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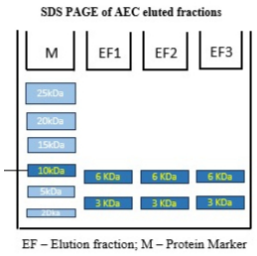
Isolation of Bioactive Insulin via C-Chain Cleavage and Inclusion Body-Based Purification from *E. coli BL21-(DE3)*



EF – Elution fraction; M – Protein Marker

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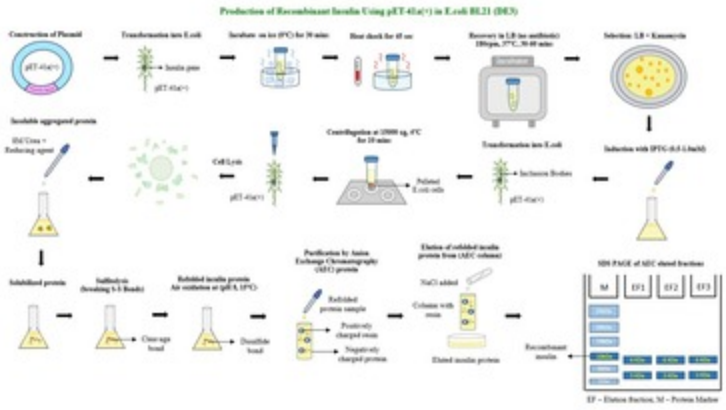
Disclaimer

This protocol was developed and executed in compliance with biosafety and ethical regulations. All recombinant DNA work was conducted using *E. coli* BL21 (DE3), a non-pathogenic laboratory strain, under approved containment conditions. Appropriate aseptic techniques and waste management protocols were strictly followed. This procedure is intended solely for research purposes and not for clinical or therapeutic use.

The authors and associated members of this project assume no responsibility or liability for any damage, injury, or loss resulting from the use, misuse, or misinterpretation of this protocol by others.

Abstract

This protocol describes the post-expression processing of recombinant human insulin in *E. coli* BL21 (DE3), focusing on the cleavage of the C-chain to obtain functional insulin composed of A and B chains. Following optimal expression using 0.5 mM IPTG in Terrific Broth, the cells were lysed via high-pressure homogenization. Inclusion bodies containing proinsulin were isolated, washed with Triton X-100 buffer, and prepared for downstream solubilization and refolding. The C-chain was cleaved during the refolding process, yielding biologically active insulin. Final purification and lyophilisation produced stable, functional insulin suitable for further biochemical and structural characterisation.



Graphical Abstract of Bioactive Insulin

Guidelines

1. Ensure recombinant gene constructs comply with institutional and national biosafety regulations.
2. Sterilize all cultureware and operate under aseptic conditions to prevent contamination.
3. All reagents (IPTG, antibiotics, urea, DTT, etc.) should be freshly prepared and validated.
4. Use cold-chain handling for proteins and include protease inhibitors during lysis and purification.
5. Buffer pH, salt concentration, and temperature must be tightly controlled to maintain insulin stability.

Materials

General Molecular Biology Reagents

- Plasmid: **pET-41a(+) vector** containing recombinant human insulin gene
- Competent *E. coli* **BL21 (DE3)** strain
- **IPTG** (Isopropyl β-D-1-thiogalactopyranoside), 1 M stock
- **LB broth** (Luria–Bertani)
- **LB agar powder**
- **Kanamycin sulfate** (stock: 50 mg/mL)

Competent Cell Preparation (TSS Method)

- **PEG 8000**, 10% (w/v)
- **$MgCl_2$**, 1 M stock
- **DMSO**
- **TSS buffer components**
- **Sterile Milli-Q water**

Lysis and Homogenization

- **Tris-HCl**, pH 8.0, 50 mM
- **NaCl**, 100 mM
- **EDTA**, 1 mM
- **Triton X-100**, 1% (v/v)
- **Lysozyme**, 1 mg/mL
- **DNase I**, 10 µg/mL
- **$MgCl_2$**, 5 mM
- **Protease inhibitor cocktail** (optional)

Inclusion Body Washing and Solubilization

- **Urea**, 8 M
- **NaCl**, 500 mM
- **Triton X-100**, 0.5–1% (w/v)
- **Tris-HCl**, pH 8.0
- **β-Mercaptoethanol or DTT** (reducing agents)

Sulfitolysis and Refolding

- **Sodium sulfite (Na_2SO_3)**
- **Sodium tetrathionate ($Na_2S_4O_6$)**
- **Glycine**, 1 M (for pH control)
- **Refolding buffer** components: Tris, EDTA, NaCl

Trypsin Digestion (C-chain cleavage)

- **Trypsin enzyme** (MS-grade, bovine/porcine)

- **$CaCl_2$**, 2 mM (enzyme co-factor)
- **Digestion buffer**: Tris-HCl, pH 8.0

Chromatography Buffers

➤ AEX Buffers (pH 10):

- **Glycine-NaOH**, 1 M stock
- **NaCl**, 1 M stock (29.2 g/L)

➤ **Reverse-Phase Chromatography:**

- **Acetonitrile (HPLC-grade)**
- **0.1% TFA (Trifluoroacetic acid)** or **Formic acid**
- **Milli-Q water**

II. Equipment and Consumables

Microbiology & Culture

- Autoclave
- Shaking incubator (37 °C and 25 °C)
- Spectrophotometer (for OD600)
- Erlenmeyer flasks (250 mL, 500 mL, 2 L, 5 L)
- Centrifuge tubes (15 mL, 50 mL)

Lysis & Homogenization

- Homogenizer (probe or Dounce)
- Centrifuge (Refrigerated, up to 10,000 rpm)
- Ice buckets and racks

Chromatography Systems

- FPLC/HPLC system (e.g., ÄKTA Pure or equivalent)
- Q-Sepharose column (5 mL)
- Resource 15 RP column (5 mL)
- Syringe filters (0.22 µm)
- Filtration units and tubing sets

Analytical Reagents and Tools

SDS-PAGE

- Pre-cast 10% SDS-PAGE gels (10 wells)
- MES Running Buffer (1×)
- Protein loading dye (Reducing and Non-reducing)
- Protein molecular weight marker
- SDS-PAGE staining solution (Coomassie Brilliant Blue or Silver stain)

Mass Spectrometry

- LC–MS grade solvents (Acetonitrile, water, formic acid)
- Sample desalt columns or ZipTips (if needed)
- Access to LC–MS instrument (ESI or MALDI-based)

Storage and Consumables

- Cryovials (for glycerol stocks)
- Sterile pipette tips (filtered)
- Sterile Petri plates
- Sterile 0.22 µm filters
- pH meter
- Vortex and heating block
- Vacuum centrifuge or lyophilizer (optional, for insulin drying)

Safety warnings

All procedures involving recombinant DNA, bacterial cultures, and high-pressure equipment must be conducted with extreme caution. Personnel must be adequately trained and fully familiar with biosafety level 1 practices before beginning any part of this protocol. Handling of *E. coli* BL21 (DE3) should be performed in a sterile environment to prevent cross-contamination or accidental release. The use of high-pressure homogenisers such as PandaPlus requires adherence to the manufacturer's safety guidelines to avoid physical injury or equipment damage. Failure to clean and sanitise equipment before and after use can result in hazardous chemical exposure or biological contamination. Triton X-100, isopropyl alcohol (IPA), and NaOH solutions are chemical irritants and should be handled with gloves and eye protection in a fume hood. Centrifugation at high speeds must only be performed using appropriate rotors and balanced loads to avoid catastrophic failure. Any deviation from the specified parameters, especially temperature, pressure, or buffer composition, may result in incomplete lysis, protein degradation, or failure to recover the functional insulin product. This protocol is intended solely for research use. The authors strictly disclaim any responsibility for injury, loss, damage, or failure arising from improper execution, misuse, or unauthorised modification of the protocol.

Ethics statement

This study does not involve any experiments on animals or humans. All experimental procedures were conducted using *Escherichia coli* BL21 (DE3), a laboratory-grade, non-pathogenic microbial strain, under biosafety level 1 (BSL-1) conditions. Therefore, ethical approval from an Institutional Animal Care and Use Committee (IACUC) or equivalent was not required. However, all protocols were carried out in strict accordance with the guidelines set forth by the Institutional Biosafety Committee (IBC) to ensure compliance with internationally accepted standards for safe handling of genetically modified organisms.

