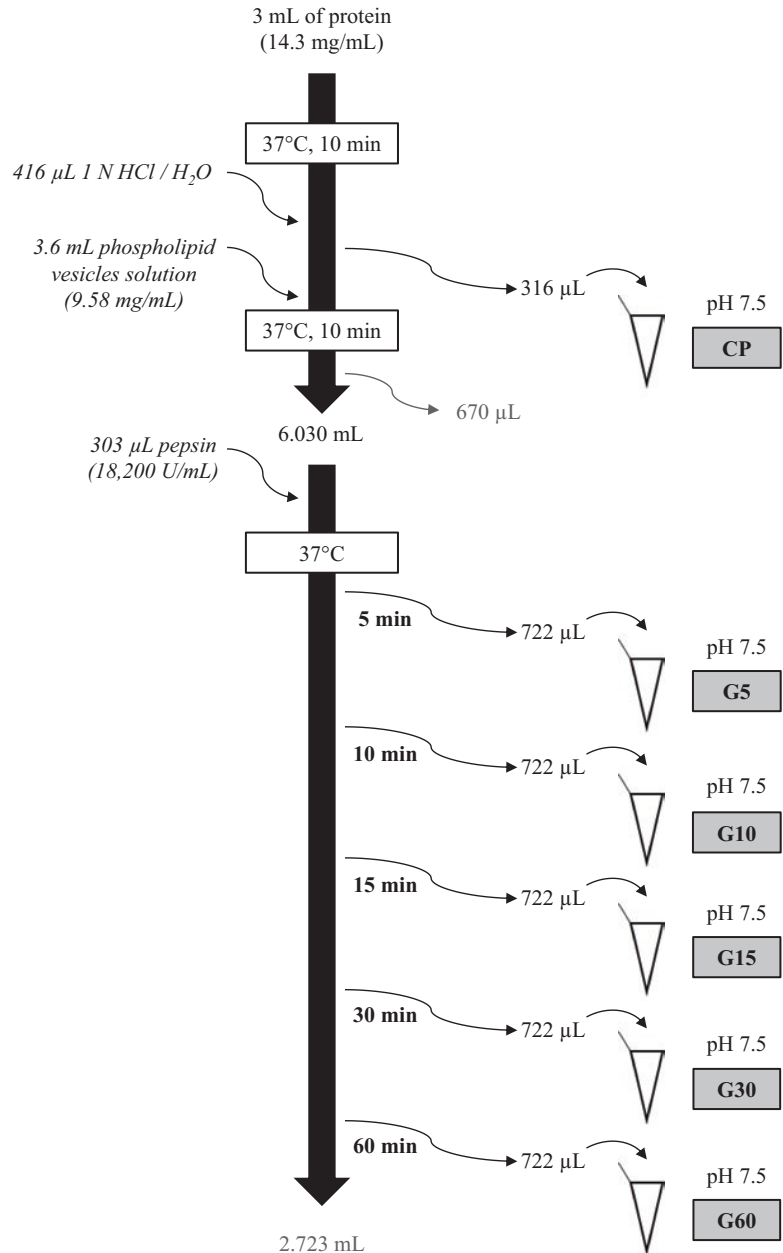


Fig. 1 Diagram of protein digestion with simulated gastric fluid

5. Add 3.6 mL of phospholipid vesicles solution and incubate for 5 min at 37 °C.
6. Prepare a solution of pepsin in simulated gastric fluid at 18,200 U/mL. Prepare immediately before use it to avoid degradation.
7. Take 6.030 mL of the mix β -casein-phospholipid vesicles solution and add 303 μL of pepsin solution (18,200 U/mL)

to obtain a physiological ratio of 182 U of pepsin per mg of β -casein.

8. Gastric digestion is performed in a shaking incubator at 37 °C.
9. After 5 min since addition of pepsin, take an aliquot of 722 μ L in a tube label as G5 containing 78 μ L of 1 M NaHCO_3 (increasing the pH to 7.5 stops pepsinolysis) and add 200 μ L of water (*see Note 5*).
10. After 10, 15, 30, and 60 min since addition of pepsin, repeat **step 9** and take aliquots in labeled tubes as G10, G15, G30, and G60 respectively (*see Note 5*).

