Recombinant Human Insulin

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Insulin is a well-characterized peptide that can be produced by recombinant DNA technology for human therapeutic use. A brief overview of insulin production from both traditional mammalian pancreatic extraction and recombinant bacterial and yeast systems is presented, and detection techniques, including electrophoresis, are reviewed. Analytical systems for insulin separation are principally based on reversed-phase chromatography, which resolves the deamidation product(s) (desamido insulin) of insulin, proinsulin, and insulin. Process-scale separation is a multistep process and includes ion exchange, reversed-phase, and size exclusion chromatography. Advantages and/or disadvantages of various separation approaches, as described by the numerous literature references on insulin purification, are presented.

Introduction

Insulin is a polypeptide hormone that is essential for the supply of energy to the cells of the body (Barfoed, 1987). It has been estimated that the disease diabetes mellitus (impaired insulin production and its complications) is the third largest cause of death in industrialized countries after cardiovascular diseases and cancer (Barfoed, 1987). Produced by the β-cells of the pancreas in response to hyperglycemia, insulin is a potent hormone directly or indirectly affecting every organ or tissue in the body. The main functions of insulin are to stimulate anabolic reactions for carbohydrates, proteins, and fats, all of which have the metabolic consequences of producing a lowered blood glucose level (Norman and Litwack, 1987).

It was estimated in 1986 that there were 60 million diabetics in the world (Johnson, 1986) and that 12 million Americans have diabetes (Barfoed, 1987). These numbers are increasing dramatically. The U.S. population is thought to be growing at a rate of 1% per year, but the rate of growth of the insulin-using diabetic population is 5-6% per year (Johnson, 1986).

Health problems relating to diabetes can be devastating. Barfoed (1987) reports that, although acute symptoms can be treated with insulin, vascular complications in the later stages of the disease reduce the life expectancy of diabetics by one-third. In addition, he reports that diabetics are 25 times more likely to go blind and have twice the risk of dying from heart disease or stroke. In the U.S. the direct and indirect costs to the economy are estimated to be over 5 billion dollars annually.

The administration of insulin as a diabetic treatment has a long and interesting history. In 1922, Frederick Banting and Charles Best successfully treated the first human patient with an insulin preparation obtained from animal pancreatic extractions (Barfoed, 1987). From 1922 to 1972 the only available insulin was purified from the pancreases of pigs and cows. This insulin was quite valuable in prolonging the lives of diabetics who otherwise would have slowly died because glucose was unavailable to their body cells. Improvements in the purification of insulin during the 1970s were successful in producing a stable drug with a predictable action time (Chance et al., 1975; Dolan-Heiting, 1981; Barfoed, 1987). In 1959, Novo Nordisk A/S (Bagvaerd, Denmark) modified the state of the insulin itself to obtain an insulin crystal with a high zinc content which had a long action time, in comparison to amorphous insulin that was quickly absorbed (Dolan-Heiting, 1981; Barfoed, 1987). The additions of protamine and zinc delayed absorption since insulin forms a stable hexamer in the presence of zinc.
human insulin, and recombinant human insulin is identical to human insulin. Recombinant human insulin is less likely to cause immunological reactions during therapeutic use than animal-derived insulin. Porcine insulin, when administered over long periods of time, may result in serious allergic reactions. Sequence variation in insulin is most common at positions 8–10 (middle of the A chain disulfide bond), and these differences can lead to antigenic responses (Norman and Litwack, 1987). One impetus for development of the recombinant human insulin process was perceived beef and pork shortage in the 1970s which, if it had actually occurred, would have restricted the availability of the pancreatic tissue from which insulin was extracted and, therefore, the amount of insulin available (Hall, 1987). At that time it was feared that the insulin demand was increasing faster than the supply. Americans continue to limit meat consumption today, thereby affecting the availability of pancreatic tissue.

Insulin was a particularly good choice to be the first therapeutic protein to be produced with DNA technology, simply because of the large amount of insulin needed. In comparison to another recombinant product, the human growth hormone which has a current usage of several kilograms/year, the market for insulin is 2 orders of magnitude larger (Norman and Litwack, 1991). Since being developed in 1982, the amount of human insulin used has increased dramatically. It is estimated that in the U.S. 73% of the patients use human insulin (Crossley, 1991).

