

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

Marcin Zieliński*, Agnieszka Romanik-Chruścielewska, Diana Mikiewicz, Natalia Łukasiewicz, Iwona Sokołowska, Jarosław Antosik, Agnieszka Sobolewska-Ruta, Anna Bierczyńska-Krzysik, Piotr Zaleski, Andrzej Płucienniczak

Institute of Biotechnology and Antibiotics, Starościńska 5, Warszawa, 02-516, Poland

ARTICLE INFO

Keywords:

Diabetes
Hyperglycemia
Recombinant human insulin
pIBAINS expression vector
Escherichia coli 20 strain
Inclusion bodies

ABSTRACT

The number of people with diabetes is estimated to be over 370 million, in 2030 it will increase to 552 million. In Poland, the number of people with diabetes is estimated to be 3.5 million (9.1%). According to the estimates of the International Diabetes Federation, the percentage of patients in the adult Polish population will increase to around 11% over the next 20 years. Despite the appearance of insulin analogues on the pharmaceutical market, insulin delivery is still the most effective method of pharmacotherapy in cases of extremely high hyperglycemia.

A new bacterial host strain (*Escherichia coli* 20) was obtained at the Institute of Biotechnology and Antibiotics and a new pIBAINS expression vector was constructed that provides greater efficiency in the production of recombinant human insulin. In the IBA Bioengineering Department, successful attempts were made to produce recombinant human insulin on a laboratory and quarter-technical scale, and several batches were performed on a semi-technical scale. The production process has been divided into several stages: 1. biosynthesis of insulin in the fermenter, 2. isolation, purification and dissolution of inclusion bodies, 3. protein renaturation, 4. enzymatic reaction with trypsin, 5. multi-stage purification of insulin using low-pressure and HPLC techniques. At each stage of insulin production, qualitative and quantitative analyses were performed to confirm identity and purity. In particular, the molecular weight of insulin, the amount of insulin and the content of protein impurities were studied. The results of these experiments are presented in this work.

1. Introduction

An insulin deficit in an organism or a lack of cell response to the produced hormone leads to diabetes mellitus. This usually incurable illness manifests as high blood sugar (hyperglycemia). Chronic hyperglycemia may lead to perturbation and dysfunction of various organs including the kidneys, the heart, blood vessels, the nervous system and the retina [1]. According to the MFD (International Diabetes Federation) estimates, the percentage of patients in the adult Polish population will increase to around 11% over the next 20 years [2]. This population may in the future be the recipient of the results of our project. Insulin replacement therapy is the standard of care for patients with type 1 and advanced type 2 diabetes mellitus. Insulin delivery is still the most effective method of pharmacotherapy in cases of extremely high hyperglycemia. Porcine and bovine pancreatic tissue was the source of the hormone for many years, followed by semisynthetic human insulin obtained by modification of animal insulin. With the

development of recombinant DNA technology, recombinant (biosynthetic) human insulin became available in large amounts by biosynthesis in microorganisms (*Escherichia coli*, yeasts) providing reliable supplies of the hormone worldwide at affordable prices. Recombinant human insulin was one of the first products of biotechnology. The purity and pharmaceutical quality of recombinant human insulin was demonstrated to be superior to animal and semisynthetic insulin and patients with diabetes could be safely and effectively transferred from animal or semisynthetic human insulin to recombinant human insulin with no change expected in insulin dosage [3].

Human insulin is a hormone produced in the β -cells of the pancreatic islets, responsible for glucose metabolism regulation [4]. Human insulin is a polypeptide with a molecular mass of 5808 Da. The hormone consists of two chains: chain A (21 amino acids) and chain B (30 amino acids) connected by two disulphide bridges. Additionally, chain A contains an intrachain disulphide bond [5]. A new bacterial host strain (*E. coli* 20) was obtained at the Institute of Biotechnology and

* Corresponding author.

E-mail address: marcinzielinski2@wp.pl (M. Zieliński).

<https://doi.org/10.1016/j.pep.2019.02.002>

Received 8 November 2018; Received in revised form 4 February 2019; Accepted 4 February 2019

Available online 05 February 2019

1046-5928/ © 2019 The Authors. Published by Elsevier Inc. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

Antibiotics. Also, a new pIBAINS expression vector was constructed. The new strain and the expression vector have been reported in the Polish patent office. The results of these experiments are presented in this work.

2. Material and methods

2.1. Materials

Escherichia coli 20 IBA 1 strain is a laboratory derivative of *E. coli* CSH50R [ATCC: 39111] [6–8]. Antifoam 204, L-proline, thiamine hydrochloride, tetracycline hydrochloride, trizma base, Triton X-100, lysozyme, EDTA, sodium bicarbonate, citraconic anhydride, ethanolamine, aprotinin were purchased from Sigma. Magnesium sulphate heptahydrate, glucose, ammonium chloride, isopropyl alcohol, acetonitrile were from Avantor Performance Materials Poland S.A. Calcium chloride dihydrate was from J.T.Baker. Sodium chloride, sodium hydroxide, di-potassium hydrogen phosphate, potassium dihydrogen phosphate, 37% hydrochloric acid, trypsin, carboxypeptidase B, zinc chloride, sodium sulphate were purchased from Merck. DEAE Sepharose Fast Flow, Q Sepharose Fast Flow, Sephadex G-25 were from GE Healthcare. Ferric ammonium citrate was purchased from Alfa Aesar. Yeast extract was from Becton Dickinson. HPLC reversed-phase C18-300 column was purchased from ACE Ltd. Bioflo celligen 310 bioreactor was from Eppendorf.

