Saptasense Protocol

Synthesis and Validation of Asn-BSA Beads

Background

We have expressed EibA on the surface of *E. coli* K12 which has subsequently been conjugated to an anti-Asn secondary antibody. This anti-Asn antibody is specific to, and will bind Asparagine in solution. Bovine Serum Albumin (BSA) can be crosslinked via glutaraldehyde to an antigen of interest, such as asparagine. Glutaraldehyde is composed of two carbonyl groups, both which can react with amine groups (i.e., those present in BSA and Asn). We will validate the success and specificity of this reaction via FTIR and SDS PAGE.

Here, we will synthesize Asn-BSA beads which form interactions with anti–Asn antibodies. Importantly, up to 30-35 asparagine molecules can be conjugated to one BSA protein. In theory, this will allow anti-Asn antibodies from several bacterial cells to bind to the same bead, thereby inducing passive bacterial agglutination (clumping). In the absence of free asparagine in solution, bacteria will bind Asn-BSA and agglutinate. Conversely, when free asparagine is present in solution, it will compete with the Asn-BSA beads and result in lower levels of agglutination. Moreover, higher concentrations of free Asparagine will inhibit BSA-mediated agglutination to a greater extent than lower concentrations, allowing for a quantifiable and specific detection mechanism.

Part I: Synthesis

Materials

Bovine Serum Albumin (BSA)
Glutaraldehyde (25% solution in H_2O)
Asparagine
Tris buffer
Sodium Acetate (anhyd.)
HCl
NaOH

FTIR (Hutch 102)
SDS-PAGE
Thermometer
pH meter

Procedure

1. Prepare the working solutions according to the table below. When working with **glutaraldehyde**, ensure that you are working in a fume hood and are wearing proper PPE (gloves, goggles, lab coat). Wash hands thoroughly after handling.

Order of Addition	Reagent	Working Stock Solution Concentration	Make using	Volume of Working Solution	In each reaction mixture	Final Concentration
1	Sodium Acetate Solution (10X reaction buffer)	1.5 M	204 mg in 1 mL dH_2O	1 mL	10 µL	0.15 M
2	dH_2O	55 M	1 mL of dH_2O	1 mL	66 µL	-
3	BSA	0.1 mM	6.643 mg in 1 mL dH_2O	1 mL	2 µL	2 µM
4	Asparagine	60 mM	7.92 mg in 10 mL ddH_2O	10 mL	2 µL	1.2 mM
5	25% Glutaraldehyde solution	0.1%	4 µL in 996 µL dH_2O	1 mL	20 µL	0.02 % = 0.2 mM
		0.25%	10 µL in 990 µL dH_2O	1 mL	20 µL	0.05 % = 0.5 mM
Total					**100 µL**	-

**The molar ratio is kept as following – BSA: Glutaraldehyde: Asparagine = 1: (100, 250): 600*

Rationale: *Excess glutaraldehyde is used in the reaction mixture to ensure enough cross-linking. Theoretically, a glutaraldehyde can cross-link BSA with Asparagine at 35 lysine residues. But it can also form a lot of by-products like cross-linking BSA with other BSA and so on. For asparagine, we need at least twice as much asparagine in the reaction mixture than the cross-linker since imine formation, an intermediate step in the reaction mechanism, will most likely occur in a statistical mixture, so we might generate a lot of the linked double asparagine*

as a byproduct but as long as we have enough asparagine to be linked to BSA, we will get our desired product in substantial amount.

2. Prepare 1 mL of 0.1 M Tris buffer pH ~7
   - ☐ Mix 12.1 mg of Tris base in 1 mL of dH_2O
   - ☐ Adjust pH to 7 (with necessary HCl or NaOH as needed)

3. Prepare reaction tubes
   - ☐ Aliquot 10 µL of reaction buffer and 66 µL of water into each of ten tubes labeled Tube 1-9.
   - ☐ Aliquot 2 µL BSA solution into each Tube 1-8.
   - ☐ Aliquot 2 µL Asparagine solution into each tube from 1-7 and 9.
   - ☐ Aliquot the 20 µL of 0.1% w/v glutaraldehyde into each Tube 1-3 and 20 µL of 0.25% w/v glutaraldehyde into each tube 4-6.
     - ☐ This step MUST be completed in a fume hood. When handling the glutaraldehyde, ensure that you are wearing gloves, googles, and a lab coat.
   - ☐ Set the pipettor to ~85uL and pipette up and down to mix the contents in each tube
     - ☐ Be sure to change tips for each reaction tube
   - ☐ Incubate each reaction in a heating block at 30C for the times indicated below.