**Background**

**Insulin Structure.** Insulin is a well-defined peptide with known amino acid sequence and structural characteristics (Watson et al., 1983; Norman and Litwack, 1987). This hormone consists of two separate peptide chains which are the A chain (21 amino acids) and the B chain (30 amino acids) joined by disulfide bridges as indicated in Figure 1. Proinsulin is the biological precursor of insulin and is a single peptide chain formed when the A and B chains are bonded by the C peptide (Figure 2). Human and porcine insulins differ by only one amino acid, while bovine and human insulins differ by three amino acids, as indicated in Figure 1.

In a recent text on hormones, Norman and Litwack (1987) detail the discovery of insulin, its sequence, and structure. Because of its small size, insulin has been an ideal molecule for the study of peptide sequence and structure. In 1956, the primary amino acid sequence was found by F. Sanger, and in 1967, D. F. Steiner and R. Chance determined the structure of proinsulin. Work continued with the elucidation of the three-dimensional structure through X-ray crystallography by D. Crowfoot-Hodgkin in 1969. Using this technique, insulin was seen to exist as a monomer, a dimer, or a hexamer. X-ray crystallography remains an important technique in confirming the identities of different types of insulin. In the structure–function relationship for insulin, three features have been found to have been conserved: (1) the precise positions of the three disulfide bonds; (2) the N- and C-terminal regions of the A chain; and (3) the hydrophobic residues of the B chain (Norman and Litwack, 1987). Recombinant human insulin is chemically, physically, and immunologically equivalent to pancreatic human insulin and is biologically equivalent to both pancreatic human insulin and purified pork insulin (Chance et al., 1981a).

**Traditional Insulin Production and Purification.** Historically, insulin has been purified from animal tissue by extraction procedures followed by chromatographic

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with the rate of dissociation of the hexamer being the rate-controlling step in the absorption of the insulin.

The development of recombinant DNA technologies resulted in the production and use of the first recombinant product, human insulin, in 1982. Then, as now, recombinant human insulin has two distinct advantages over traditional insulin obtained from pancreatic extraction: there is virtually an unlimited supply of recombinant
Structures of Various Insulins

A-Chain:

Sheep

Asp-Arg-Val

Reef

Asp-Arg-Val

Human

Port

B-Chain:

Human

Asp-Arg-Val

Reef

Asp-Arg-Val

Sheep

Asp

Figure 1. Comparison of A and B chains of insulin from different sources. Reprinted with permission from Dolan-Heitlinger, J. Recombinant DNA and Biosynthetic Human Insulins: A Source Book, Eli Lilly and Co., Indianapolis, IN, 1982. Copyright 1982 Eli Lilly and Co.

Techniques (Dolan-Heitlinger, 1981; Barfoed, 1987). Frozen bovine or porcine pancreases are dried, extracted with ethanol, and acidified to pH 2 with HCl or H2SO4. These steps both inactive and remove the enzyme trypsin, which could degrade the insulin. Calcium carbonate is then added to neutralize the solution, the extract is concentrated by vacuum extraction at low temperatures, and the insulin is precipitated by salt addition. The precipitate is redissolved in water and precipitated by adjusting the pH to the isoelectric point of insulin.

Chromatography steps for further purification of the insulin may include gel filtration followed by ion exchange systems (Dolan-Heitlinger, 1981). Application of insulin to a gel filtration column gives a major peak composed of insulin, desamido insulin(s), arginine insulin(s), insulin ethyl ester(s), glucagon, pancreatic polypeptide, and somastatin. A small amount of larger molecular weight material elutes ahead of the insulin peak and contains proinsulin, and proinsulin intermediates, plus a mixture of covalent insulin dimers. Proinsulin-like materials may be immunogenic and therefore must be removed during the purification procedure (Chance, 1972; Chancé et al., 1975). Gel filtration followed by ion exchange chromatography separates insulin from these other materials.