2.2. Host strain

The *E. coli* 20 strain IBA 1 (F- lambda-ara Δ(pro lac) rpsL thi recA cytR) is a derivative of the *E. coli* CSH50R strain. It is a laboratory strain derived from *E. coli* K-12, Gram (-) negative bacterium belonging to the *Enterobacteriaceae* family. It is a strain used for transformation. It grows in the form of a uniform suspension in LB (lysogeny broth) liquid medium. The optimal growth temperature is 37 °C. The strain also grows on minimal medium (MM) in the presence of thiamine (2 µg/ml) and L-proline (in the concentration range 50–200 µg/ml). The strain forms round, flat, smooth-edged colonies on the rich TSA (trypticase soy agar) substrate. The *E. coli* strain IBA 1 has a deletion in the proline-lactose operon. The mutation in the rpsL gene encoding the ribosomal S12 protein is associated with streptomycin resistance (growth on the medium with streptomycin at a final concentration of 50 mg/ml). The strain also has a mutation in the cytR gene encoding a transcriptional regulator that binds to the DNA. From the gene sequence, 1026 bp in length, 145 bp were excised. An efficient method to produce recombinant human insulin using *E. coli* 20 bacterial strain lacking the cytR repressor is described in Polish patent No. PL 180,818 [9].

2.3. Construction of human insulin gene

The expression plasmid pIBAINS is a derivative of plasmid pBR322 [ATCC: 31344]. Briefly, pIBA was prepared by ligation of two *HindIII*/*SmaI* fragments and amplified by PCR, using pBR322 plasmid as a template. Then, using a PCR method, 63 nucleotides with short molecular cloning sites and a synthetic transcription terminator trpA (Pharmacia Biotech 1995) were inserted into this plasmid. The resulting plasmid pIBA-1 was cleaved with *EcoRI*/*NdeI* and then a 945 bp PCR fragment comprising the *deoPIP2* promoter (also digested with *EcoRI*/*NdeI*) was ligated in the clockwise orientation to give the pIBA-2 plasmid. The sequence of the *deoPIP2* promoter region was amplified from the chromosomal DNA of *E. coli* K12 strain based on the nucleotide sequence from the gene database [GenBank: [AP009048](#)]. The pIBA-2 plasmid was further treated with *NdeI*/*XbaI* and ligated with the 363 bp *NdeI*/*XbaI* insert, comprising a hybrid protein SOD_INS [10]. The codon usage of the hybrid protein gene was optimized for expression in *E. coli*. The resulting plasmid pIBAINS carries resistance to tetracycline. Transcription initiation is under control of the *E. coli* *deoPIP2* promoter. The

constructed plasmid pIBAINS was used to transform the *E. coli* 20 strain obtained in our Institute. We used IBA's *E. coli* 20 strain and IBA's vector because ownership of a patented strain allows the invention to be commercialized.

2.4. Cell culture

Bacterial cells for culture came from glycerol stock stored at –80 °C. *E. coli* 20 cells, harboring plasmid pIBAINS with the human insulin gene, were grown for 18 h at 30 °C in shaking flasks containing 50 mL GMS medium [11] supplemented with tetracycline (100 µg/mL), proline (600 µg/mL) and thiamine (2 µg/mL) until the optical density at 600 nm was about 0.5–1.0. The flasks were used as an inoculum for a Bioflo Celligen 310 7.5 L fermenter (starting GMS medium volume 4 L). In the fermenter antibiotics were not used. Cells were grown for 15–16 h at 37 °C. During the growth stage of the culture, 40% glucose and 12 mg/mL proline were exponentially added as the carbon source (to maintain glucose concentration range 70–120 mg/dL). The pH was controlled during the entire run by addition of 16% NH₄OH. When OD 600 nm reached about 25–35, the glucose feeding was limited until its concentration in the medium was reduced to 0 g/dL. Then, the glucose feeding was connected to a pump with acid (pH-stat control using cascade). Glucose was automatically added by the controller whenever the pH exceeded the set point (dead band was 0.01). The glucose level was maintained by the pH-stat in the concentration range of 30–50 mg/dL. After induction, the culture was grown for 4–5 h until OD 600 nm was about 50–60, reaching the stationary phase of growth.

2.5. Isolation of inclusion bodies

Cells were harvested by centrifugation at 15,000 × g for 15 min at 4 °C. The pelleted cells were suspended in lysis buffer (50 mM Tris-HCl pH 8.0, 500 mM NaCl, 1 mM EDTA, 0.043% lysozyme). The bacterial suspension was gently mixed for 30 min at room temperature. Then, 20 ml of Triton X-100 was added and the suspension was stirred for 10 min. Cells were lysed by a high-pressure homogenizer, and subjected to centrifugation at 18,400 × g for 15 min at 4 °C. The resulting pellets were washed with 50 mM Tris-HCl pH 8.0, 500 mM NaCl and 1% Triton X-100 and centrifuged again at 18,400 × g for 15 min at 4 °C. Next, the pellets were washed twice with 50 mM Tris-HCl pH 8.0, 500 mM NaCl buffer. Finally, the suspension of inclusion bodies was centrifuged at 15,000 × g for 15 min at 4 °C. The obtained inclusion bodies were frozen at –30 °C for further preparation.

