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(54) Titre : PROCESSUS D'ISOLEMENT DE L'INSULINE PAR CHROMATOGRAPHIE LIQUIDE HAUTE PRESSION
(54) Title: A PROCESS FOR ISOLATING INSULIN BY HIGH-PRESSURE LIQUID CHROMATOGRAPHY

(57) Abrégé/Abstract:
A process for isolating insulin is described and entails carrying out the separation with pressure-stable acidic cation exchange material under a pressure of from 1.1 MPa to 40 MPa.

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Abstract

A process for isolating insulin by high-pressure liquid chromatography

A process for isolating insulin is described and entails carrying out the separation with pressure-stable acidic cation exchange material under a pressure of from 1.1 MPa to 40 MPa.

Description

A process for isolating insulin by high-pressure liquid chromatography

In the preparation of recombinant insulins, the microbial biosynthesis of the insulin takes place in bacteria such as transformed Escherichia coli via a single-chain precursor molecule which, besides the natural insulin sequence of the A and B chains, comprises a connecting peptide and a fusion protein sequence. The latter is connected to the N-terminal end of the complete protein for genetic engineering reasons, and is responsible for the expressed insulin-containing product resulting in the form of inclusion bodies in the transformed E. coli. The aim of the working up is to make the insulin available from this fusion protein in multistage protein-chemical process steps. This entails in every case, just like the natural biosynthesis of insulin in the beta cell of the pancreas, passing through the stage of folding to (pre)proinsulin. The (pre)proinsulin is converted by enzymatic cleavage (for example with trypsin) into a cleavage mixture which comprises another insulin precursor, which is di-Arg-(B31-32)-insulin, mono-Arg-B31-insulin and the connecting peptide (up to 35 amino acid residues). Also produced are byproducts which are generated by the protease activity of the trypsin, such as incomplete intermediates, de-Thr-B30-insulin or else Arg-A0-insulin and preinsulin. US 5 101 013 discloses the separation of said mixtures of insulin and insulin derivatives by atmospheric pressure or medium pressure chromatography on strongly acidic ion exchangers such as S-Sepharose®, Fraktogel®TSK or SP Trisacryl®. The known chromatography materials are not pressure-stable and become compressed in the chromatography columns under a pressure of above 1 MPa, and separation of the insulin-containing mixtures is then no longer possible.

Another known process for isolating insulin is high-pressure liquid chromatography on lipophilically modified silica gel (US 5 245 008, EP 0 547 544).

Cationic exchange purification processes using atmospheric pressure do not

achieve, even with optimized traditional gel materials, the separation efficiencies necessary to attain the required degree of purification for the recombinant insulin. Chromatography with modern preparative gel materials, for example Poros 50 µm/Perseptiv, Source 30 µm/Pharmacia or Makropep 50 µm/BioRad, is carried out in a medium pressure process. Medium pressure chromatography with gels of smaller particle size (for example Source 15 µm) affords only slight selectivity improvements in respect of the separation efficiency, so that in this case too the last purification stage inevitably remains high-pressure liquid chromatography (HPLC) to eliminate extremely small impurities in the insulins. The disadvantages of reverse phase HPLC, such as risk of denaturation of the protein, bleeding of the RP silica gel phase and unsatisfactory cleaning in place measures, must be accepted for this and require above-average expenditure of money and time.