Production of Human Insulin. The desire to not be restricted to animal tissue sources for insulin production led to the interest in manufacturing human insulin. Human insulin can be obtained by extraction from the human pancreas, chemical synthesis from individual amino acids, conversion of porcine insulin to human insulin "semisynthesis", or fermentation of genetically engineered microorganisms (Barfoed, 1987). Extraction from a human pancreas is only feasible for research purposes, and chemical synthesis (although it has been accomplished) is currently not economical. The third and fourth methods have been developed for commercial production of insulin for therapeutic use and will be discussed.

Conversion of Porcine Insulin. Semisynthetic human insulin was commercially developed in the 1970s by Novo Nordisk A/S and is produced by substituting the B-30 alanine residue of porcine insulin with a threonine residue. Five steps result in human monocomponent insulin (Barfoed, 1987). Insulin is first extracted from frozen porcine pancreas glands. The second step of


processing entails conventional purification processes, which have been described in the previous section. Next,
the purified porcine insulin is converted to human insulin in a medium containing only a small amount of water and trypsin and a large quantity of organic solvent and threonine ester. The tryptic hydrolyses insulin at Arg-Arg-Asp, while at the same time catalyzing the reverse reaction in which the threonine ester displaces alanine from position B-30 in the insulin molecule. This transeptidation of porcine insulin to human insulin was optimized to 97% yield using soluble trypsin (Markussen et al., 1983).

Transpeptidation is also catalyzed by immobilized trypsin, although the yield is lower at 80% (Usui and Morihara, 1987). This is followed by chromatographic purification to reduce measurable levels of proinsulin and remove the other reagents. Finally, the product is formulated and then filled under sterile conditions, packaged, and distributed.

**Insulin Produced by Recombinant Methods.** Human insulin was the first animal protein to be made in bacteria in a sequence identical to that of the human pancreatic peptide (Watson et al., 1983). This was accomplished by Eli Lilly and Co. (Indianapolis, IN) and Genentech (San Francisco, CA). These companies worked together to achieve expression of recombinant human insulin in 1978 in Escherichia coli (E. coli) K-12 using genes for the insulin A and B chains synthesized at the City of Hope National Medical Center (Chance et al., 1981a). Scientists at Genentech cloned the genes in frame with the β-galactosidase gene of plasmid pBR322; the recombinant plasmid was simplified in E. coli (Chance et al., 1981b). The first successful expression was announced in 1978, with scale-up and approval by the appropriate drug regulatory agencies achieved by 1982 (Johnson, 1983). Insulin's small size and the absence of methionine (Met) and tryptophan (Trp) residues in the A and B chains were critical elements in the decision to undertake the cloning of this peptide, as well as in the achievement of rapid development of the manufacturing process. The Met and Trp residues produced as a consequence of engineering and expression in E. coli are hydrolyzed by the reagents used during the insulin recovery process. The presence of these amino acids in insulin would have resulted in the hydrolysis and destruction of the product.

**Two-Chain Method.** Although recombinant human insulin is now produced in several ways, the first successful method, illustrated schematically in Figure 3 (Watson et al., 1983), was accomplished on a laboratory scale by Genentech followed by successful scale-up by Eli Lilly and Company (Chance et al., 1981a,b,c). Each insulin chain was produced as a β-galactosidase fusion protein in separate fermentations using E. coli transformed with plasmids containing either the A or B insulin peptide sequence. The products were intracellular and appeared in prominent cytoplasmic inclusion bodies (Williams et al., 1982). The method used to extract the peptides from the inclusion bodies is proprietary information. Recombinant proteins produced in E. coli usually represent 10–40% of the total protein (Burgess, 1987).

Once removed from the inclusion bodies, chemical cleavage by CNBr at the Met residue between the β-galactosidase (abbreviated β-gal) and the A or B chain, followed by purification, gave separate A and B peptides. The peptides were then combined and induced to fold at a ratio of 2:1 of A:B chains (S-sulfonated forms) in the presence of limited amounts of mercapto in order to obtain an active hormone (Chance et al., 1981c; Frank and Chantler, 1981). After 24 h, the yield was approximately 60% based on the amount of B chain used (Chance et al., 1981a; Johnson, 1986). Goodell et al. (1979) obtained similar results with 50% of the total cellular protein expressed as either the A or B chain fusion protein. Subsequent folding of S-sulfonated chains gave 50–80% correct folding.