2.6. Dissolution of inclusion bodies

The inclusion bodies containing insulin were dissolved in 12 mM carbonate buffer (NaHCO₃) with 0.2 mM EDTA. The pH of the solution was adjusted to about 11.9 with 5 M NaOH and stirred for 45 min at room temperature. Then the pH was adjusted to 10.8 with 2 M HCl. Finally, the suspension was centrifuged at 24,000 × g for 15 min at 4 °C to remove insoluble debris.

2.7. Renaturation

After dissolving, the sample was allowed to fold during the subsequent 18 h period with vigorous stirring and aeration. At the end of renaturation, the pH was adjusted to 9.0 with 2 M HCl. Renaturation was studied at room temperature and at 8 °C.

2.8. Citraconylation reaction

The insulin precursor was treated with citraconic anhydride in order to control enzymatic digestion required for forming insulin and to prevent insulin degradation. 1 ml of citraconic anhydride and 5 M NaOH were added to the protein solution in portions so that the pH of

the solution remained in the range 8.7–9.3. The amount of anhydride was calculated according to the formula: $0.2 \times V \text{ solution (dm}^3\text{)} \times A280 \text{ nm}$. After the addition of the total amount of citraconic anhydride, the solution was stirred for 2.5 h. Then 3 ml of a 2 M ethanolamine solution was added. The quantity was calculated according to the formula: $3 \times V \text{ citraconic anhydride}$ and mixed for 45 min.

2.9. Trypsinization

After citraconylation the pH of the solution was adjusted to 8.8 with 2 M HCl and diluted so that the conductivity was below 5 mS. Then 70 µl of a 1% trypsin solution was added and the solution was mixed for about 16–18 h at room temperature. The amount of trypsin was calculated according to the formula: $A280 \text{ nm} \times V \text{ solution (dm}^3\text{)}/75$. The reaction was inhibited with 1 mg/ml aprotinin. The amount of added aprotinin was calculated from the formula: $V \text{ trypsin}/21$.

2.10. Low-pressure chromatography on DEAE Sepharose

A protein sample was applied to a column packed with DEAE Sepharose and equilibrated with 0.5 M Tris pH 8.6, followed by 20 mM Tris pH 8.6, a protein sample was applied. After the application, the column was rinsed with buffers: DW I - 20 mM Tris pH 8.6 (conductivity 6 mS) and DW II - 20 mM Tris pH 8.6 + 20% isopropanol (conductivity 3 mS). Insulin was eluted with DE buffer - 20 mM Tris pH 8.6 + 30% isopropanol (conductivity 6 mS).

2.11. Decitraconylation

The main fraction eluted from the column was diluted 2 times with H₂O and the pH was lowered to 2.9 with 2 M HCl. The sample was stirred for 1 h and left at 4 °C overnight.

2.12. Precipitation of insulin

1 M zinc chloride solution was added to the sample and the pH was adjusted to 5.9. The solution was stirred for 1 h. The amount of added zinc chloride was calculated according to the formula: $2 \times V \text{ cm}^3 \text{ solution}/100$. Then the sample was centrifuged at $18,400 \times g$ for 15 min at 4 °C. The precipitate can be stored at 4 °C for up to 30 days.

2.13. Dissolving the zinc salt

The precipitate of insulin was suspended in water. The volume was calculated from the formula: total amount of protein obtained after DEAE/7. Then 1 M Tris pH 8.6 was added to a concentration of 30 mM and 0.2 M EDTA (the amount calculated from the formula: total amount of protein obtained after DEAE/190). Conductivity of the sample was below 3 mS.

2.14. Low-pressure chromatography on Q Sepharose

The protein sample was applied to a column packed with Q Sepharose and equilibrated with 0.5 M Tris pH 8.6, followed by 20 mM Tris pH 8.6. After the application, the column was rinsed with buffers: QW - 20 mM Tris pH 8.6 + 20% isopropanol (conductivity 0.5 mS). Proteins were eluted with QE1 buffer - 20 mM Tris pH 8.6 + 30% isopropanol (conductivity 0.42 mS) and QE2 buffer - 20 mM Tris pH 8.6 + 30% isopropanol (conductivity 3 mS).

2.15. Reaction with carboxypeptidase B

The main fraction eluted from the column was diluted 2 times with H₂O and 50 µl of carboxypeptidase B (2.5 mg/ml) was added (10 µl for every 150 AU of total protein determined in the main fraction). The pH of the sample was adjusted to 8.8. The reaction was carried out for

16–18 h at room temperature.