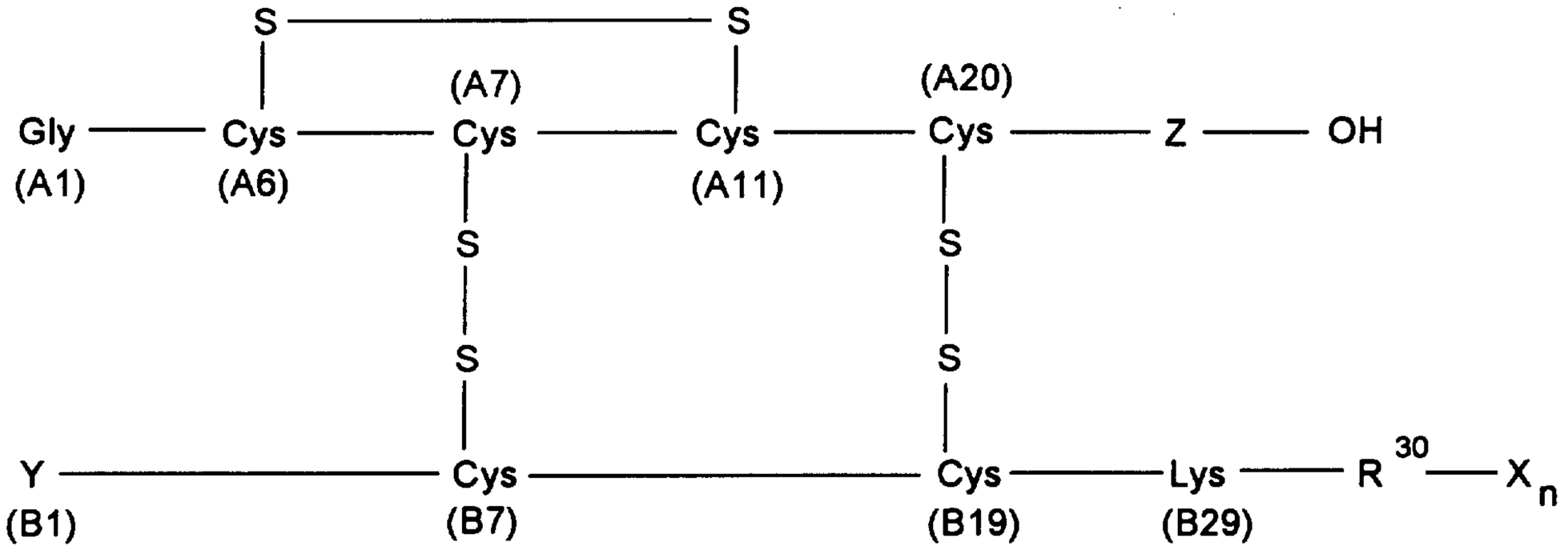
In the endeavor to provide improved separation and isolation processes for obtaining insulin from enzymatic cleavage reactions, it has now been found that the same can be achieved by chromatography of the insulin and insulin derivative mixtures of pressure-stable acidic cation exchangers under a pressure of from 1.1 MPa to 40 MPa. The insulin isolated in this way is suitable for direct use, without further purification steps, in injection solutions for treating diabetes mellitus.

The invention therefore relates to a process for isolating insulin by chromatography, wherein the separation is carried out with pressure-stable acidic cation exchange materials under a pressure of from 1.1 MPa to 40 MPa.

The term insulin means compounds which are of animal or human origin, for example human insulin or porcine insulin, insulin precursors such as proinsulins or preinsulins, or recombinant insulins or insulin derivatives expressed by genetically modified microorganisms. Insulins can also be modified by chemical or enzymatic derivatization, for example de-Phe-B1-insulin, diarginine-insulin (B31, B32), monoarginine-insulin, diphenylalanine-insulin (B31,B32) (US 4 601 852), or Gly^{A21}-Arg^{B31}-Arg^{B32}-human insulin (EP 368 187).

The insulins preferably employed in the process according to the invention have the

formula I



R^{30} is the residue of a genetically encodable L-amino acid,

X is a hydroxyl group, a genetically encodable L-amino acid residue,

n is an integer from 0 to 10,

Y is hydrogen atom or L-phenylalanine residue, and

Z is a genetically encodable L-amino acid residue, and

residues A2-A20 correspond to the amino acid sequence of the A chain of human insulin, animal insulin or an insulin derivative and residues B2-B29 correspond to the amino acid sequence of the B chain of human insulin, animal insulin or an insulin derivative.

Preferred insulins have the formula I where

R^{30} is a residue from the group of L-alanine and L-threonine,

X is an L-amino acid residue from the group of L-arginine, L-lysine and L-phenylalanine,

n is an integer from 0 to 6,

Z is a residue from the group of glycine, L-alanine, L-serine, L-threonine, L-aspartic acid and L-glutamic acid, and

A1 to A20 or B2 to B29 represent the amino acid sequence of human, porcine or bovine insulin.

It is particularly preferred to isolate human insulin and Gly^{A21}-Arg^{B31}-Arg^{B32}-human insulin.