3.2 Electrophoresis SDS-PAGE

1. Dilute all samples (CP, G5, G10, G15, G30, and G60), 1:4 (v:v) in sample buffer.
2. Heat the diluted samples at 90–95 °C for 5 min.
3. Remove the gel from the package.
4. Remove the combs by pulling them upward firmly and smoothly and rinse the wells with running buffer (*see Note 6*).
5. Remove the tape from the bottom of the cassette.
6. Insert the cassette into the slot in the Criterion cell tank. The criterion cell tank allows running two gels at once.
7. Fill the upper chamber with running buffer.
8. Fill the lower buffer tank with running buffer to the marked fill line.
9. Load 30 μ L of the sample or 10 μ L of molecular mass marker using a pipet with gel-loading tips (*see Note 7*).
10. Place the lid on the tank, aligning the color-coded plugs with corresponding jacks on the lid.
11. Turn on power supply and run the gels at 150 V. Check for bubbles at the cathode side to ensure that gel rig is running.
12. After electrophoresis is complete (*see Note 8*), turn off the power supply, disconnect the electrical leads, and remove the lid.
13. Remove the gels from the cell and break the seals on both sides of the cassette using the Criterion cell lid (see instruction from manufacturer) or a spatula.
14. Pull the two halves of the cassette apart and remove the gel by floating the gel into deionized water.
15. Rinse the gel carefully in deionized water for 1 min to remove the traces of running buffer.
16. Cut the gel in two parts (*see Fig. 2*) and proceed to Subheadings [3.3](#) or [3.4](#).

3.3 Visualizing the Gel

1. Wash the right half of the gel for 5 min in water (*see Note 9*).
2. Discard all the water and add the fixative solution to completely cover the gel.