2.16. RP-HPLC chromatography

The separation was carried out on an ACE 5C18-300 column using an acetonitrile gradient. Mobile phase A: 2000 ml of 0.2 M sodium sulphate solution pH 2.3 preheated in warm water to 25 °C - 30 °C with 440 ml of acetonitrile, pH 2.3. Mobile phase B: 1250 ml of 0.2 M sodium sulphate solution pH 2.3 preheated in warm water to 25 °C - 30 °C with 1250 ml acetonitrile, pH 2.3. The main fraction eluted from the column was diluted 3 times with H₂O and then the insulin was precipitated with zinc chloride.

2.17. Sephadex G-25 chromatography

The precipitate of insulin was suspended in water and the pH was adjusted to 3.0. To exchange the sample buffer, the protein sample was applied on a column packed with Sephadex G-25 equilibrated with 5 mM ammonium acetate pH 4.0. Insulin was eluted with the same buffer.

2.18. Protein concentration measurement

The protein concentration was measured at a wavelength of 280 nm. The spectrophotometer was calibrated with water.

3. UHPLC chromatography tandem MALDI-TOF/TOF mass spectrometry

3.1. UHPLC chromatography

Sample separation was performed using Waters Acquity UHPLC System with Empower 3 Software, equipped with an ACQUITY UPLC[®] CSH[™] C18 1.7 µm, $2.1 \times 50 \text{ mm}$ column. The analyses were carried out at 23 °C at a flow rate 0.713 ml/min and spectrophotometric detection at 220 nm. Injection volume was 2 µl. A gradient of two buffers A1 and B1 was employed. Eluent A1 was 9.66 g NaH₂PO₄ × H₂O and 24.58 g NaClO₄ × H₂O in 1000 ml of water, pH 2.5. Eluent B1 was 9.66 g NaH₂PO₄ × H₂O in 1000 ml of water, pH 2.5, mixed with acetonitrile 1:1 (v/v).

3.2. MALDI-TOF/TOF mass spectrometry

Mass spectra were acquired using a 4800 MALDI TOF/TOF analyzer (Applied Biosystems). The instrument was operated in positive ion reflector mode. 0.5 µl of sample was spotted onto a 384 Opti-TOF MALDI plate and mixed with 0.5 µl of a saturated solution of alpha-cyano-4-hydroxycinnamic acid (CHCA) matrix from Sigma-Aldrich, dissolved in 50:50 water/acetonitrile with 0.1% TFA – final concentration (Sigma-Aldrich). Calibration was achieved with a 4700 proteomics analyzer calibration mixture provided by Applied Biosystems. Data Explorer Software, Version 4.9, was applied to process the acquired spectra.

The whole process of producing recombinant insulin is illustrated in Fig. 1.

4. Results and discussion

4.1. Genetic construction of pIBAINS plasmid

The human insulin gene was composed according to the following formula: L-B-X-A. In the formula, L is a leader peptide represented by the modified SOD gene, B is human insulin chain B, X is a short peptide Lys-Arg connecting chain B with chain A, A is human insulin chain A. This is described in our patent [12]. The plasmid pIBAINS, size 4354 bp, is made up of the following regulatory sequences and genes: from 7 bp to 942 bp a regulatory region with the deoP1P2 promoters is located,

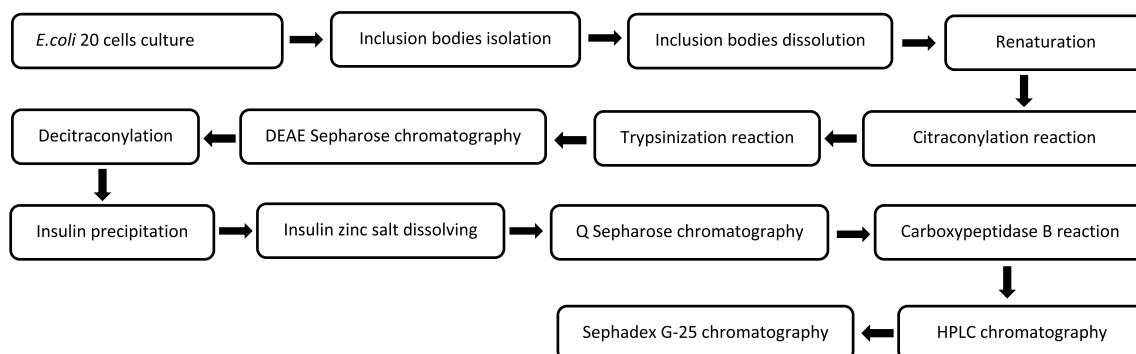


Fig. 1. The block diagram of the process for obtaining recombinant human insulin from *E. coli* 20 cells.