Before start

- Confirm that your protein expression has been optimised (e.g., 0.5 mM IPTG induction at 25 °C for 16 hours) and yields sufficient biomass.
- Prepare all media (LB, Terrific Broth) and sterilise by autoclaving.
- Prepare the stock solutions
- Calibrate and clean all equipment, including the centrifuge, homogeniser (PandaPlus), and spectrophotometer.
- Have Oak Ridge centrifuge tubes, homogeniser accessories, and ice available before starting.
- Ensure access to a -22 °C freezer for pellet handling.
- Obtain protease inhibitor tablets and Triton X-100 for lysis and washing steps.
- Ensure that the lyophilisation equipment is functioning and cleaned if you plan to proceed immediately after purification.

Construct of Plasmid

4d

1 In this protocol, the expression vector pET-41a(+) was employed for the production of recombinant insulin.

4d

Note

The gene of interest encoding insulin was already cloned into the plasmid b

efore the initiation of our work. The specific insulin gene sequence integrated into the vector may vary depending on the intended application or insulin type (e.g., human insulin, insulin analogues). In our case, the recombinant construct in pET-41a(+) was provided by a separate department. Our role began with the utilisation of this construct, starting from the preparation of competent *E. coli* BL21 (DE3) cells, followed by transformation and subsequent downstream processing, including inclusion body isolation and lyophilisation.

Competent Cells Preparation

1d 0h 26m

2 Chemically competent *Escherichia coli* BL21 (DE3) cells were prepared using the TSS method (Transformation and Storage Solution), a single-step, time-efficient alternative to the conventional $CaCl_2$ method. We will use the TSS Buffer to get our desired competent cells.

1m

3 **Buffer Preparation: TSS Buffer (pH: Not Required to adjust, 5 mL)**

30m

	A	B
	Reagent	**Final Concentration**
	PEG 8000	10% (w/v)
	$MgCl_2$	10 mM
	DMSO	5% (v/v)

Table (1.1): Composition of TSS Buffer

4 Prepare 10 mL of Autoclaved L.B Broth Media for Culture Preparation. 10m

Note

Calculations (Media Preparation)
0.25 grams -> 10 mL of Water

5 Inoculate a single colony (For better results, use single isolated colonies) of *E. coli* BL21 (DE3) into 10 mL of Autoclaved L.B Broth Media 5m

Note

For optimal results, select a single, well-isolated colony of medium size rather than clustered colonies. Careful selection at this stage is critical for achieving consistent and efficient protein expression

6 Incubate the culture overnight at 200 rpm, 37°C, 16:00:00 16h 30m

7 Prepare 100 mL of L.B Broth for sub-culturing 10m

Note

Calculation (Sub-Culturing)
2.5 grams -> 100 mL of Water

8 Dilute the overnight culture in a 1:100 ratio to 100 mL of Autoclaved L.B Broth Media 100 mL of Autoclaved L.B Broth Media 10m

Note

Take 1 mL of Culture from STEP 6 and dilute in 99 mL of Autoclaved L.B Broth Media

Note

Take 1 mL of Culture from STEP 6 and dilute in 99 mL of Autoclaved L.B Broth Media

9 Incubate at 180 rpm, 37°C until the optical density (OD_{600}) reaches 0.4–0.5 (mid-log phase). Check the OD_{600} by a UV Spectrophotometer, take L.B Broth as a BLANK. 5h 30m

Note

In this step, incubation typically requires approximately 5 hours to reach an OD_{600} of **0.5**. However, the time may vary depending on the specific insulin analogue and growth conditions.

10 Immediately place the culture on ice for 00:30:00 minutes 30m

11 Pellet the cells by centrifugation at 4000 rpm, 4°C, 00:10:00 10m

12 Gently resuspend the cell pellet in 1 mL of TSS Buffer (Should be placed in ice-cold). Avoid vortexing to preserve cell membrane integrity. 10m

13 Dispense 50 µL aliquots into sterile, pre-chilled microcentrifuge tubes. Store at –80 °C for long-term use. 30m

Transformation 19h 2m 45s

14 Transformation was performed using chemically competent *E. coli* BL21 (DE3) cells prepared via the TSS method. This strain, commonly used in industrial biotechnology, harbours the T7 RNA polymerase gene under control of the lacUV5 promoter, allowing high-level IPTG-inducible expression.

Note

The recombinant plasmid utilised in this experiment carried the gene of interest under a T7 promoter along with a kanamycin resistance marker for selection. The transformation procedure was optimised to ensure high efficiency while preserving cell viability.

15 Remove a 50 µL aliquot of TSS-prepared competent *E. coli* cells from –80 °C and thaw on ice for 00:15:00 Minutes 15m

16 Add 0.1 µL of Plasmid DNA in an aliquot competent cells 10m

Note

This plasmid DNA is designed with a certain concentration for which the required volume we add is 100ng 0.1 µL PLASMID DNA

17 The mixture was incubated on ice for 00:15:00 minutes to facilitate the interaction between plasmid DNA and the bacterial membrane, promoting uptake. 15m

18 While the TSS method generally does not require heat shock, a brief 42 °C Celsius heat shock for 00:00:45 Seconds can sometimes enhance transformation efficiency. In our case, the heat shock was applied. 45s

19 Immediately return the tubes to ice for 00:02:00 minutes 2m

20 Prepare 1000 µL of Autoclaved L.B Broth Medium 10m

CALCULATION
2.5 grams →100 mL
0.025 grams → 1 mL

21 Add 800 µL of Autoclaved L.B Broth of sterile LB broth to the transformation mixture Eppendorf tube. 10m

22 Incubate 180 rpm, 37°C, 01:00:00 1h
This step is known as regeneration

23 Prepare 100 mL of L.B Agar for plating 10m

Note

Make 2 Agar plates containing 25 mL of L.B Agar each.

24 Add [M] 50 mg/mL of Kanamycin Antibiotic to the agar plate 10m

CALCULATION
50mg →1 mL

25 After the solidification of agar, add 100 µL of culture . Spread evenly using a sterile glass spreader or L-spreader. 10m

26 Incubate the plates inverted 180 rpm, 37°C, 16:00:00 16h 30m

Colony Forming Screening 5m

27 After 18 hours, well-isolated colonies appeared on the agar surface, indicating successful uptake and expression of the recombinant plasmid. 5m

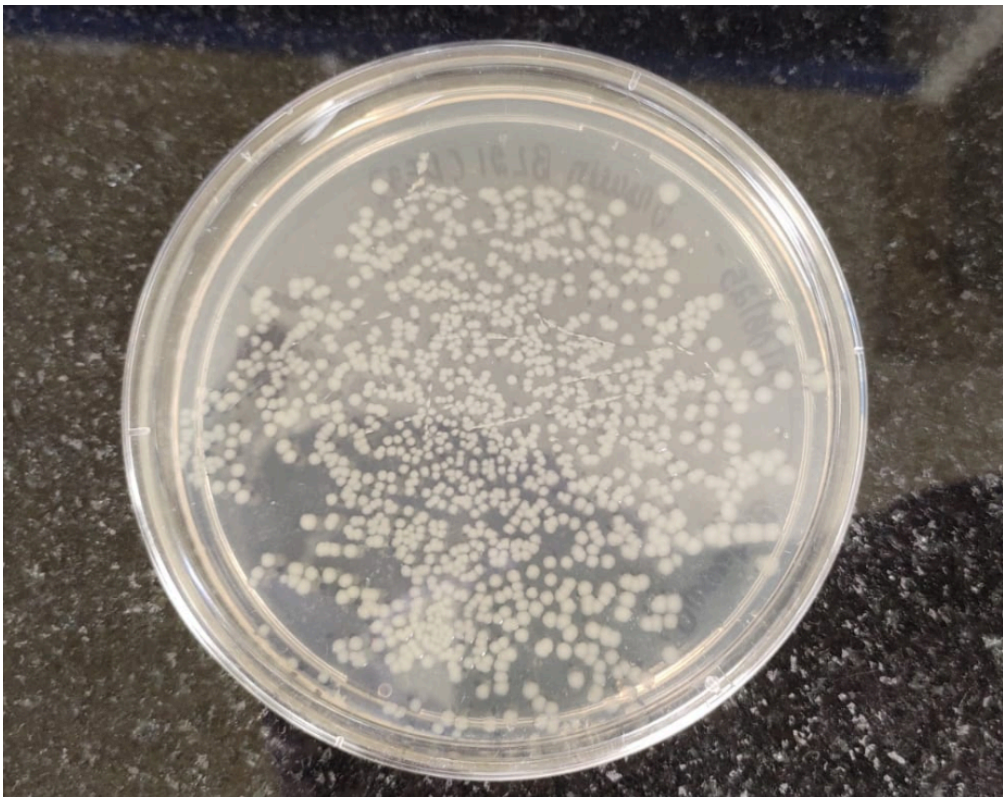


Figure (1.1): Colonies of *E.coli* BL-21 (DE3)