The amino acid sequence A1 to A20 of human insulin is:

Gly Ile Val Glu Gln Cys Cys Thr Ser Ile Cys Ser

Leu Tyr Gln Leu Glu Asn Tyr Cys (SEQ ID NO: 1)

The amino acid sequence B1 to B29 of human insulin is:

Phe Val Asn Gln His Leu Cys Gly Ser His Leu

Val Glu Ala Leu Tyr Leu Val Cys Gly Glu Arg

Gly Phe Phe Tyr Thr Pro Lys (SEQ ID NO: 2)

Insulin can be employed both in the relatively contaminated state and in prepurified form (for example by gel chromatography). Insulin is still contaminated, after repeated crystallization and even after gel chromatography, with insulin-like concomitant substances which are very similar in molecular weight and which, at a suitably chosen pH, differ in their charge state from one another and from insulin but form complexes with insulin (US 4 129 560). Examples of such substances are:

Deamidoinsulin, arginine- and diarginine-insulin and insulin ethyl ester.

The term pressure-stable acidic cation exchange materials means, for example, materials such as a copolymer of polystyrene and divinylbenzene, which are modified with sulfo groups, in particular with R-O-CH_2-CHOH-CH_2-O-CH_2-CHOH-CH_2-SO_3^- groups. The following products are particularly preferred.

- Source®30S supplied by Pharmacia Biotech AB, Uppsala, Sweden, pressure-stable, spherical and porous materials with a particle diameter of about 30 µm (Downstream, No 19, (1995), Pharmacia Biotech AB, S-751 82 Uppsala, Sweden, pages 3-8)

- Source®15S supplied by Pharmacia Biotech AB, Uppsala, Sweden, pressure-stable, spherical and porous materials with a particle diameter averaging 15 µm

- Poros 50 µm, supplied by Perseptiv

- Makroprep 50 µm, supplied by Biorad

The eluents contain a buffer substance which keeps the pH of the eluent constant, water and organic solvents. Suitable buffer substances are disclosed in the literature, for example phosphates, alkali metal or alkaline earth metal salts such as potassium acetate, ammonium citrate, sodium citrate, acetate, sulfate or chloride. The eluents furthermore contain water-miscible organic solvents such as alcohols, ketones, methyl acetate, dioxane or acetonitrile. (C_1-C_4) alcohols such as n- or iso-propanol, methanol, ethanol or butanol are preferably employed.

The concentration of the water-miscible organic solvents for the chromatography is 10 to 50% by volume, preferably 20 to 40% by volume, particularly preferably 25 to 35% by volume. The concentration of the buffer substance is about 1 mmol/l to 140 mmol/l, based on water as solvent, preferably 2 mmol/l to 120 mmol/l. Further additives which can be added to the buffer solution are, for example, salt, preferably physiologically tolerated mineral salt, one or more organic acids such as formic acid, acetic acid, lactic acid or citric acid, preferably lactic acid, a base, preferably NaOH, and/or preservatives. The preferred pH of the buffer solution is about 2.5 to 5.5, particularly preferably from about 3.5 to 4.0. The concentration of the organic acid may vary within a wide range. Advantageous amounts are from 10 to 100 mmol/l, based on water as solvent, preferably from 25 to 50 mmol/l.

The temperature during the chromatography is 0°C to 50°C, preferably 15 to 30°C, particularly preferably 15 to 20°C. The operating pressure during the chromatography is substantially constant. The chromatography can be carried out using different pressures, for example the chromatography can be carried out under a pressure of from 1.1 to 40 MPa, in particular under 1.5 to 10 MPa. The eluent flow rates are 200 to 1000 cm/h, maximum 2000 cm/h.

The loading of the columns, chromatography and elution of the insulins and insulin derivatives take place by known, conventional technical methods. The loading of the column with the insulin solution to be purified preferably takes place using aqueous/alcoholic or purely aqueous buffer solution. The insulin solution has a

protein content of about 1 to 10%, preferably 3%.

The loading of the pressure-stable acidic cation exchanger can take place, for example, by dissolving the insulin mixture in a buffer solution - preferably having the composition described previously and having the pH described previously - and bringing the resulting solution into contact with the pressure-stable acidic cation exchanger.