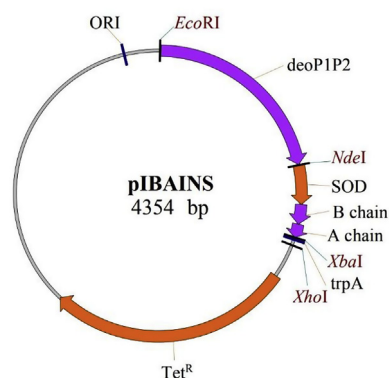


Fig. 2. The construction scheme for the pIBAINS plasmid. The pIBAINS plasmid contains a hybrid protein SOD:INS gene under the control of the *deoP1P2* promoter. Abbreviations used: *deoP1P2* - *deoP1P2* promoter; *Tet^R* - tetracycline resistant; *trpA* - transcription terminator *trpA*; SOD - superoxide dismutase gene; A chain, B chain - chains of the human insulin gene; ORI - replication origin.

from 946 bp to 1137 bp, there is a sequence encoding a fragment of the human SOD gene, from 1138 bp to 1299 bp the sequence encoding the A, B chains and C chain (in the form of the Lys-Arg linker) of recombinant human insulin INS is included (the nucleotide sequence of insulin has been changed according to the frequency of codons in *E. coli*), from 1312 bp to 1338 bp, the coding region of the *trpA* transcription terminator is located, from 1508 bp to 2698 bp, the tetracycline resistance gene *Tet* is located. The structure of the pIBAINS plasmid containing the gene coding for the recombinant human insulin protein (insulin INS) is shown schematically in Fig. 2.

4.2. Cell culture

As a result of *E. coli* 20 cell culture in a fermenter (described in Material & Methods) we obtained about 224 g of wet biomass (56 g from 1 L of the culture media). An example of the growth curve is shown in Fig. 3. Maximum OD 600 nm was 54.4.

4.3. Isolation of inclusion bodies

After isolation performed according to the protocol described in Material & Methods, we obtained about 58 g of inclusion bodies (14.5 g from 1 L of the culture media).

4.4. Dissolution of inclusion bodies

An aliquot of 17.9 g of inclusion bodies was dissolved as described in Material & Methods and we obtained 5460 AU of protein.

4.5. Renaturation

In the case of renaturation at room temperature, the yield was around 95–96% and at 7–8 °C the yield was around 99%. We obtained 5450 AU of protein. The refolding process was completed after 18–19 h and was shown to depend on the redox potential and the pH value, but not on the temperature.

4.6. Citraconylation reaction and trypsinization

Citraconic anhydride allowed for the reversible blocking of Lys/Arg amino acid residues to carry out controlled tryptic digestion. Correctness of citraconylation and trypsinization reactions was checked by mass spectrometry.

4.7. Low-pressure chromatography on DEAE Sepharose

Immediately after reaction with trypsin the protein was applied to a column filled with DEAE Sepharose. As a result, 250 ml of the insulin fraction was obtained. The protein concentration was 8.57 AU/ml. A chromatogram from DEAE Sepharose Fast Flow chromatography of insulin is shown in Fig. 4.

4.8. Decitraconylation

The main fraction eluted from the column was diluted 2 times with H₂O and the pH was lowered to 2.9 with 2 M HCl. Citraconic anhydride (2-methylmaleic anhydride) is used to block primary amine groups at alkaline pH values (pH 7–9). At acidic pH (3–4), the amide linkage is hydrolyzed and citraconic acid is released, yielding the original amine.

4.9. Precipitation of insulin and dissolving the zinc salt

10 ml of 1 M zinc chloride solution was added to the sample and the pH was adjusted to 5.9. The solution was stirred for 1 h. After centrifugation (as described in Material & Methods) the precipitate of insulin was suspended in 285 ml of water. Then 9.2 ml of 1 M Tris pH 8.6 was added to a concentration of 30 mM and 11.3 ml of 0.2 M EDTA.

4.10. Low-pressure chromatography on Q Sepharose

Immediately after dissolving the zinc salt the protein was applied to a column filled with Q Sepharose. As a result, 226 ml of the insulin fraction was obtained. The protein concentration was 2.77 AU/ml. Chromatogram from Q Sepharose Fast Flow chromatography of insulin is shown in Fig. 5.

4.11. Reaction with carboxypeptidase B and RP-HPLC chromatography

Carboxypeptidase B catalyzed the hydrolysis of lysine and arginine

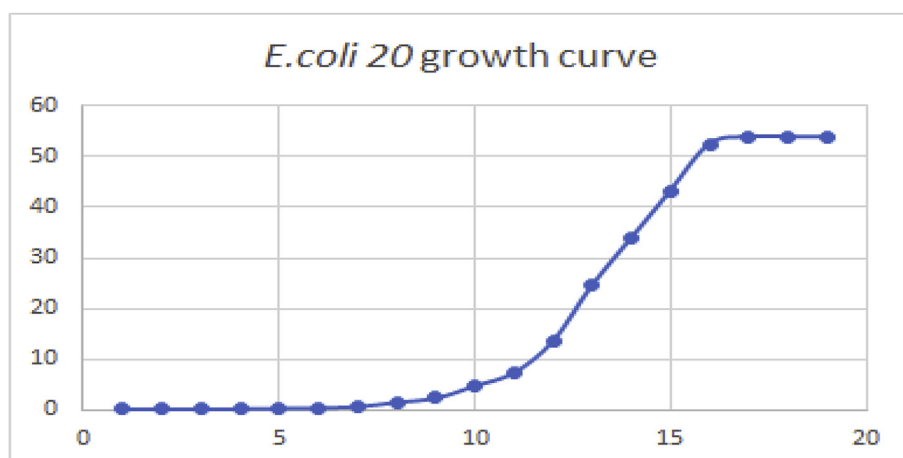


Fig. 3. *Escherichia coli* 20 growth curve. X-axis values are hours of culture, y-axis values are optical density (OD) 600 nm.