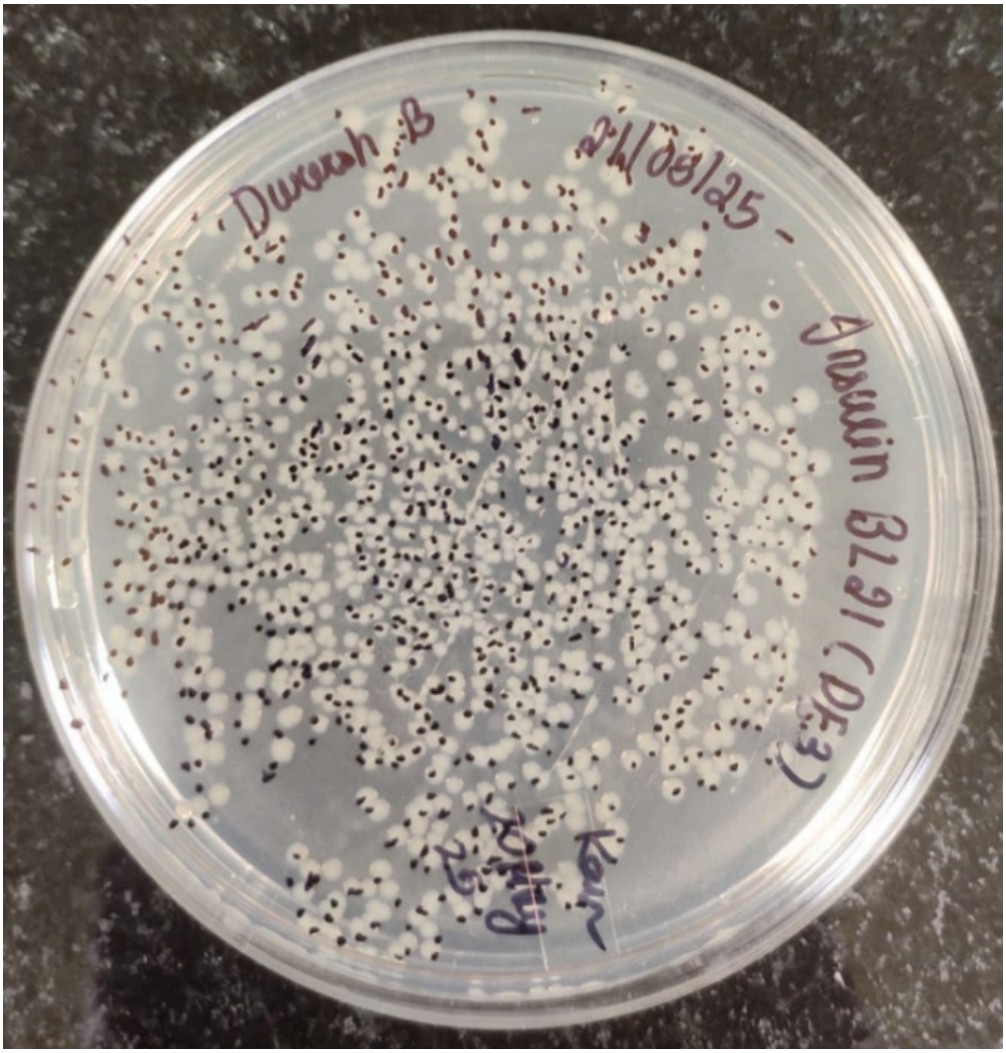


Figure (1.2): Colony Counting by classic method

Note

Figure (1.2) The photograph shows a lawn of discrete, circular colonies obtained after plating *E. coli* BL21 (DE3) cells transformed with the recombinant plasmid and incubating overnight at 37 °C. The plate was subsequently used to determine transformation efficiency by manual colony counting ("classic method").

$$\text{Transformation efficiency (CFU}\,\mu g^{-1}\,\text{DNA)} = \left(\frac{\text{Number of transformant colonies}}{\mu g\ \text{plasmid DNA plated}}\right) \times \left(\frac{\text{Total recovery volume}\ (\mu L)}{\text{Volume plated}\ (\mu L)}\right)$$

Figure (1.3): Formula to calculate transformant efficiency

Expression Screening (IPTG Induction)

22h 10m

28 Following successful transformation and colony selection, expression screening was conducted to identify optimal conditions for maximal recombinant protein production. This involved systematically varying IPTG concentration and incubation temperature, two key parameters known to influence the expression yield and solubility of recombinant proteins in *E. coli*. The goal was to determine the condition that favours high-level expression of the target protein, preferably as inclusion bodies to facilitate downstream purification.

Note

We selected two incubation temperatures—25 °C and 37°C—to observe how a slower, low-temperature expression might affect solubility compared to rapid expression at a higher temperature.

Simultaneously, we tested two concentrations of Isopropyl β-D-1 thiogalactopyranoside (IPTG), a molecular mimic of allolactose that induces the expression of genes under the lac operon.

The concentrations used were 0.1 mM and 0.5 mM, giving us a matrix of four different induction conditions:

(1) 25 °C at 0.1 mM IPTG,
(2) 25 °C at 0.5 mM IPTG,
(3) 37 °C at 0.1 mM IPTG, and
(4) 37 °C at 0.5 mM IPTG.

29 Prepare 10 mL of L.B Broth Medium for primary inoculation 10m

CALCULATION
2.5 grams for 100 mL
0.25 grams for 10 mL

30 Pick a single, well-isolated colony from the transformation plate and inoculate it into 10 mL of Primary Culture and incubate 180 rpm, 37°C, 16:00:00 10m

31 Prepare 10 mL of Autoclaved L.B Broth Media for different Batch each (x5) 5m

CALCULATIONS
2.5 grams → 100 mL
0.25 grams → 10 mL (BATCH 1)
1.25 grams → 50 mL (BATCH 5)

32 Dilute 1% of the overnight culture (**e.g., 100 µL into 10 mL**) into five 10 mL cultures of fresh LB broth (with kanamycin). Make an extra L.B Broth culture as a reference 10m

CALCULATIONS

100 µL of Primary Culture → 10 mL of Batch Culture

33 Monitor OD_{600} until cultures reach 1.0 (post-log phase).

34 Add IPTG (Isopropyl β-D-1-thiogalactopyranoside) after reaching 0.6 OD_{600} at different conditions mentioned in **Table 1.2** 10m

35

Condition	Temperature	IPTG Concentration	Time	Expression	
				Soluble fraction (Supernatant)	Insoluble fraction (Pellet)
A	25 °C	0.1 mM	16 h	❌	✓
B	25 °C	0.5 mM	16 h	❌	✓
C	37 °C	0.1 mM	4 h	❌	✓
D	37 °C	0.5 mM	4 h	❌	✓

Table (1.2): Different conditions for Expression screening with Results

36 Incubate the Induced Batch (x4) & One Uninduced Reference batch (x1)
Total 5 Batch

37 The 4-hour induced batch (x2) was incubated 180 rpm, 37°C, 04:00:00 4h

38 The 4-hour induced batch (x2) was harvested 4000 rpm, 4°C, 00:10:00 & stored at -20 °C Deep Freeze 15m

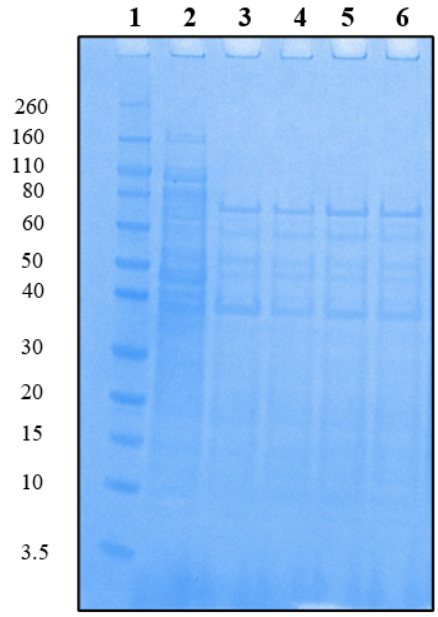
39 The 16-hour induced batches (x2) were incubated 180 rpm, 25°C, 16:00:00 16h

40 The 16-hour induced batches (x2) were harvested 4000 rpm, 4°C, 00:10:00 10m

41 The uninduced sample was directly analysed with SDS-PAGE after completion of 16-hour batches

42 Samples were then analysed using SDS-PAGE to evaluate the expression level and solubility of the protein under each condition. 1h