The elution solution, which can in principle have a composition similar to that of the buffer solution described previously, preferably has a pH of from 3.5 to 4.0. A particularly suitable elution process is one in which the elution solution displays a time gradient of the salt concentration, preferably with a linear course. This concentration gradient can be applied, for example, by a low salt concentration (zero in the limiting case) being present in the elution solution at the start of the elution, and by increasing the salt concentration during the elution process. It is possible in this way to achieve a particularly effective separation of the protein mixture. A preferred salt concentration gradient varies from near 0 mol of salt/l (at the start of the elution) to about 0.8 mol of salt/l (at the end of the elution), particular preferably from about 0.10 (at the start of the elution) to about 0.25 mol/l (at the end of the elution). Suitable as added salt are many organic and inorganic salts. Physiologically tolerated salts such as ammonium and alkali metal salts are preferred, particularly preferably sodium salts, in particular sodium chloride.

The separation process according to the invention takes place in a column process. The temperature, which is preferably kept constant during the ion exchange chromatography, may be varied within a wide range. A preferred temperature range is from about -10°C to about 50°C, in particular from about 15 to about 25°C.

Concentrating the insulin after the chromatography from the eluates takes place by precipitation with zinc salt or by crystallization. It is moreover possible optionally for the solvent to have previously been substantially removed from the solution by distillation under reduced pressure, or for its concentration to have been reduced by dilution with water. In any event, the solvent concentration should be 10% or less

before the precipitation or crystallization, in order to keep the protein content in the supernatant at < 50 mg/l. The resulting insulin precipitates can be isolated by decantation, centrifugation or filtration and be dried. The process according to the invention is suitable not only for analytical chromatography but also for preparative chromatography, in particular when the process according to the invention is carried out with a preparative high-pressure liquid chromatography (HPLC) system.

The term "preparative chromatography" means a purification process with the aim of isolating, and not merely analyzing, pure products in each chromatography run. The amount of pure products may vary within wide limits, for example from 1 mg to 5.0 kg, preferably from 50 mg to 2.5 kg.

The process according to the invention is described in detail in the following examples. Percentage data are based on volume unless indicated otherwise.

Example 1

Buffer A: 30% n-propanol, 50 mM lactic acid, 0.01 M NaCl in water, pH 3.5

Sorbent: Source® S15 µm

Column dimensions: 5 cm x 25 cm.

A preparative HPLC column (5cmx25cm, column volume approximately 500 ml) is packed with a suspension of Source S 15 µm in 50% aqueous ethanol and equilibrated with Buffer A. For this purpose, the buffer is pumped onto the column at a flowrate of 98 ml/minute. A pressure of 1.9 MPa builds up during this. The column is in this case part of a chromatography system with a fractionation collector and UV detector (254/280 nm).

5 g of the crude recombinant human insulin to be purified are dissolved in 500 ml of

Buffer A and pumped onto the column at the same flowrate as above. Renewed equilibration is carried out with 1000 ml of Buffer A during a subsequent washing period.

For elution, a linear NaCl gradient composed of Buffer A and Buffer B (Buffer B = Buffer A + 0.15 M NaCl) is pumped via a gradient mixing system onto the low pressure side of the column, and a UV elution diagram is obtained. The eluate is fractionated, and the individual fractions are checked by a conventional analytical HPLC method. Fractions which correspond to the required purity are combined. After dilution with water (1 vol. of eluate + 2 vol. of water), the highly purified insulin is isolated by crystallization as Zn^{2+}insulin by known processes.
Yield: 3.8 g; purity > 98%

HPLC analysis

12.5 mg of protein containing insulin are dissolved in 25 ml of eluent C (see eluents). 0.02 ml is loaded onto a high-pressure liquid chromatography column.

Column: ET 250/8/4 ®Nucleosil 300-5 C_{18} (Macherey & Nagel, Aachen, Germany)

Eluents: stock solution: 41.4 g of sodium dihydrogen phosphate * H_2O
1800 ml of double-distilled water
adjust to pH 2.5 with 85% phosphoric acid and make up to 2000 ml with double-distilled water

Eluent C: 500 ml of stock solution
500 ml of acetonitrile
1000 ml of double-distilled water

Eluent D: 500 ml of stock solution
1300 ml of acetonitrile
200 ml of double-distilled water
6.4 g of sodium chloride

Gradient:

Time	% C	% D
0 min	96	4
6 min	91	9
15 min	91	9
25 min	85	15
30 min	65	35
32 min	10	90
35 min	96	4
45 min	96	4

The slope of the gradient should be adjusted so that the main peak of the insulin is eluted after 17 to 21 min.