Chromatogram

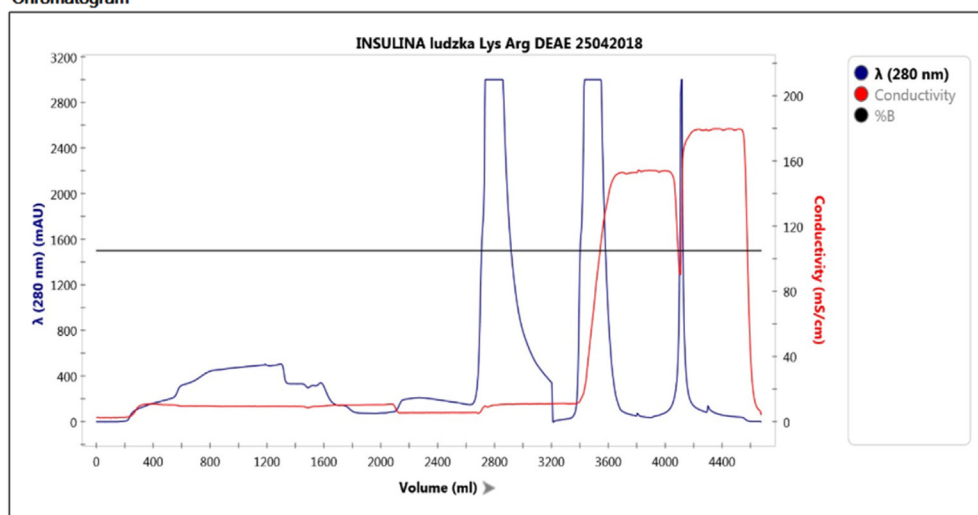


Fig. 4. Chromatogram from DEAE Sepharose Fast Flow chromatography of insulin. The first step was 3 CV of 100% DW I and 3 CV of 100% DW II to elute unbound proteins. The second step was 2 CV of 100% DE to elute the protein of interest. The insulin peak is from about 2650 to 3200 ml (x-axis). The next step was 2 CV of 1 M NaCl and then 0.5 M NaOH to elute all proteins from the column. Buffer DW I: 20 mM Tris pH 8.6 (conductivity 6 mS), buffer DW II: 20 mM Tris pH 8.6 + 20% isopropanol (conductivity 3 mS), buffer DE: 20 mM Tris pH 8.6 + 30% isopropanol (conductivity 6 mS), flow: 15 ml/min.

Chromatogram

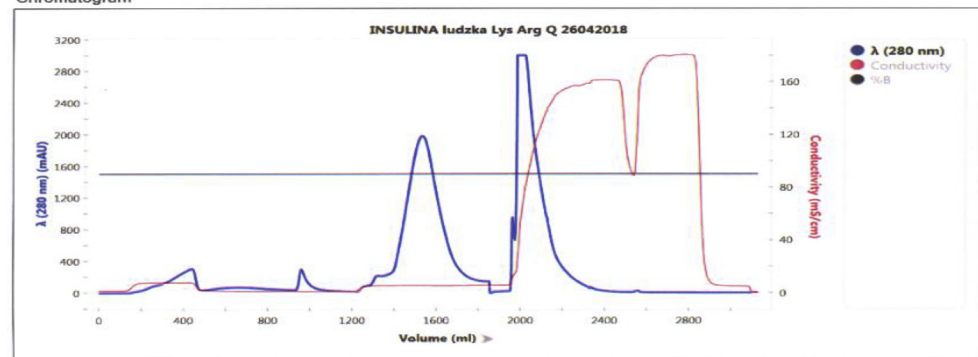


Fig. 5. Chromatogram from Q Sepharose Fast Flow chromatography of insulin. The first step was 2 CV of 100% QW to elute unbound proteins. The second step was 2 CV of 100% QE I to elute weakly bound proteins and 2 CV of 100% QE II to elute the protein of interest. The insulin peak is from about 1400 to 1800 ml (x-axis). The next step was 1 CV of 1 M NaCl and then 0.5 M NaOH to elute all proteins from the column. Buffer QW: 20 mM Tris pH 8.6 + 20% isopropanol (conductivity 0.5 mS), buffer QE I: 20 mM Tris pH 8.6 + 30% isopropanol (conductivity 0.42 mS), buffer QE II: 20 mM Tris pH 8.6 + 30% isopropanol (conductivity 3 mS), flow: 10 ml/min.

(a short peptide Lys-Arg connecting chain B with chain A). After reaction with carboxypeptidase B described in Material & Methods, the sample was applied to a RP-HPLC ACE 5C18-300 column. As a result, 97 ml of the insulin fraction was obtained. The protein concentration was 2.42 AU/ml. A chromatogram from RP-HPLC chromatography of insulin is shown in Fig. 6.

4.12. Sephadex G-25 chromatography

To exchange the sample buffer and get rid of residual acetonitrile, the protein sample was applied to a column packed with Sephadex G-25 equilibrated with 5 mM ammonium acetate pH 4.0. As a result, 31 ml of the insulin fraction was obtained. The protein concentration was 3.59 AU/ml.