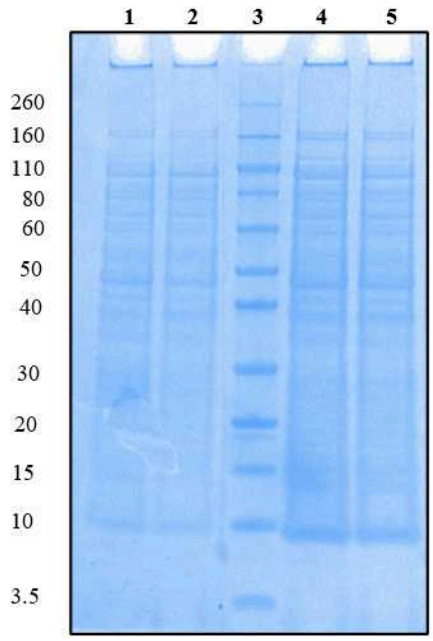
Running Buffer: MES (1X)
Running Time: 35 Minutes (Depends on Volts)
Volts: 170 V
Wells: 10 well gel
Loading Dye: Non-Reduced
Gel Type: Pre-Cast (10 %) SDS



Lanes	Samples
1	Marker
2	Un-induced WCL
3	37°C, 0.5 mM, supernatant
4	37°C, 1 mM, supernatant
5	25°C, 0.5 mM, supernatant
6	25°C, 1 mM, supernatant

Figure (1.4): Effects of IPTG and temperature on expression of recombinant protein analysed by SDS-PAGE (Soluble fractions or supernatant)

RESULTS (Figure 1.3): No Bright Bands in the soluble fraction were visible



Lanes	Samples
1	37˚C, 0.5 mM, pellet
2	37˚C, 1 mM, pellet
3	Marker
4	25˚C, 0.5 mM, pellet
5	25˚C, 1 mM, pellet

Figure (1.5): Effects of IPTG and temperature on expression of recombinant protein analysed by SDS PAGE (insoluble fraction or pellet)

RESULTS Figure (1.4): Bright Band at 10Kda was visible

Scale-Up (Pilot Scaling to 2 Litre Batch)

1d 9h 30m

43 Based on the results from expression screening, the optimal condition for recombinant protein production was selected and used for pilot-scale expression. The goal of this step is to scale up the bacterial culture volume to obtain **sufficient biomass for downstream processes** such as inclusion body isolation, C-chain cleavage, and insulin purification. All pilot-scale fermentations were conducted using LB medium supplemented with kanamycin, induced with IPTG, and incubated under previously optimised conditions.

44 Prepare 25 mL of L.B Broth Media for primary culture 10m

CALCULATE
2.5 grams → 100 mL
0.625 grams →25 mL

45 Inoculate a single confirmed positive colony (Kanamycin Resistant) into 25 mL of Autoclaved L.B Broth Media grow overnight at 180 rpm, 37°C, 16:00:00 16h

46 Prepare 500 mL of Autoclaved T.B Broth (x4) Batch for secondary culture. 10m

Note

T.B Broth media is used for higher growth of bacteria in secondary culture. 500 mL of broth was stored in a 2-litre flask. We made 4 batches containing 500 mL TB Broth in a 2-Litre flask each. So the total was 2 Litres of TB Broth Media

CALCULATION
47.6 grams → 1000 mL (1 L)
23.8 grams → 500 mL (BATCH 1)
95.2 grams → 2000 mL (2 L)- (BATCH 4)

47 After the overnight incubation, 1% of the primary culture was used to inoculate four 2-litre Erlenmeyer flasks, each containing 500 mL of Terrific Broth (TB) supplemented. 10m

CALCULATION
0.25 mL of Primary Culture in 500 mL of Secondary Culture (1 BATCH)
1 mL Total → 2 Litres

48 Incubate the secondary culture at 180 rpm, 25°C till 0.95 O.D is reached.

49 Add 0.5 mM IPTG when O.D reaches 0.95 5m

CALCULATION

Stock (1M) of IPTG
C1 V1 = C2 V2
1000 mM X x mL = 0.5 mM X 500 mL

x = (0.5 mM x 500 mL) / 1000
x = 0.25 mL IPTG → 500 mL of Secondary Culture
x = 1 mL of Total IPTG → 2 Litres 0f Total Secondary Culture

x = Required volume of IPTG

50 Incubate the secondary culture at 180 rpm, 25°C, 16:00:00 16h

51 Harvest the induced Culture at 8000 rpm, 4°C, 00:35:00 35m

52 **RESULT:** The resulting cell pellet weighed approximately, **18 grams** from the total **2-litre culture (4 flasks).** This pellet served as the starting material for all downstream steps, including lysis and inclusion body preparation. 20m

Preparation and Use of Lysis Buffer (Inclusion Body Preparation) 10m

53 **Buffer Preparation: Lysis Buffer (pH 8, 300 mL)**

	A	B	C	D
	Reagent	**Stock**	**Final Conc.**	**Volume Used**
	Tris-HCl	2 M	50 mM	7.5 mL
	NaCl	5 M	500 mM	30 mL
	EDTA	0.1 M	1 mM	3 mL
	Milli-Q Water	—	—	259.5 mL
	Protease Inhibitor Tablet	—	—	1 tablet

Table (1.3): Components required for making lysis buffer for cell lysis.

Note

For each gram of pellet, we need 10 mL of Lysis Buffer
In our case, 18 grams of pellets need at least 180 mL of Lysis Buffer. We made up to 250 mL. Protease tablet should be added after adding all the components to prevent protein degradation by endogenous proteases released during lysis.. Adjust the pH to 8.0

54 This step should be performed in a homogeniser at 6000 rpm, 00:10:00 . **This step is not centrifugation, strictly performed in a homogeniser**. 10m

Note

This pre-homogenisation allowed proper dispersion of the pellet and better accessibility of buffer components to the cells, which significantly improved the efficiency of the subsequent high-pressure lysis step.

55 Now our next step is to properly crush the cell with the help of High-Pressure cell disruption (Pandaplus Homogeniser) at

Homogenization of Lysate for Enhanced Inclusion Body Recovery

1h 35m

56 This step should be performed in a homogeniser at 6000 rpm, 00:10:00 . **This step is not centrifugation, strictly performed in a homogeniser**.

20m

Note

This pre-homogenisation allowed proper dispersion of the pellet and better accessibility of buffer components to the cells, which significantly improved the efficiency of the subsequent high-pressure lysis step.

57 Prepare [M] 0.5 Molarity (M) NaOH for removing residual protein & debris from previous runs, and add to thehigh-pressure homogeniser.

10m

CALCULATIONS
C1 V1 = C2 V2
5N V1 = 0.5 N 500 mL
V1 = 50 mL of NaOH in 500 mL of Milli-Q water

58 Add the solution mixture to the high-pressure homogeniser at 1000 Bar and repeat this step at least 5 cycles.

30m

Note

The pressure-driven disruption from both vertical and horizontal planes crushed the cells thoroughly.

59 Centrifuge the solution mixture at 9000 rpm, 4°C, 00:20:00 20m

Note

Use Oak Ridge tube for centrifugation. This step separated the insoluble fraction (containing inclusion bodies) from the soluble components. The efficient preparation of cell lysate and the separation of aggregates are vital for maintaining product purity and improving downstream yield.

60 After centrifugation of the lysate, the supernatant was discarded. The pellet, consisting primarily of inclusion bodies, was retained for further processing. 5m

Note

The inclusion bodies were visually observed as white or light beige in colour, typical of protein aggregation.

61 Weigh the pellet mass. In our case, the weight obtained was approximately 3 grams (2.968 grams Precise). 10m

Note

This weight was important for determining the buffer volumes required for the washing step.

Washing of Inclusion Body 1h 10m

62 0.5 % ()A wash buffer was freshly prepared for cleaning the inclusion bodies. pH was adjusted to 8.0 using NaOH or HCl

Buffer Preparation: Wash Buffer (30 mL)

Component	Stock	Final Conc.	Volume Used
Tris-HCl	2 M	50 mM	0.75 mL
Triton X-100	—	0.5% (v/v)	1.5 mL

Milli-Q Water	—	—	27.75 mL
Total Volume			30.00 mL

Table (1.4): Wash buffer components.

Note

The total volume was calculated based on a 1:10 w/v ratio, meaning 10 mL of wash buffer per gram of pellet. Since the pellet weighed 3 g, the total wash buffer volume was 30 mL. Triton X-100 (0.5 %), a non-ionic detergent, helped solubilise membrane components and remove hydrophobic contaminants. Tris-HCl provided buffer stability and maintained the protein environment during washing.