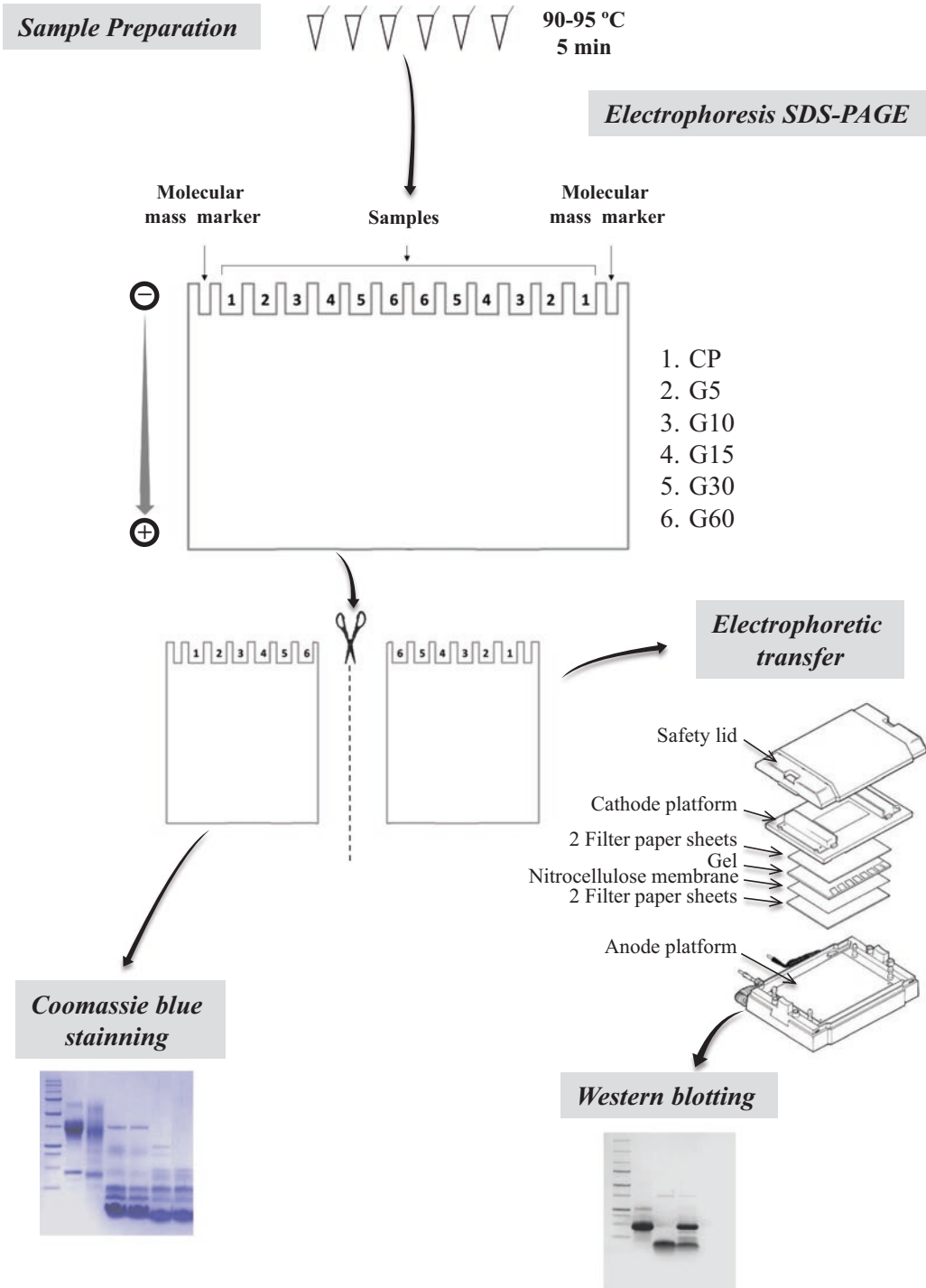


Fig. 2 Schematic of the western blotting workflow for assessment of IgE reactivity of milk and egg allergens after digestion with simulated gastric fluid

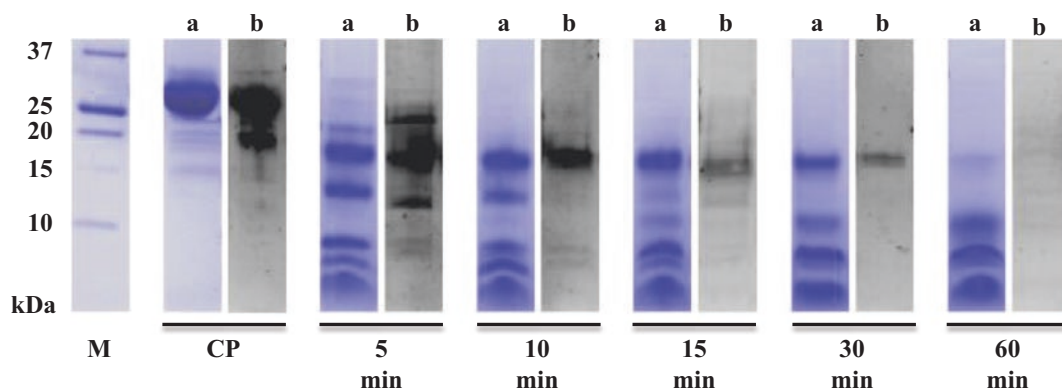


Fig. 3 SDS-PAGE patterns (a) and subsequent western blotting (b) of β -casein after 5, 10, 15, 30, and 60 min of in vitro gastric digestion with simulated gastric fluid. Electrophoresis was performed using bis-tris 12% acrylamide gels with reducing agents. *M* molecular mass marker, *CP* control protein

3. Gently shake on an orbital shaker for 30 min.
4. Remove all the fixation solution and rinse the gel in deionized water for 1 min.
5. Add enough Coomassie blue G-250 stain (Bio-Rad) to completely cover the gel.
6. Gently shake on an orbital shaker for 1 h (*see Note 10*).
7. Rinse the gel in deionized water for at least 1 h (*see Note 11*).
8. Acquire the image immediately using the Gel Doc system (*see Fig. 3*).

3.4 Electrophoretic Transfer

1. Wash the left half of the gel for 5 min in water (*see Note 12*).
2. Cut the nitrocellulose membrane and four pieces of filter paper to the exact shape of the left half of the gel (*see Note 13*).
3. Soak the left half of the gel (*see Note 14*), the nitrocellulose membrane (*see Note 15*), and the filter paper sheets in western blot transfer buffer for 20 min.
4. Remove the safety cover and the stainless-steel cathode assembly from the transfer cell and place two presoaked sheets of filter paper onto the platinum anode (*see Note 16*).
5. Place the pre-wetted nitrocellulose membrane on top of the filter paper.
6. Carefully place the equilibrated gel on top of the nitrocellulose membrane, aligning the gel on the center of the membrane (*see Note 17*).
7. Place the other two sheets of presoaked filter paper on the top of the gel.
8. Carefully place the cathode onto the stack. Press to engage the latches with the guideposts without disturbing the filter paper stack.

9. Place the safety cover on the unit and plug the unit into the power supply.
10. Turn on the power supply and transfer the gel for 30 min at 18 V.