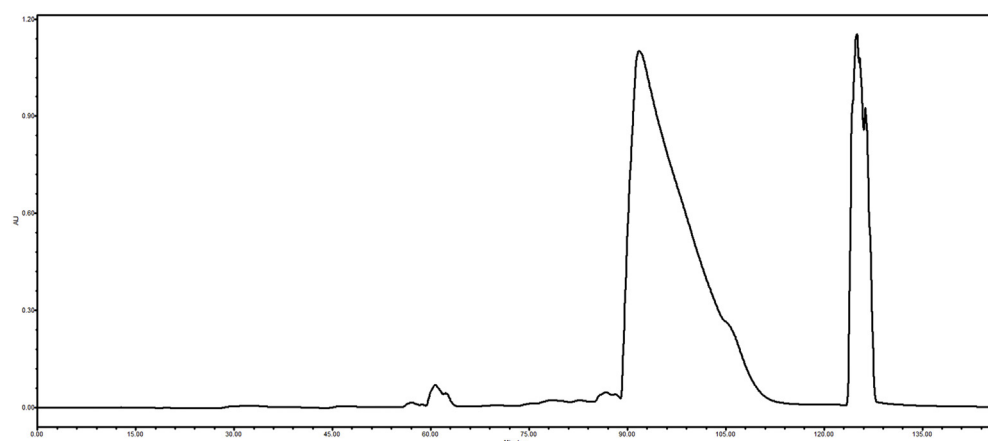


Fig. 6. Chromatogram from RP-HPLC. The insulin was purified under isocratic conditions - mobile phase A: 2000 ml of 0.2 M sodium sulphate solution pH 2.3 preheated in warm water to 25 °C - 30 °C with 440 ml of acetonitrile, pH 2.3. Mobile phase B: 1250 ml of 0.2 M sodium sulphate solution pH 2.3 preheated in warm water to 25 °C - 30 °C with 1250 ml acetonitrile, pH 2.3. Detector wavelength: 214 nm. The insulin peak is from about 90 to 105 min (x-axis).

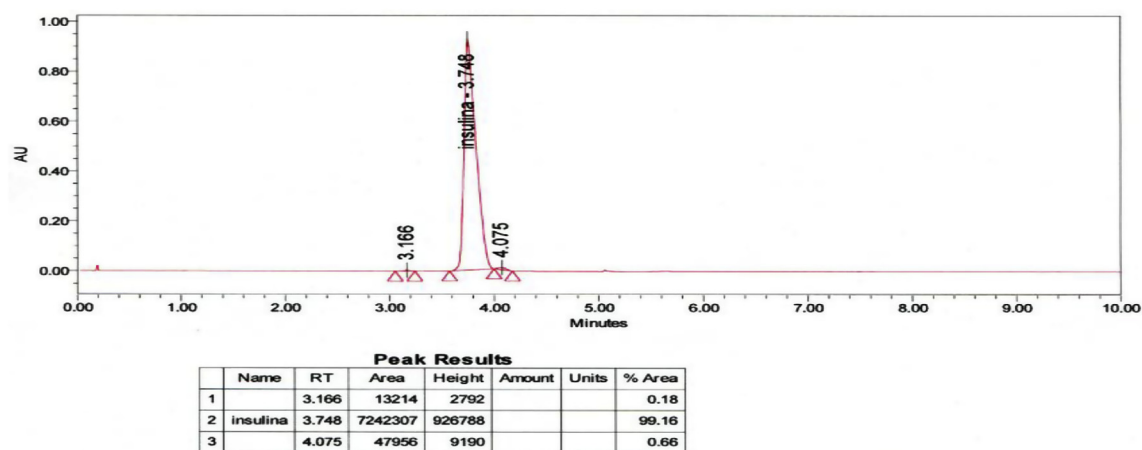


Fig. 7. UHPLC analysis of purified recombinant human insulin. RT value of insulin is 3.748 min. Protein purity is about 99%.

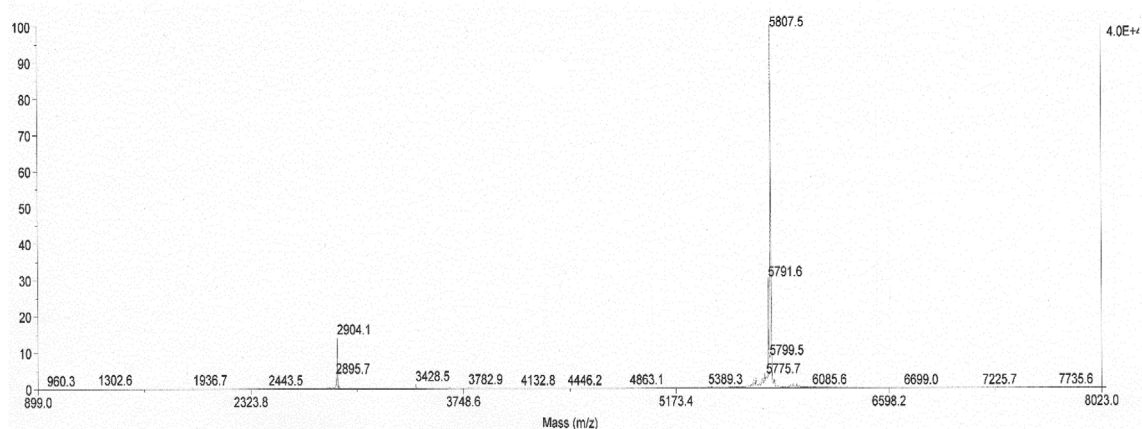


Fig. 8. Mass spectrum of recombinant human insulin. The molecular weight obtained for insulin by the ESI-MS method was 5807.5 Da. Theoretical calculated mass is 5807.63 Da.