Temperature: 40°C

Total running time: 45 min,

Flow rate: 1 ml/min

Detection: 210 nm

Example 2

5 g of a mixture of de-Thr^{B30}-human insulin (insulin from Seq ID No. 1 and No. 3 with correct cystine bridges), Arg-B31-human insulin (insulin from Seq ID No. 1 and No. 5 with correct cystine bridges), and Gly^{A21}-Arg^{B31}-Arg^{B32}-human insulin (insulin from Seq ID No. 6 and No. 4 with correct cystine bridges) are purified as in Example 1. The mixture is typically produced in the isolation of recombinant insulin. Said mixture furthermore contains, because of non-specific enzymatic cleavages, very small amounts of other impurities whose concentration must be reduced to give the medicinal substance ready for use.

The various components are eluted as in Example 1. The UV diagram shows a selective separation of the individual components according to increasing isoelectric

point with increasing NaCl gradient (de-Thr^{B30}-human insulin:I; Arg^{B31}-human insulin: II; Gly^{A21}-Arg^{B31}-Arg^{B32}-human insulin: III). The fractionated column eluate is analyzed as in Example 1, and the required high-purity product Gly^{A21}-Arg^{B31}-Arg^{B32}-human insulin is isolated by crystallization.

Yield: 1.78 g; purity > 98.5%

Example 3

Comparative example

5 g of a mixture as in Example 2 are purified under the conditions of medium pressure chromatography under 1 MPa (10 bar) with an eluent flow rate of 39 ml/min. The UV elution diagram shows a similar separation of the components as in Example 2. However, it is evident merely from the course of this diagram that the sharpness of separation of the individual compounds is less. The HPLC analysis confirms a distinct overlap of the individual insulin impurities. The purified product is isolated by crystallization. Despite a lower yield, the purity does not comply with the required specification of the medicinal substance. Preparation thereof requires a subsequent preparative HPLC purification on reverse phase silica gel.

Yield: 1.5 g of Gly^{A21}-Arg^{B31}-Arg^{B32}-human insulin; purity: 97.6%.

SEQUENCE LISTING

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(ii) TITLE OF APPLICATION: A process for isolating insulin using high-pressure liquid chromatography

(iii) NUMBER OF SEQUENCES: 6

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(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 Amino acids
(B) TYPE: Amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Protein

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Escherichia coli

(ix) FEATURES:
(A) NAME/KEY: Protein
(B) LOCATION: 1..21

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

Gly Ile Val Glu Gln Cys Cys Thr Ser Ile Cys Ser Leu Tyr Gln Leu
1 5 10 15

Glu Asn Tyr Cys Asn
20

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 Amino acids
(B) TYPE: Amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Protein

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Escherichia coli

(ix) FEATURES:
(A) NAME/KEY: Protein
(B) LOCATION: 1..30

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

Phe Val Asn Gln His Leu Cys Gly Ser His Leu Val Glu Ala Leu Tyr
1 5 10 15

Leu Val Cys Gly Glu Arg Gly Phe Phe Tyr Thr Pro Lys Thr
20 25 30

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 Amino acids
(B) TYPE: Amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Protein

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Escherichia coli

(ix) FEATURES:
(A) NAME/KEY: Protein
(B) LOCATION: 1..29

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

Phe Val Asn Gln His Leu Cys Gly Ser His Leu Val Glu Ala Leu
1 5 10 15

Tyr Leu Val Cys Gly Glu Arg Gly Phe Phe Tyr Thr Pro Lys
20 25

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 32 Amino acids
(B) TYPE: Amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Protein

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Escherichia coli

(ix) FEATURES:
(A) NAME/KEY: Protein
(B) LOCATION: 1..32

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

Phe Val Asn Gln His Leu Cys Gly Ser His Leu Val Glu Ala Leu Tyr
1 5 10 15

Leu Val Cys Gly Glu Arg Gly Phe Phe Tyr Thr Pro Lys Thr Arg Arg
20 25 30

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 Amino acids
(B) TYPE: Amino acid
(D) TOPOLOGY: linear

