Title

Human αB-crystallin as fusion protein and molecular chaperone increases the expression and folding efficiency of recombinant insulin Dual role of human αB-crystallin in insulin production

Author

The authors of the research article are Mohsen Akbarian and Reza Yousefi. The authors of the content are Mohsen Akbarian and Reza Yousefi.

Summarising culturing conditions

The study used Escherichia coli XL1-blue and BL21 (DE3) cells for expression of the fusion proteins. The cells were cultured overnight in Luria-Bertani (LB) medium containing kanamycin at 37°C. For protein expression, the cells were grown in LB medium supplemented with antibiotics and induced with isopropyl-β-D-thiogalactopyranoside (IPTG) at a final concentration of 0.25 mM. The cells were grown overnight and then harvested by centrifugation. The harvested cells were stored at -20°C before further processing. The cell culture conditions for the inoculum or start culture were not mentioned in the provided text. The culture conditions in the fermentor or bioreactor were also not mentioned.

Summarise purification conditions

1) The fusion proteins, αB-AC and αB-BC, were expressed in Escherichia coli cells and were found to be completely insoluble.

2) The insoluble inclusion bodies containing the fusion proteins were isolated by low-speed centrifugation and washed with Triton X-100 and urea.

3) The inclusion bodies were then solubilized in 8M urea and further purified using anion exchange chromatography (for αB-BC) and immobilized metal affinity chromatography (for αB-AC).

4) Cleavage of the fusion proteins was performed using CNBr, resulting in the release of the insulin peptides.

5) The peptides were then purified using gel filtration chromatography and further analyzed using SDS-PAGE to confirm their purity.

6) The purified insulin peptides underwent oxidative sulfitolysis to prevent improper disulfide bonding.

7) After sulfitolysis, the peptides were subjected to one-step gel filtration chromatography to separate them from residual fragments.

8) The final purified insulin peptides were analyzed using analytical RP-HPLC to confirm native folding.

9) The purified insulin peptides were then subjected to phenyl sepharose hydrophobic interaction chromatography to further purify natively folded insulin.

10) The natively folded insulin was collected and dialyzed against acetic acid before final purification using gel filtration chromatography.

11) The purified natively folded insulin was characterized using fluorescence, circular dichroism, and near infrared spectroscopy to confirm its structural and functional similarity to standard insulin. In the Materials and methods section, the authors described the purification process of the insulin chains. The process starts with the isolation and dissolution of the inclusion bodies, which contain the fused insulin chains. The inclusion bodies were isolated from Escherichia coli cells through cell lysis and subsequent washing steps.

The dissolved inclusion bodies were then subjected to a series of purification steps. First, the fusion proteins (αB-AC and αB-BC) were purified using a single-step purification approach, resulting in proteins with a purity of more than 48%. The purification step involved the use of phenyl sepharose column and

elution at 250mM ammonium sulfate.

Following the purification of the fusion proteins, the insulin peptide chains were released from the fusion proteins using a chemical cleaving agent (CNBr cleavage) and purified using a single-step gel filtration.

The purity of the purified insulin chains was assessed using RP-HPLC analysis, and the structural characteristics were analyzed using fluorescence, CD, and NIR spectroscopy. The aggregation and oligomerization patterns of the insulin samples were studied using ThT fluorescence measurement and size exclusion chromatography.

Finally, the in vivo activity of the insulin product was assessed using an insulin tolerance test (ITT) in mice. The experiment involved the subcutaneous injection of the insulin samples into mice, followed by the measurement of blood glucose levels at regular intervals.

Overall, the purification process involved multiple steps, including isolation and dissolution of inclusion bodies, purification of fusion proteins, release and purification of insulin chains, and assessment of structural and biological activities. The specific quantities of chemicals, buffers, and other reaction conditions were not mentioned in the provided information.