63 Resuspended the wash buffer with the inclusion body using a homogeniser at 6000 rpm to ensure thorough mixing and even distribution. 20m

64 The mixture was centrifuged at 15000 x g, 4°C, 00:10:00 10m

65 The supernatant was discarded. 5m

66 Repeat Steps 62, 63 & 64 once. 25m

67 The final Inclusion Body pellet was collected and weighed. The weight had reduced slightly to approximately 2.74 grams, reflecting the removal of non-target material. 10m

Solubilisation of Inclusion Bodies 3h

68 The washed inclusion body pellet (2.74 g) was subjected to solubilization using a chaotropic and reducing buffer system (Solubilisation Buffer). 30m

Buffer Preparation: Solubilisation Buffer (pH 9.0, 50 mL)

	A	B	C	D
		Stock	**Final Conc.**	**Volume/Weight Used**
	Glycine-NaOH	1 M	50 mM	2.5 mL
	Urea	—	8 M	24 g
	DTT	1 M	10 mM	0.5 mL
	EDTA	0.1 M	1 mM	0.5 mL
	Milli-Q Water	—	—	To make up to 50 mL

Table (1.5): Components required to make solubilisation buffer

Note

Urea disrupts hydrogen bonds, thereby unfolding the protein into linear polypeptides. DTT (dithiothreitol) reduces disulfide bridges, ensuring a fully denatured protein. EDTA chelates divalent metal ions (e.g., Mg^{2+}, Ca^{2+}) that may otherwise catalyse unwanted oxidation. The entire 2.74 g inclusion body pellet was resuspended in 50 mL of solubilization buffer.

69 The mixture was gently stirred for 02:00:00 at room temperature until complete dissolution of the pellet occurred. 2h

70 After dissolution, the sample was centrifuged at 15000 x g, 4°C, 00:30:00 30m

71 The supernatant obtained was taken forward for the sulfitolysis reaction. Discard the pellet

Oxidative Sulfitolysis 3h 20m

72 Prepare the Sulphitolysis solution

10m

Sulphitolysis Solution (pH 9.0)

	A	B	C	D
	Reagent	**Stock/Type**	**Final Conc.**	**Volume**
	Sodium sulfite (Na_2SO_3)	1 M	200 mM	10 mL
	Sodium tetrathionate	0.5 M	20 mM	2 mL

Table (1.6): Components required for oxidative sulfitolysis

73 [M] 200 millimolar (mM) of sodium sulfite and [M] 200 millimolar (mM) of sodium tetrathionate was added to the solubilised sample. 10m

74 Stirred gently at room temperature for 03:00:00 in the dark, as light exposure can degrade tetrathionate. 3h

Note

These processes were essential to preserving the protein's native conformation during the high-risk refolding stage. Proper buffer design, redox control, and pH maintenance during these steps significantly influenced the success of the final product.

Anion-Exchange Chromatography - FIRST CAPTURE (AEX-1) 3h 45m

75 Following sulfitolysis, the denatured and chemically stabilised recombinant protein was subjected to a first-stage purification using anion exchange chromatography (AEX).

76 **CHROMATOGRAPHY CONDITIONS** 20m

A pre-packed Q-Sepharose Fast Flow column was used for the run. The column was equilibrated with 5 mL column volumes of equilibration buffer at a flow rate of 3 mL/min. UV absorbance at 280 nm. Use NaCl to elute protein bound to the column in a linear gradient manner (Low to High Concentration).

Buffer Preparation: Equilibration Buffer (pH 8.0, 500 mL)

	A	B	C	D
	Component	**Stock Conc.**	**Final Conc.**	**Volume Used**
	Tris-HCl	2 M	50 mM	12.5 mL
	Urea	—	6 M	180 g
	Milli-Q Water	—	—	To 500 mL

Table (1.7): Composition of Equilibration Buffer for AEX

Elution Buffer (pH 8.0, 500 mL)

	Component	**Stock Conc.**	**Final Conc.**	**Volume Used**
	Tris-HCl	2 M	50 mM	12.5 mL
	NaCl	—	1 M	29.2 g
	Urea	—	6 M	180 g
	Milli-Q Water	—	—	To 500 mL

Table (1.8): Composition of Elution Buffer for Anion Exchange

Note

Following sulfitolysis, the denatured and chemically stabilised recombinant protein was subjected to a first-stage purification using anion exchange chromatography (AEX). This step plays a critical role in selectively capturing the sulfonated form of the protein while eliminating excess salts, urea, small molecules, and misfolded or truncated proteins. This chromatographic technique exploits the net negative charge of the sulfonated protein under alkaline conditions, allowing it to bind to a positively charged resin matrix in the column. Proper buffer design and pH optimisation ensured selective binding and efficient elution. In this protocol, the sulfonated recombinant protein (negatively charged at pH 8.0) binds effectively to the column matrix and is eluted using a high-salt buffer.

77 The sulfonated sample was diluted 10–fold with water in order to stop the sulfitolysis reaction and load onto the column. 10m

78 Load the column with the sample first, and use the wash buffer (Equilibration Buffer). 10m

Note

Loosely bound impurities were removed by washing with 5 mL column volumes of equilibration buffer

79 Gradient elution was performed to elute out the bound protein using elution buffer (Linear gradient of NaCl, low to high).

80 Fractions were collected and monitored using UV absorbance at 280 nm.

81 **Anion-Exchange Chromatogram (AEX-1)** 3h

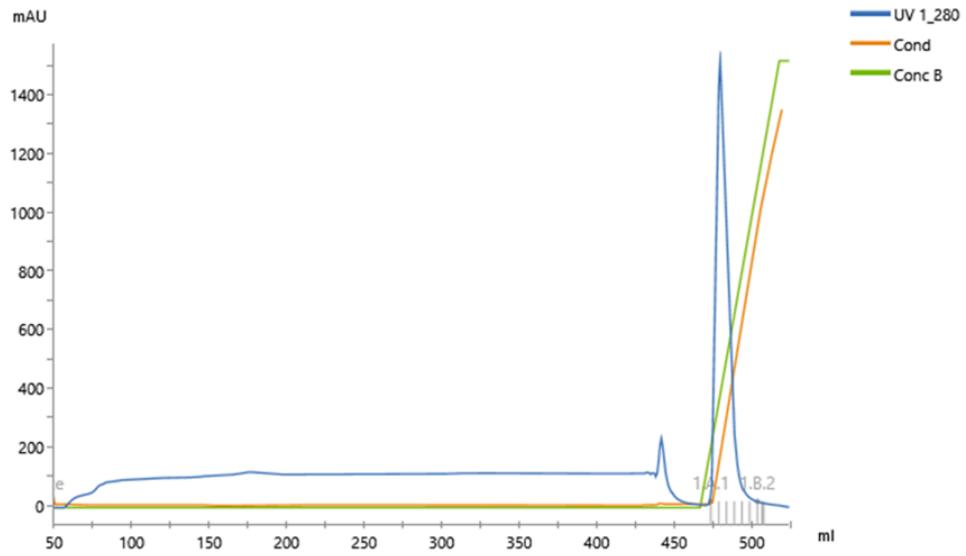


Figure Data (1.6): Chromatogram representing the purification of insoluble sulphonated recombinant protein by Anion-Exchange Chromatography; **Y-axis:** Absorbance (mAU) 280 nm; **X-axis:** volume (mL); **Column:** Q- Sepharose 5 mL

Interpretation: The blue line represents absorbance at 280 nm (UV_1 280), which correlates with the presence of proteins in the eluate. A sharp and significant peak observed between 470–500 mL indicates the elution of the target insulin protein, which was retained on the column and subsequently eluted under the applied gradient. The orange line (Cond) and green line (Conc B) represent the conductivity and buffer B (elution buffer) concentration, respectively. The sharp insulin peak coincides with a steep increase in elution buffer concentration, suggesting that the protein eluted under high salt, typically consistent with ion exchange, specifically Anion-exchange chromatography purification of inclusion body-derived proteins. This peak confirms successful expression and partial purification of bioactive insulin, which can be further verified by SDS-PAGE.

82 95 mg of Refolded protein was obtained **(Before Digestion)** 5m

Note

By performing chromatography, we obtained a curve. We use the area under the curve formula to get a rough idea about the amount of protein (mg) obtained by the chromatography.