(ii) TYPE OF MOLECULE: Protein

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Escherichia coli

(ix) FEATURES:
(A) NAME/KEY: Protein
(B) LOCATION: 1..31

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

Phe Val Asn Gln His Leu Cys Gly Ser His Leu Val Glu Ala Leu Tyr
1 5 10 15

Leu Val Cys Gly Glu Arg Gly Phe Phe Tyr Thr Pro Lys Thr Arg
20 25 30

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 Amino acids
(B) TYPE: Amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Protein

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Escherichia coli

(ix) FEATURES:
(A) NAME/KEY: Protein
(B) LOCATION: 1..21

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

Gly Ile Val Glu Gln Cys Cys Thr Ser Ile Cys Ser Leu Tyr Gln Leu
1 5 10 15

Glu Asn Tyr Cys Gly
20

THE EMBODIMENTS OF THE INVENTION IN WHICH AN EXCLUSIVE PROPERTY OR PRIVILEGE IS CLAIMED ARE DEFINED AS FOLLOWS:

1. A process for isolating an insulin by chromatography comprising the steps of:

a) obtaining a column comprising pressure-stable acidic cation exchange material;

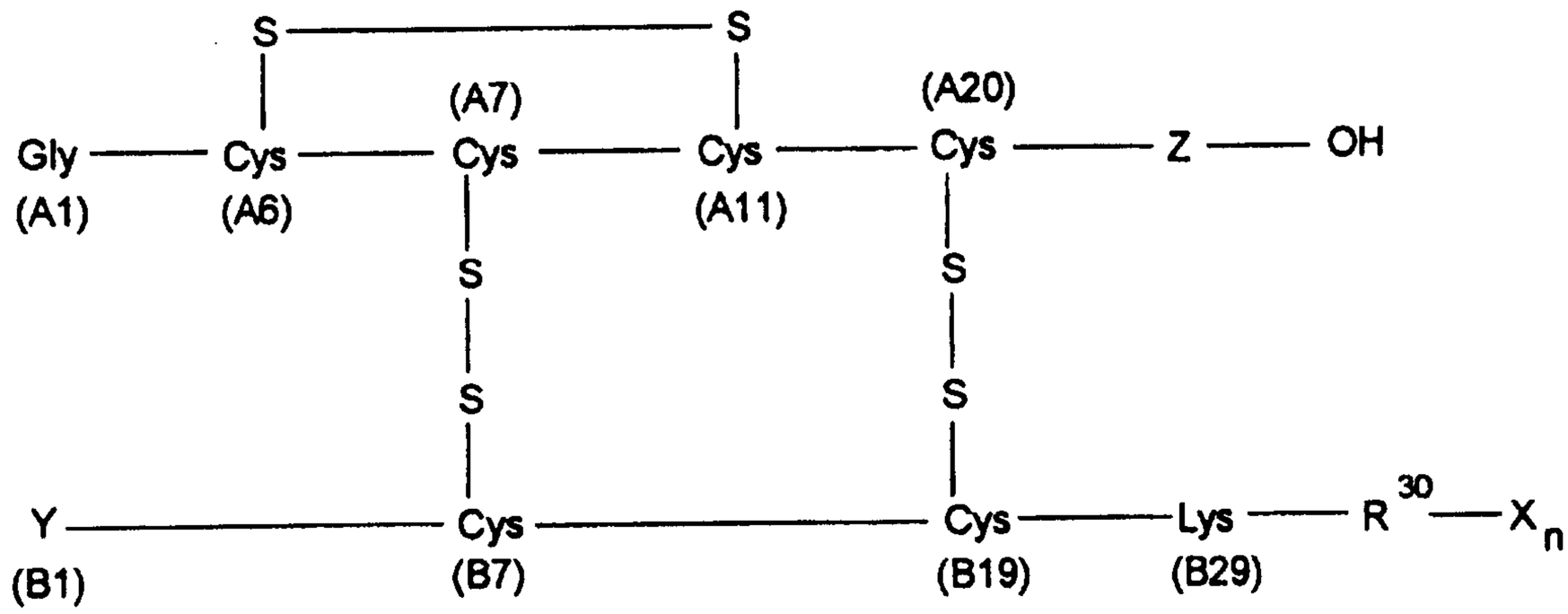
b) loading the column with a sample solution including an insulin;

c) performing chromatography; and

d) eluting the insulin from column with an eluting solution;

said process being performed under pressure of from about 1.1 MPa to about 40 MPa.