The large size of the β-gal fusion protein limited yields since the fusion protein of β-gal (~1000 amino acids) and insulin A or B chain (21 or 30 amino acids, respectively) became detached from the cell's ribosome (premature chain termination during translation) and therefore yielded incomplete insulin peptides (Burnett, 1983; Hall, 1987). A key improvement to this approach was the use of the tryptophan (Trp) operon in place of the lac operon (β-gal system) to obtain a smaller fusion protein. The Trp operon consists of a series of five bacterial genes which sequentially synthesize the enzymes responsible for the anabolism of tryptophan. One of these enzymes, Trp E, has only 190 amino acids compared to β-gal's 1000 amino acids. The Trp E gene followed by genes for the A or B chains of insulin has the added advantage of enhancing fusion protein production from 5–10% to 20–30% of the total protein (Hall, 1987) since the Trp promoter is a strong promoter in E. coli. This leads to at least 10-fold greater

**Figure 3.** Production of insulin in bacteria by using synthetic insulin genes. The bacteria produce hybrid proteins consisting of the N-terminal portion of the β-gal protein fused to either the A or B chain of insulin. Reprinted with permission from Watson, J. D., et al. Recombinant DNA—A Short Course. W. H. Freeman and Co.: New York, 1985; pp 231–235. Copyright 1985 W. H. Freeman Co.
expression of polypeptide when compared to the lac (i.e., β-gal) system (Burnett, 1983). The Trp operon is turned on when the E. coli fermentation runs out of tryptophan (Hall, 1987; Etienne-Decant, 1988). This characteristic is beneficial during fermentation since cell mass can first be maximized. Then, when appropriate, the cell’s insulin production system can be turned on by allowing the fermentation media to become depleted in Trp. Since the insulin fusion protein is an intracellular product (i.e., inclusion body), productivity is proportional to cell mass. If the inclusion bodies were formed prematurely, cell growth would stop, and therefore, total cell mass would be lower than the maximum possible. Consequently, the “Trp switch” is a very important practical tool in maximizing production.

The advantage of the Trp LE system in terms of potential productivity is clear from Figure 5. The smaller Trp protein, like the large lac protein, is highly insoluble. Consequently, the Trp E fusion protein also accumulates intracellularly in the form of insoluble inclusion bodies, which help to retard the proteolytic degradation of the product during fermentation and initial recovery (Burnett, 1983). The structure of the insulin coding plasmid is shown in Figure 4, and the relative molecular size of various human insulin chimeric proteins is shown in Figure 5 (Burnett, 1983).

Details of the two-chain method of insulin production are given in the literature. Separate fermentations of specific recombinant E. coli strains are performed to produce each chain with the inclusion bodies processed to yield partially purified S-sulfonate forms. These are subsequently used in the combination reaction (Frank and Chance, 1985; Johnson, 1984; Johnson, 1986). Both of the fermentations produce a chimeric protein composed of the specific A or B chain linked through the amino acid methionine (Met) to a Trp-LE promoter peptide (Frank and Chance, 1985).

After fermentation is completed, the cells are recovered and disrupted. The cell debris is then separated from the inclusion bodies, and the inclusion bodies are dissolved in a solvent, although specific are not known (Wheelwright, 1991). Inclusion bodies are sometimes dissolved in 6 M guanidine HCl and 0.1 mM dithiothreitol (Burgess, 1987). Next, the Trp-LE-Met-A chain and the Trp-LE-Met-B chain undergo a CNBr cleavage to release the A and B insulin chains. Further modifications of the A and B chains include oxidative sulfhydryl, purification, and combination to produce crude insulin. This crude insulin is subjected to ion exchange, size exclusion, and reversed-phase high-performance liquid chromatography to produce the purified recombinant human insulin (Frank and Chance, 1983).