SDS-PAGE Analysis of First Capture

50m

83 **SDS-PAGE Condition** 50m

Running Buffer: MES (1X)
Running Time: 35 Minutes (Depends on Volts)
Volts: 170 V
Wells: 10 well gel
Loading Dye: Non-Reduced
Gel Type: Pre-Cast (10 %) SDS

L: Load Sample
FT: Flow through Sample
M: Marker
EF1: Elution Fraction 1
EF2: Elution Fraction 2
EF3: Elution Fraction 3

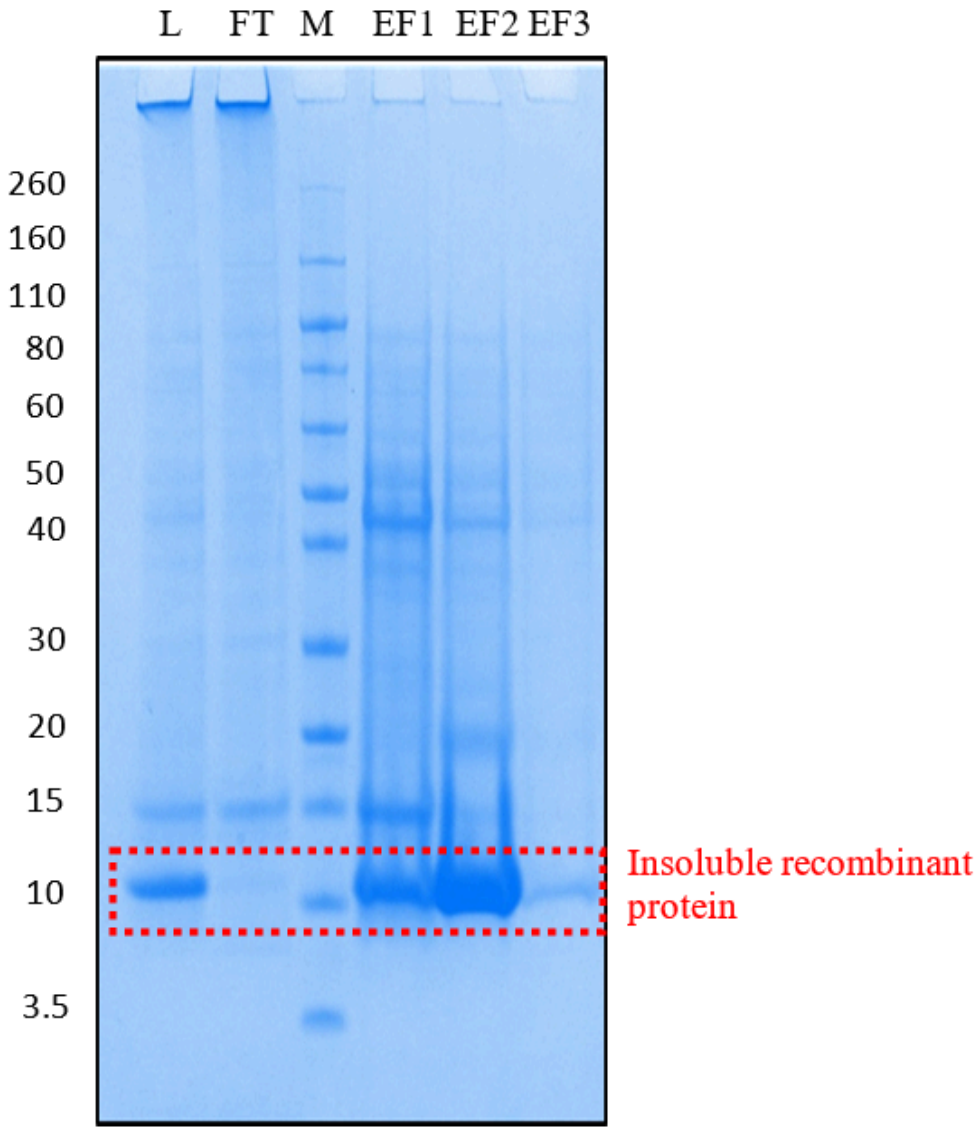


Figure (1.7): SDS-PAGE of purified insoluble sulfonated insulin recombinant protein.

Interpretation: The SDS-PAGE gel shown above depicts the analysis of samples collected from the Anion Exchange Chromatography (AEC-1) purification of sulfonated recombinant insulin expressed in *E. coli* BL21 (DE3). The gel was run under non-reducing conditions using a 10% pre-cast SDS-polyacrylamide gel with MES running buffer at 170 V for 35 minutes. A prominent protein band is observed in EF1, EF2, and EF3 at approximately 10 kDa, which corresponds to the expected size of sulfonated recombinant insulin. The absence of this band in the flow-through lane (FT) confirms successful retention and binding of the protein to the anion exchange column matrix. Its strong presence in the elution fractions indicates effective elution under high-salt or pH-shifted conditions.

Analysis: This analysis demonstrates that the AEC-1 step was successful in isolating the target protein from the crude lysate. The protein remained in the insoluble/sulfonated form, suitable for downstream refolding and purification processes.

Refolding

2d 0h 40m

84 **Buffer Preparation: Refolding Buffer (pH 9.0, 500 mL)**

20m

Reagent	Stock Conc.	Final Conc.	Volume / Weight Used
Glycine-NaOH	1 M	50 mM	25 mL
NaCl	5 M	20 mM	2 mL
β-Mercaptoethanol	14.3 M	0.3 mM	10.5 µL
Cystine	Solid	1 mM	155 mg (Calculated from 46.5 mg/150 mL)
Cysteine	Solid	5mM	0.43 g (based on 130 mg/150 mL)
Milli-Q Water	—	—	Adjusted to 500 mL

Table (1.9): Refolding buffer composition

Note

Dissolve cystine separately because it is insoluble, and gently vortex help to dissolve.

85 Cystine was dissolved in a separate 15 mL Falcon tube due to its low pH (not really dissolved in pH 9.0) and then added to the refolding buffer.

10m

86 The eluted sulfonated protein was diluted 10-fold in refolding buffer (Glycine-NaOH). 10m

Note

This step reduces urea concentration and initiates proper folding kinetics.

87 Incubate the solution at 4-10 °C for 48:00:00 2d

Note

Gentle stirring to allow disulfide reshuffling and protein refolding. Visual turbidity was monitored to detect aggregation. The solution remained mostly clear, indicating successful refolding.

Enzymatic Cleavage 50m

88 Prepare the Digestion solution 10m

(pH 9.0)

Enzyme	Dosage (per 8 mg)	For 95 mg of protein
Trypsin	35 µg	415.6 µg (approx.)
Carboxypeptidase B	0.6 µg	7.05 µg (approx.)

Table (1.10): Calculations and components required for enzymatic cleavage

89 Prepare [M] 50 millimolar (mM) Glycine Buffer **(pH 10)** 10m

90 Prepare [M] 1 Molarity (M) of NaOH **(pH 10)** 10m

91 Add both the component and the make-up to 150 mL of Total Buffer solution) 10m

92 Add 95 mg Refolded protein to the buffer and add trypsin & carboxypeptidase **(pH 10)** 10m

93 Run SDS-PAGE. In this experiment digested gel is shown after the completion of the second capture.

Anion-Exchange Chromatography-(Second Capture)-AEX-II 2h

94 **Chromatography Conditions:** 2h

Column used: Q-Sepharose 5 mL;
Absorbance: 280 nm

Buffer Preparation: Equilibration Buffer (pH 10, 500 mL):

Component	Stock Conc.	Final Conc.	Volume Used
Glycine-NaOH	1 M	50 mM	25 mL
Milli-Q Water	—	—	To 500 mL

Table (1.11): Composition of Equilibration Buffer for second AEX capture

Buffer Preparation: Elution Buffer(pH 10, 500 mL):

Component	Stock Conc.	Final Conc.	Volume Used
Glycine-NaOH	1 M	50 mM	25 mL
NaCl	—	1 M	29.2 g

	Milli-Q Water	—	—	To 500 mL

Table (1.12): Composition of elution buffer for secondary capture

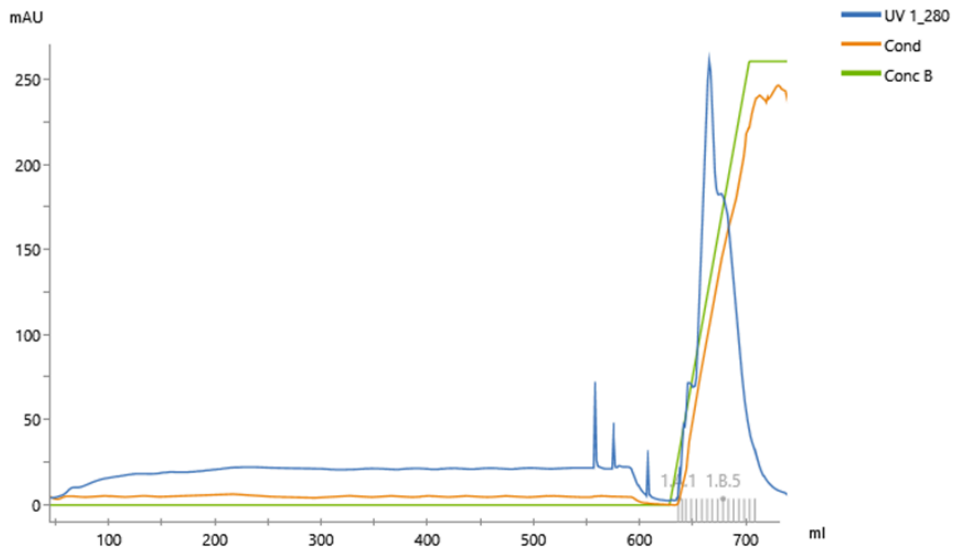


Figure (1.8): Chromatogram representing purification of renatured recombinant protein after trypsin digestion; **Y-axis:** Absorbance (mAU) 280 nm; **X-axis:** volume (mL).