2. The process as claimed in claim 1, wherein an insulin of the formula I



where

R^{30} is the residue of a genetically encodable L-amino acid,

X is a hydroxyl group, or a genetically encodable L-amino acid residue,

n is an integer from 0 to 10,

Y is hydrogen atom or L-phenylalanine residue, and

Z is a genetically encodable L-amino acid residue, and

residues A2-A20 correspond to the amino acid sequence of the A chain of human insulin, animal insulin or an insulin derivative and residues B2-B29

correspond to the amino acid sequence of the B chain of human insulin, animal insulin or an insulin derivative, is isolated.

3. The process as claimed in claim 2, wherein an insulin derivative of the formula I where R^{30} is L-alanine or L-threonine, and X is an L-amino acid from the group of L-arginine, L-lysine or L-phenylalanine, Z is glycine, L-alanine, L-serine, L-threonine, L-aspartic acid or L-glutamic acid, n is an integer from zero to 6 and A1 to A20 or B2 to B29 represents the amino acid sequence of human, porcine, or bovine insulin, is isolated.

4. The process as claimed in any one of claims 1 to 3, wherein human insulin is isolated.

5. The process as claimed in any one of claims 1 to 4, wherein the eluting is carried out using an $H_2O/(C_1\text{-}C_4)$ alkanol mixture which comprises from 10 to 50 percent by volume of $(C_1\text{-}C_4)$ alkanol.

6. The process as claimed in any one of claims 1 to 4 wherein the eluting is carried out using an $H_2O/(C_1\text{-}C_4)$ alkanol mixture which comprises from 20 to 40 percent by volume of $(C_1\text{-}C_4)$ alkanol.

7. The process as claimed in any one of claims 1 to 4 wherein the eluting is carried out using an $H_2O/(C_1\text{-}C_4)$ alkanol mixture which comprises from 25 to 35 percent by volume of $(C_1\text{-}C_4)$ alkanol.

8. The process as claimed in any one of claims 1 to 7, wherein a crosslinked polymer of polystyrene and divinylbenzene with sulfo groups is used as pressure-stable acidic cation exchanger.

9. The process as claimed in any one of claims 1 to 8, wherein the pressure-stable acidic cation exchanger is loaded with from 5 to 15 g of protein per liter column volume at a pH of from 2.5 to 5.5.

10. The process as claimed in any one of claims 1 to 8, wherein the pressure-stable acidic cation exchanger is loaded with from 5 to 15 g of protein per liter column volume at a pH of from 3.5 to 4.0.

11. The process as claimed in any one of claims 1 to 10, wherein the $H_2O/(C_1-C_4)$ alkanol mixture used for the eluting comprises ethanol, propanol or isopropanol as (C_1-C_4) alkanol.

12. The process as claimed in claim 11 wherein the $H_2O/(C_1-C_4)$ alkanol mixture used for the eluting comprises propanol.

13. The process as claimed in any one of claims 1 to 12, wherein the pH of the eluting solution is adjusted to 3.5 to 4.0.

14. The process as claimed in any one of claims 1 to 13, wherein the loading and eluting solution comprises a buffer substance.

15. The process according to claim 14 wherein the buffer substance is based on an organic acid.

16. The process according to claim 15 wherein the organic acid is lactic acid.

17. The process as claimed in any one of claims 1 to 16, wherein eluting is carried out with an ammonium or alkali metal salt gradient from 0 to 0.8 mol/l.

18. The process as claimed in any one of claims 1 to 16, wherein eluting is carried out with an ammonium or alkali metal salt gradient from 0.10 to 0.25 mol/l.

19. The process as claimed in any one of claims 1 to 18, wherein the chromatography is carried out under a pressure of from 1.5 to 10 MPa.