4.13. Purification summary

At the end of the whole process we obtained on the average 2700 IU of up to 99% purity recombinant human insulin from 1L of the culture media by a three-step purification procedure consisting of DEAE/Q Sepharose and RP-HPLC chromatography. The molecular weight obtained for insulin by the ESI-MS method was 5807.5 Da. Theoretical calculated average mass is 5807.63 Da. Examples of UHPLC and mass spectrometry results are shown in Fig. 7 and Fig. 8.

5. Conclusions

Diabetes belongs to so called “lifestyle” or “civilization diseases” – it is a major threat to the human health and well-being globally. Up to now, the most effective method to treat this disease in the case of hyperglycemia is still insulin delivery. Thus, it is meaningful to establish an effective and economical protocol for insulin production. To conclude, we reported here the construction of a new pIBAINS expression vector and the establishment of a new bacterial host strain *E. coli* 20, which is able to produce human insulin with high efficiency. The

plasmid does not integrate into the genome and remains stable for at least 80 generations without antibiotic selection. We described the whole process from cell culture through inclusion body isolation and dissolution to protein purification. In the case of renaturation at room temperature, the yield was around 95–96% and at 7–8 °C temperature the yield was around 99%. The refolding process was completed after 18–19 h and was shown to depend on the redox potential and the pH value, but not on the temperature. As a result of bacterial culture we obtained an average of 56 g wet biomass and 14.5 g inclusion bodies, while at the end of the whole process on the average 2700 IU of up to 99% purity recombinant human insulin from 1 L of the culture media by a three-step purification procedure consisting of DEAE/Q Sepharose and RP-HPLC chromatography, which is a comparable result to other expression systems [13]. Benefits of using our *E. coli* 20 strain is that ownership of a patented strain allows the invention to be commercialized on the pharmaceutical industry market. According to the MFD estimates, the percentage of diabetes patients in the adult Polish population will increase to around 11% over the next 20 years. This population may in the future be a recipient of the results of our project.

References

- [1] M. Bliss, *The Discovery of Insulin*, University of Chicago Press, Chicago, 1982 155 p.
- [2] <https://www.idf.org>.
- [3] W. Landgraf, J. Sandow, Recombinant human insulins – clinical efficacy and safety in diabetes therapy, *Eur. Endocrinol.* 12 (1) (2016) 12–17.
- [4] P. Wang, N.M. Fiaschi-Taesch, R.C. Vasavada, D.K. Scott, A. García-Ocaña, A.F. Stewart, Diabetes mellitus-advances and challenges in human β -cell proliferation, *Nat. Rev. Endocrinol.* 11 (4) (2015) 201–212.
- [5] R. Hilgenfeld, G. Seipke, H. Berchtold, D.R. Owens, The evolution of insulin glargine and its continuing contribution to diabetes care, *Drugs* 74 (8) (2014) 911–927.
- [6] Y.S. Cheng, D.Y. Kwok, T.J. Kwok, B.C. Soltvedt, D. Zipser, Stabilization of a degradable protein by its overexpression in *Escherichia coli*, *Gene* 14 (1–2) (1981) 121–130.
- [7] Y.E. Cheng, Density separation of protein overproducing bacteria, United States patent US 4, 480, 038, 1984.
- [8] J.H. Miller, *Experiments in Molecular Genetics*, Cold Spring Harbor N. Y., Cold Spring Harbor Laboratory Press, 1972 466 p.
- [9] Hartman J.R., Mendelovitz S., Gorecki M.: Sposób Wytwarzania Insuliny Ludzkiej, Oraz Polipeptyd Hybrydowy. BIO-TECHNOLOGY GENERAL CORR, Iselin (US), Patent PL 180818.
- [10] R.A. Hallewell, F.R. Masiarz, R.C. Najarian, J.P. Puma, M.R. Quiroga, A. Randolph, et al., Human Cu/Zn superoxide dismutase cDNA: isolation of clones synthesizing high levels of active or inactive enzyme from an expression library, *Nucleic Acids Res.* 13 (6) (1985) 2017–2034.
- [11] J.R. Hartman, S. Mendelovitz, M. Gorecki, Generation of Human Insulin, International publication number: Worldwide International Property Organization WO96/20724 A1.
- [12] P. Borowicz, A. Plucienniczak, J. Mikołajczyk, T. Głąbski, D. Kurzynoga, D. Mikiewicz-Syguła, et al. New insulin analogs of prolonged activity. European Patent Application EP 2321344A2, 2009.
- [13] N.A. Baeshen, M.N. Baeshen, A. Sheikh, et al., Cell factories for insulin production, *Microb. Cell Factories* 13 (2014) 141.