Interpretation: The chromatogram represents the elution profile of the refolded and trypsin-digested recombinant insulin purified using Q-Sepharose (strong anion exchanger, 5 mL column) under a linear salt gradient at pH 10. Absorbance was monitored at 280 nm to track protein content. The blue line (UV 280 nm) indicates the presence and concentration of proteins in the elution profile, while the green line (Conc B) and orange line (Conductivity) represent the increasing concentration of NaCl in Buffer B, which facilitates the elution of negatively charged proteins. A sharp and distinct peak is observed between 650–700 mL, coinciding with the increase in conductivity and salt concentration. This peak corresponds to the elution of the mature insulin protein, which carries a net negative charge at pH 10, allowing it to bind to the Q-Sepharose resin and elute under high-salt conditions. The absence of significant absorbance peaks prior to this region indicates successful washing and removal of non-binding impurities. This confirms that the second AEX capture step effectively isolated the bioactive insulin form after C-chain cleavage and refolding.

SDS-PAGE Analysis of Second Capture (AEX-2)

40m

95 **SDS-PAGE Condition** 40m

Running Buffer: MES (1X)
Running Time: 35 Minutes (Depends on Volts)
Volts: 170 V
Wells: 10 well gel
Loading Dye: Non-Reduced
Gel Type: Pre-Cast (10 %) SDS

UD: Undigested Protein
L: Load
FT: Flow Through
M: Molecular Marker
1: Sample 1 of the elution Fraction
2: Sample 2 of the elution Fraction
3: Sample 3 of the elution Fraction
4: Sample 4 of the elution Fraction
5: Sample 5 of the elution Fraction
6: Sample 6 of the elution Fraction
7: Sample 7 of the elution Fraction
8: Sample 8 of the elution Fraction

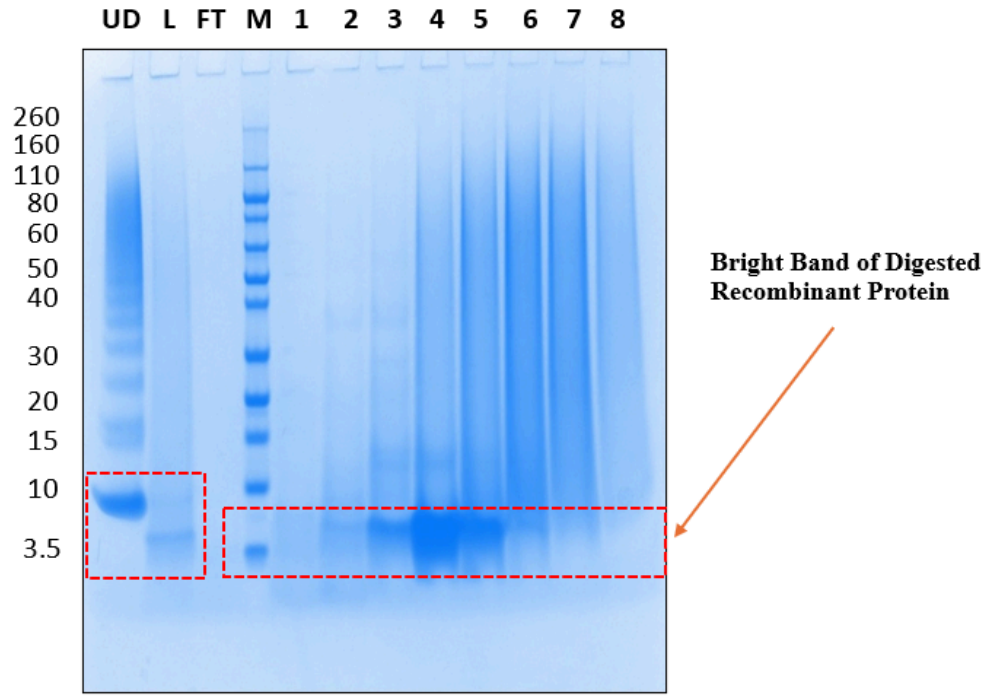


Figure (1.9): SDS-PAGE (non-reducing) analysis of recombinant insulin before and after trypsin digestion. Prominent band of Digested protein at 6 KDa, whereas band near 9 KDa represents the precursor undigested protein.

Interpretation: The bright, sharp band in lanes 1–8 at the ~6 kDa position confirms successful C-chain cleavage by trypsin and efficient recovery of the mature insulin product. The absence of higher molecular weight bands indicates minimal undigested or aggregated forms, affirming high enzymatic cleavage. efficiency.

Reversed-Phase Chromatography (RPC)- Third Capture - Polishing Step

4h 30m

96 **Buffer Preparation: Equilibration Buffer (pH 4.0, 300 mL)**

15m

97 **Buffer Preparation: Elution Buffer (pH 4.0, 150 mL)**

15m

	A	B	C
	Component	**Final Conc.**	**Notes**

	A	B	C
	TFA	0.05%	Ion-pairing agent
	ACN	80%	Organic solvent for elution
	Milli-Q Water	—	Make up to 150 mL

Table (1.13): Components required for elution buffer in reverse-phase chromatography

98 The column was washed with 5–10 column volumes of equilibration buffer to remove any non-retained or weakly bound contaminants. 45m

99 The digested and purified protein was loaded onto the equilibrated RPC column. 10m

100 **Chromatography Conditions:** 3h

X-Axis: Milli-Absorbance at 280 nm
Y-Axis: Volume (mL)
Column: Resource 15; 5 mL

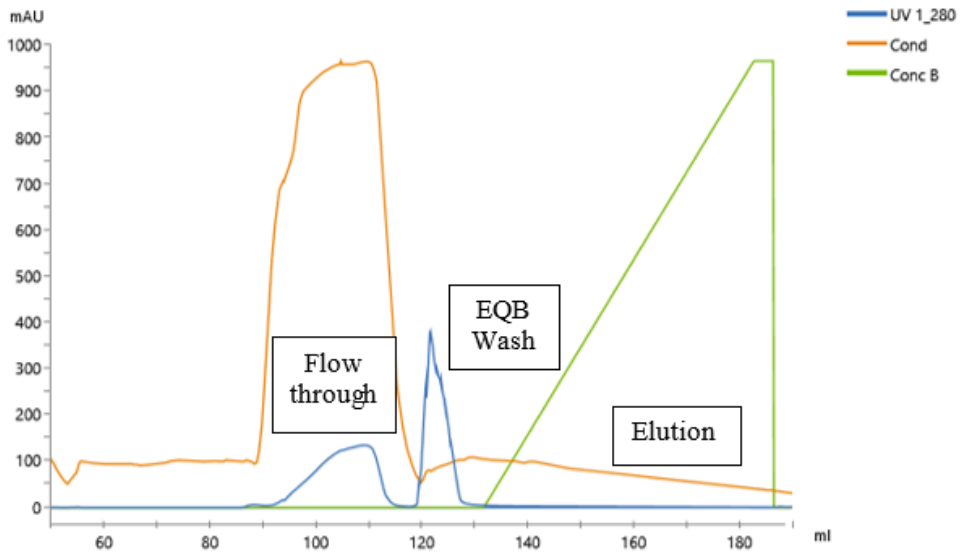


Figure (1.10): Chromatogram showing the loose binding of the target protein to the column. The recombinant protein was obtained in the washing step, which was collected in bulk. The wash sample was then analysed using SDS-PAGE and estimated.

101 The recovered weight was 11.67 mg of protein 5m

This recovered mass was calculated by the area under the curve from Reverse Phase Chromatography.