Reaction	Temp	pH	BSA	Asn	Glutaraldehyde (all 20 µL)	Incubation (min)
1	30C	7-9	2 µL	2 µL	0.02%	10
2	30C	7-9	2 µL	2 µL	0.02%	20
3	30C	7-9	2 µL	2 µL	0.02%	30
4	30C	7-9	2 µL	2 µL	0.05%	10
5	30C	7-9	2 µL	2 µL	0.05%	20
6	30C	7-9	2 µL	2 µL	0.05%	30
7	30C	7-9	2 µL	2 µL	-	20
8	30C	7-9	2 µL	-	-	20
9	30C	7-9	-	2 µL	-	20

Rationale for the controls: *The controls will be most valuable for assaying where the different reactants run on a gel and what their FTIR peaks look like. Missing one of the reactants will lead to a pretty weird XL reaction, and we don't know if we will learn much from it.*

No controls were made without glutaraldehyde. Glutaraldehyde is our only "high hazard" chemical that we are using, and we will need to be sure that we neutralize it, otherwise the solution will continue to need to be treated as hazardous. After quenching, we won't need to treat it like chemical waste or a hazardous chemical.

- ☐ At the end of the incubation period, promptly add Tris (refer to Step 4).

4. At the end of the incubation period, add 5 µL of 0.1M Tris (pH 7.5-8) buffer to each reaction mixture. Tris should be added in excess to the glutaraldehyde to quench the reaction and prevent undesired crosslinking products.

- ☐ Mix with pipette thoroughly. (always pipette to mix proteins instead of vortexing, since you don't want to add oxygen (in the form of bubbles) to the proteins - that can denature them).
- ☐ Incubate for 10 minutes.

Rationale: *(Calculation for 0.5X Tris Buffer) Number of moles of glutaraldehyde used in the reaction (max): 10 µL of 0.025% solution has 0.0000025 g/100 gmol^{-1} = 2.5*10^{-8} mol of glutaraldehyde*

*Tris buffer needed = 2.5*10^{-8}*100 (100x excess than glutaraldehyde) = 2.5*10^{-6} mol ≡ to 2.5*10^{-6} L of 1 M Tris buffer (since C=n/V) ≡ 0.25 µL of 1 M Tris buffer or 2.5 µL of 0.1 M Tris buffer*

5. Run an FTIR analysis of the product from each reaction.

6. Run a SDS PAGE on a 12% gel for each reaction as per the following protocol.

- ☐ Observe the ratio of expected complex product (~73 kDa) to side products (e.g., larger or smaller bands representing unreacted reagents and partially-crosslinked or inappropriately-crosslinked products.

SDS PAGE Protocol

Materials

PAGE

Ladder

Visualization

Cellophane

2X Sample Buffer: (Keep frozen at -20°C until ready to use)

7.5 mL 0.5 M Tris-HCl pH 6.8 = 0.3 M
3 mL 10% SDS = 2.4 %
15 mL 50% glycerol = 60 %
0.1925 g DTT = 100 mM
0.45 mg Bromophenol blue

For 1 L: 30.2 g Tris base = 0.25 M
144 g glycine = 2.0 M
10 g SDS = 0.035 %

Instruments and Equipment

Graduated cylinders (100 mL)
Hot & ice water bath
Thermometer
Gel rig
Precast TGX stain-free 12% Polyacrylamide gel
Stain-free gel imager

10X Running Buffer (*keep at RT)*

Procedure

I. Sample Preparation

- To 10 µL volume of protein sample, add 10 µL of sample buffer.
- Boil the above mixture at 95 °C for 5 min. Picofuge at 4000 rpm for 10 seconds.
- These samples can be stored at -20 °C or may be used to proceed with gel electrophoresis.

II. Gel electrophoresis

- Place the 12% polyacrylamide premade gel into the apparatus gel chamber
- Add freshly prepared 1x running buffer (300 ml) to both chambers of the apparatus.
- Load the prepared samples into the wells of the gel.
    - First lane, protein ladder, next 9 lanes our loading buffer mixed products. Any empty lane should be filled with a 2x sample buffer, at the same volume as the samples.
- Run the gel at 100 V until the dye front migrates into the running gel (~15 min), and increase to 180 V until the dye front reaches the bottom of the gel (~45 min).
    - Ensure that the dye front DOES NOT RUN OFF of the gel. It should be right at the bottom of the gel.
- Image gel.