3.5 Transfer Effectiveness Assessment

1. Following transfer, turn the power supply off, and disconnect the unit from the power supply.
2. Remove the safety cover and the cathode assembly. Discard the filter paper and the gel and take the nitrocellulose membrane. Do not allow the membrane to dry out.
3. Immerse the nitrocellulose membrane into Ponceau S solution and place it on an orbital shaker for 5 min at room temperature (*see Note 18*).
4. Rinse membrane with deionized water for 2 min to remove the background staining.
5. After seeing the protein bands to verify that the transfer is successful, wash the nitrocellulose membrane with the membrane washed-out solution for 10–20 s until the membrane is clean.
6. Rinse the nitrocellulose membrane in deionized water for 5 min.

3.6 Western Blotting

1. Block the membrane with blocking solution for 1 h at room temperature.
2. Wash with TBST for 5 min.
3. Add appropriate amount of sera from milk allergic patients to cover the membrane (usually 1:100 dilution but depends on the IgE levels in the sera. Diluted in diluent solution) and incubate overnight at 4 °C on an orbital shaker.
4. Wash 5× with TBST, 5 min each time.
5. Add anti-human IgE Fc biotin conjugate antibody (1:500 dilution, diluted in diluent solution) and incubate overnight at 4 °C on an orbital shaker.
6. Wash 5× with TBST, 5 min each time.
7. Add streptavidin-horseradish peroxidase conjugate (1:3000 dilution, diluted in diluent solution) and incubate 1 h at room temperature on an orbital shaker.
8. Wash 5× with TBST, 5 min each time.
9. Drain excess liquid by lightly blotting the membrane on dry filter paper.
10. Add Amersham ECL prime western blotting detection reagent (*see Note 19*) onto the membrane and make sure it completely covers the membrane and incubate 5 min at room temperature, protected from exposure to the light.
14. Drain off excess detection reagent by holding the membrane edge gently against a filter paper and using the image acquisition system, acquire the image immediately to avoid fluorescence degradation (*see Fig. 3*).

4 Notes

1. We routinely reuse the running buffer at least twice or thrice.
2. The final pH should be around 9.2. Do not use salts to adjust the pH because they could modify the conductivity of the buffer.
3. Tween 20 has a high density. Use a syringe, instead of a pipette, to ensure the addition of the right volume.
4. Sonication will reduce the crude lecithin solution into a suspension of single shelled liposomes. Place the container with the solution of phosphatidylcholine in an ice bath to cool the solution if it becomes hot during sonication.
5. Addition of water adjusts the final volume in all samples to 1 mL. The amount of digested protein in 1 mL of all samples (G5, G10, G15, G30, and G60) corresponds to the digestion of 4 mg of β -casein.
6. Do not pull comb out too quickly as wells will form holes, resulting in the loss of your sample.
7. Work as fast as you can without sacrificing accuracy and safety. The longer your samples sit in the buffer, the more diffuse your bands will be when you visualize the gel.
8. Electrophoresis is complete when the dye front has reached the anode.
9. Washing removes remaining SDS, which will interfere with the staining.
10. Protein bands will be visible within 20 min and reach maximum intensity within 1 h. Longer incubations will increase background.
11. Rinsing the gel overnight in water after staining will further reduce background. Stained gels can be stored in water.
12. Washing removes the remaining salts. Salts will increase the conductivity of the transfer buffer and the amount of heat generated during the transfer.
13. Transfer will be incomplete if any portion of the gel is outside the membrane and paper filters.
14. Do not soak for more than 20 min. Low molecular weight macromolecules (10,000 Da) may diffuse out of gels more readily. Twenty minutes will help to limit diffusion of low molecular weight macromolecules while providing efficient salt reduction.
15. To avoid membrane contamination, always use forceps or wear gloves when handling membranes.
16. Roll a pipet or test tube over the surface of the filter paper (like a rolling pin) to exclude all air bubbles. These air bubbles can block transfer of molecules.

17. Remember the orientation of the gel in the transfer cell when put in the chamber.
18. Since Ponceau S staining is reversible, it allows further immunological detection. This stain does not produce a deleterious effect on the sequencing of blotted polypeptides.
19. Allow the detection solutions to equilibrate to room temperature for 20 min. Mix detection solutions A (luminol) and B (peroxide) in a ratio of 1:1 to a working solution. Protect the mix reagent from exposure to the light.

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