SDS-PAGE Analysis of RPC - Third Capture 50m

102 Samples from Reverse Phase Chromatography were analysed in SDS-PAGE 50m

SDS-PAGE Conditions:

Running Buffer: MES (1X)
Running Time: 35 Minutes (Depends on Volts)
Volts: 170 V
Wells: 10 well gel
Loading Dye: Non-Reduced
Gel Type: Pre-Cast (10 %) SDS

M: Molecular Marker
R.P (N.R): Reverse phase sample (Non-Reducing Dye)
R.P-(R): Reverse Phase Sample (Reducing Dye)

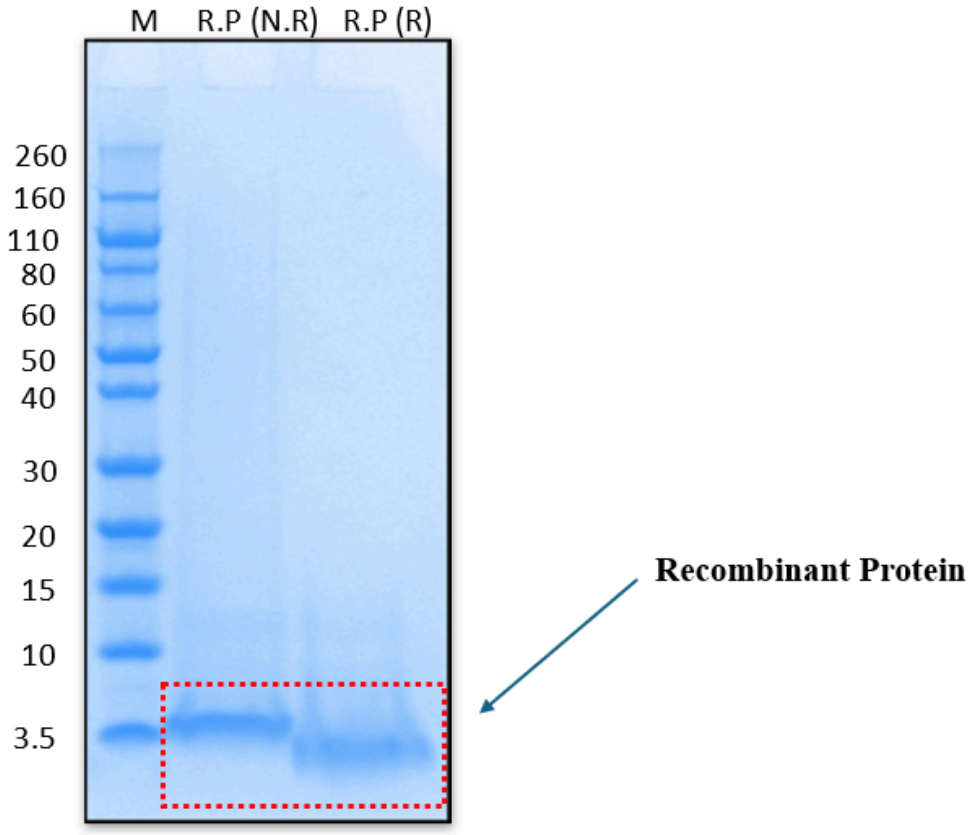


Figure (1.11): SDS-PAGE analysis showing the non-reducing lane (R.P N.R), a single intense band appears between ~5 kDa, representing the folded insulin with intact disulfide bonds between A and B chains. In the reducing lane (R.P R), two bands are clearly visible at approximately 5 kDa and 3 kDa, corresponding to the B chain and A chain, respectively, resulting from complete reduction and cleavage of disulfide bonds.

Interpreation: This band pattern confirms the presence of correctly folded, biologically relevant insulin and the successful cleavage of the C-peptide during trypsin digestion. The shift from one to two bands under reducing conditions validates the disulfide-linked heterodimeric nature of mature insulin.

MASS SPECTROMETRY Analysis & Conformation

1d

103 **Chromatogram of LC-MS (Q-TOF)**

1d

X-Axis: Deconvoluted Mass (amu)
Y-Axis: Intensity (Counts)

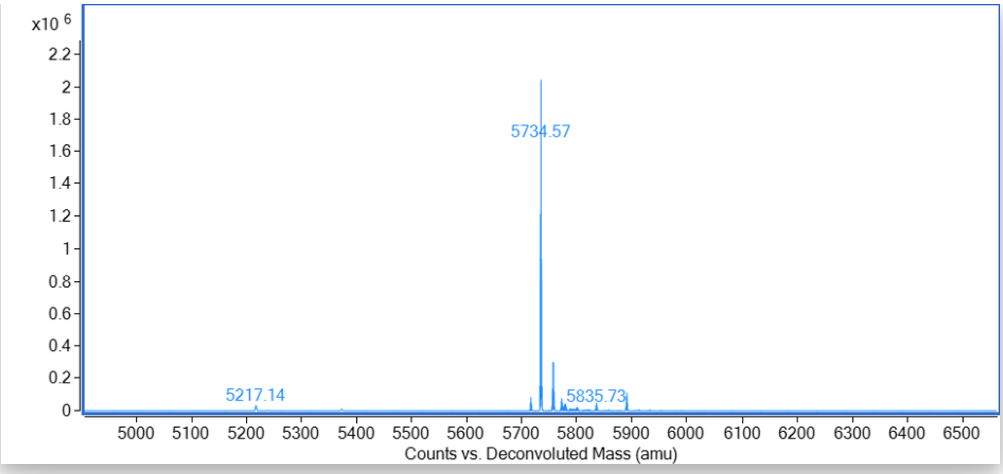


Figure (1.12): LC–MS deconvoluted mass spectrum of the final purified recombinant insulin. The main peak at **5734.57 Da** confirms the expected molecular weight of biologically active insulin following C-peptide removal and correct disulfide bond formation. Minor peaks at **5217.14 Da** and **5835.73 Da** likely represent byproducts or minor variants. ΔM = −6 Da, indicating high mass accuracy and structural integrity.

Interpretation: The deconvoluted mass spectrum reveals a dominant peak at 5734.57 Da, corresponding precisely to the expected molecular weight of mature human insulin after C-chain cleavage and disulfide bond formation. This peak confirms the successful processing of the insulin precursor into its biologically active heterodimeric form. The observed mass in MS was 5734.57 Da is within a ±6 Da deviation from the theoretical Value, which is acceptable and indicates accurate folding with the correct intra- and inter-chain disulfide linkages. Minor peaks at 5217.14 Da and 5835.73 Da likely represent degradation products or post-translational variants but are negligible compared to the dominant species. The LC–MS results conclusively validate the molecular identity, folding integrity, and purity of the recombinant insulin product.

Lyophilisation (Additional Step)

1d

104 The recovered weight was 11.67 mg of protein was subjected to lyophilized, also known as freeze-drying.

1d

Note

After achieving the final purified form of the recombinant protein via Reverse Phase Chromatography (RPC), the next step was lyophilisation, also known as freeze-drying. This technique is critical for long-term storage and stabilisation of protein products, especially when they are to be reconstituted later for analytical, formulation, or therapeutic use. Lyophilisation involves the removal of water under low temperature and vacuum, transforming the purified protein solution into a stable, dry powder without causing denaturation or aggregation. Lyophilisation consists of three main steps: Freezing – the sample is frozen below its eutectic point to form ice crystals. Primary Drying (Sublimation) – water is removed in the form of vapour under vacuum. Secondary Drying (Desorption) – remaining bound water molecules are removed to achieve the final dryness. This technique retains the protein's secondary and tertiary structure and is particularly useful for hydrophobic or aggregation-prone biomolecules. After lyophilisation, the final yield of pure, dry recombinant protein was approximately 11 mg, confirming efficient recovery from 2 L of bacterial culture. This yield reflects cumulative losses during solubilization, refolding, cleavage, and purification, and is considered industrially viable for small-scale therapeutic or research use.

Reconstitution (Additional Step)

40m

105 Add 1 mg of Recombinant Insulin Protein to an Eppendorf. 10m

106 Add 250 µL distilled water and then add [M] 0.005 Molarity (M) HCl in the Eppendorf containing the lyophilised protein. 15m

Note

It depends on the respective experimentation on how much concentration to make. In this experiment, the concentration was [M] 1 mg/mL of Recombinant insulin

107 Once the protein was completely dissolved, the pH was carefully monitored and adjusted to neutral (around pH 7.4) using [M] 5 Molarity (M) of NaOH solution 15m

It depends on the respective experimentation on how much concentration to make. In this experiment, the concentration was [M] 1 mg/mL of Recombinant insulin

DATA INTERPRETATION

108 11 mg of Recombinant Insulin Protein was purified from 2 L of Culture & the C-Chain was successfully cleaved.