Extracting material and manufacturers

Materials used in this research article include:

- Bis-1-anilino-8-naphthalenesulfonate (bis-ANS)
- Thioflavin T (ThT)
- Sodium tetrathionate
- Sodium sulfide
- Cyanogen bromide

- Standard insulin (from Sigma)

- Goat anti-rabbit IgG peroxidase

- Dialysis tube (2kDa cut-off) from Spectrum Scientific Company

- Anti αB-Cry antibody (a gift from Professor Samuel Zigler)

- Gelfiltration media and Ni-NTA matrix (from GE Healthcare and Qiagen)

Please note that the manufacturer for each chemical was not specified in the article. - Materials and chemicals used:

1. Thioflavin T (ThT) fluorescence dye.

2. Insulin samples.

3. Zinc.

4. Size exclusion chromatography column.

5. Saline.

6. CNBr cleavage site.

7. Glycine.

8. Phenyl sepharose column.

9. Ammonium sulfate.

- Manufacturers:

1. Thioflavin T (ThT) fluorescence dye: Not specified.

2. Insulin samples: Not specified.

3. Zinc: Not specified.

4. Size exclusion chromatography column: Not specified.

5. Saline: Not specified.

6. CNBr cleavage site: Not specified.

7. Glycine: Not specified.

8. Phenyl sepharose column: Not specified.

9. Ammonium sulfate: Not specified.

Title

Expression and purification of recombinant human insulin from E. coli 20 strain

Author

The authors of the content are Marcin Zieliński, Agnieszka Romanik-Chruścielewska, Diana Mikiewicz, Natalia Łukasiewicz, Iwona Sokołowska, Jarosław Antosik, Agnieszka Sobolewska-Ruta, Anna Bierczyńska-Krzysik, Piotr Zaleski, and Andrzej Płucienniczak.

Summarising culturing conditions

In the cell culture section, the researchers used E. coli 20 cells, which harbored the pIBAINS plasmid with the human insulin gene. The cells were initially grown in shaking flasks containing GMS medium supplemented with tetracycline, proline, and thiamine. Once the cells reached an optical density at 600 nm (OD600) of about 0.5-1.0, they were used as an inoculum for the fermentor/bioreactor.

In the fermentor/bioreactor, the cells were grown for 15-16 hours at 37°C. During the growth stage, glucose and proline were added as carbon sources to maintain glucose concentration within a specific range. The pH of the culture was controlled throughout the run by adding NH4OH. Once the cells reached an OD600 of approximately 25-35, glucose feeding was limited until its concentration in the medium was reduced to 0 g/dL. Glucose feeding was then maintained at a set level using a pH-stat control system. The culture was grown for an additional 4-5 hours until reaching the stationary phase of growth.

Specific details such as exact concentrations of glucose, proline, and tetracycline, as well as agitation and aeration conditions, were not mentioned in the summary or the original text.

Summarise purification conditions

Sure! Here is a detailed summary of the purification process from the Materials and Methods section:

1. Isolation of inclusion bodies:

- Cells were harvested by centrifugation and suspended in lysis buffer (50 mM Tris-HCl pH 8.0, 500 mM NaCl, 1 mM EDTA, 0.043% lysozyme).
- Triton X-100 was added, and the suspension was stirred for 10 minutes.
- Cells were lysed using a high-pressure homogenizer and centrifuged to remove insoluble debris.
- Pellets were washed with Triton X-100 and centrifuged again.
- Pellets were washed twice with buffer and centrifuged.
- The resulting inclusion bodies were frozen at -30 °C.

2. Dissolution of inclusion bodies:

- Inclusion bodies were dissolved in 12 mM carbonate buffer ($NaHCO_3$) with 0.2 mM EDTA.
- The pH was adjusted to approximately 11.9 with NaOH and stirred for 45 minutes.
- The pH was then adjusted to 10.8 with HCl, and the suspension was centrifuged to remove insoluble debris.

3. Renaturation:

- The dissolved protein sample was allowed to fold during an 18-hour period with stirring and aeration.
- At the end of renaturation, the pH was adjusted to 9.0 with HCl.
- Renaturation was performed at either room temperature or 7-8 °C.

4. Citraconylation reaction:

- The insulin precursor was treated with citraconic anhydride to control enzymatic digestion.
- Citraconic anhydride and NaOH were added to the protein solution, maintaining a pH range of 8.7-9.3.
- The solution was stirred for 2.5 hours.

- Ethanolamine was added, and the mixture was mixed for 45 minutes.

5. Trypsinization:

- The pH of the solution was adjusted to 8.8, and trypsin solution was added.

- The solution was mixed for approximately 16-18 hours at room temperature.

- Aprotinin was added to inhibit the reaction.

6. Low-pressure chromatography on DEAE Sepharose:

- The trypsinized protein sample was applied to a column filled with DEAE Sepharose.

- Unbound proteins were eluted with buffer.

- Insulin was eluted using a buffer with increased salt concentration.

- The insulin fraction was collected.

7. Decitraconylation:

- The main fraction eluted from the column was diluted and the pH was lowered to 2.9 with HCl.

- The sample was stirred and left overnight at 4 °C.

8. Precipitation of insulin:

- Zinc chloride solution was added to the sample, and the pH was adjusted.

- The solution was stirred for 1 hour and centrifuged.

- The precipitate of insulin was suspended in water.

9. Dissolving the zinc salt:

- The precipitate of insulin was suspended in water.

- Tris pH 8.6 and EDTA were added to adjust the solution concentration and remove the zinc salt.

10. Low-pressure chromatography on Q Sepharose:

- The protein sample was applied to a column filled with Q Sepharose.
- Unbound proteins were eluted with buffer.
- Insulin was eluted using buffers with increasing isopropanol concentrations.
- The insulin fraction was collected.

11. Reaction with carboxypeptidase B and RP-HPLC chromatography:

- Carboxypeptidase B was added to the main fraction eluted from Q Sepharose.
- The reaction was carried out for 16-18 hours.
- The sample was applied to an RP-HPLC column for further separation and purification.
- Insulin was eluted using a gradient of sodium sulfate and acetonitrile.

12. Sephadex G-25 chromatography:

- The purified insulin sample was applied to a column packed with Sephadex G-25.
- The sample buffer was exchanged with ammonium acetate.
- Insulin was eluted with the same buffer.

It's important to note that the quantities of chemicals, buffers, and other reagents used in the study were not explicitly mentioned in the provided text.

Extracting material and manufacturers

In the material section of the article, the following materials and chemicals were used:

1. Antifoam 204 (Sigma)
2. L-proline (Sigma)
3. Thiamine hydrochloride (Sigma)
4. Tetracycline hydrochloride (Sigma)

5. Trizma base (Sigma)

6. Triton X-100 (Sigma)

7. Lysozyme (Sigma)

8. EDTA (Sigma)

9. Sodium bicarbonate (Sigma)

10. Citraconic anhydride (Sigma)

11. Ethanolamine (Sigma)

12. Aprotinin (Sigma)

13. Magnesium sulphate heptahydrate (Avantor Performance Materials Poland S.A.)

14. Glucose (Avantor Performance Materials Poland S.A.)

15. Sodium chloride (Merck)

16. Sodium hydroxide (Merck)

17. Di-potassium hydrogen phosphate (Merck)

18. Potassium dihydrogen phosphate (Merck)

19. Hydrochloric acid (37%) (Merck)

20. Trypsin (Merck)

21. Carboxypeptidase B (Merck)

22. Zinc chloride (Merck)

23. Sodium sulfate (Merck)

24. Yeast extract (Becton Dickinson)

25. Acetonitrile (Becton Dickinson)

These materials and chemicals were obtained from the respective manufacturers mentioned in parentheses.