There is a recent patent (Robbit and Manetto, 1999) on the chiroptropic and sulfitolytic solubilization of inclusion bodies of heterologous proteins. This particular process includes sulfonation followed by a warming step, allowing the protein S-sulfonate to precipitate in high purity; 95% pure compared to the starting material. This method seeks to avoid improper disulfide bond formation or intermolecular cross-linking which can occur in the conventional processing following cell lysis.

Proinsulin Method (Intracellular). Human insulin can also be made with recombinant microorganisms that produce intact proinsulin instead of the A or B chains separately. The process routes the current method of choice for insulin production (Kroeff et al., 1989) and entails one sequence of fermentation and purification steps rather than two sets of sequences (i.e., one for the A chain and one for the B chain). This approach is summarized by Kroeff et al. (1989) with the basis of the recombinant technology described by Watson (Watson et al., 1980), as shown in Figure 2. Initially, mRNA is copied into cDNA, and a methionine codon is chemically synthesized and attached to the 5’ end of the proinsulin cDNA. The cDNA is inserted into a bacterial gene in a plasmid vector that is introduced and then grown in E. coli. Proinsulin can be released from the bacterial enzyme (β-gal) fragment (or alternatively from the Trp-LE-Met-proinsulin (Trp proinsulin)) by destroying the methionine linkers. The proinsulin chain is subjected to a folding process to allow intermolecular disulfides to form, and the C peptide can then be cleaved with enzymes to yield human insulin (Frank and Chance, 1983). In comparison, the two-chain method previously described is more complex (Figure 3). Human insulin, derived from proinsulin generated through the Trp LE promoter, is discussed by Kroeff et al. (1989). After recovery, the Trp proinsulin undergoes CNBr cleavage to yield proinsulin. The proinsulin is subjected to oxidative sulfhydration, folding conditions in the presence of a mercaptoan, several purification steps, and then enzymatic treatment to form the crude insulin. Ion
exchange, reversed-phase, and size exclusion chromatography steps result in the purified recombinant human insulin.

Proinsulin (Secreted). Ville-Komaroff et al. (1978) were first to describe a secretion system for human proinsulin in E. coli. Watson (1983) suggested that the ideal situation for recombinant protein production would be to have large amounts of the foreign protein efficiently secreted into the medium by the bacteria. In the specific case of insulin, the recombinant protein could consist of $\beta$-lactamase (an enzyme that inactivates penicillin, which is naturally secreted by bacteria into the culture media) and proinsulin. Yeasts are also attractive for this type of system since they secrete only a few of their own proteins. Therefore, fewer extraneous proteins would need to be removed in purification. Yeasts also are able to facilitate the formation of disulfide bonds. However, for glycosylated proteins, yeasts tend to overglycosylate.

Novo Nordisk A/S used baker's yeast or Saccharomyces cerevisiae to secrete insulin as a single-chain insulin precursor. Both the process and the product were approved by the Danish Parliament in 1986 (Diers et al., 1991). Diers et al. (1991) describe the unfolded peptide as a leader or presequence, next a Lys-Arg sequence (recognized by the processing enzyme), the B chain (amino acids 1-29), a short peptide bridge, followed by the A chain (amino acids 21-28). In this precursor, amino acid 29 of the B chain of insulin is connected to amino acid 30 of the A chain by a short connecting peptide containing one basic amino acid adjacent to the A chain. Human insulin is produced through transpeptidation followed by hydrolysis of the ester bond formed. Several chromatography steps follow for further purification (Diers et al., 1991).

Another process for producing human proinsulin intracellularly in the yeast S. cerevisiae has recently been described (Trotter and Carlens, 1990). Using this yeast system in an optimized batch-fed fermentation, yields of the fusion protein of superoxide dismutase-human proinsulin (SOD-PI) were reported to be 1660 mg/L. SOD-PI would be the starting material for the production of recombinant human insulin; yields of the final product have not been reported. Apparently, the expression of heterologous polypeptides in yeast has sometimes been lower than desirable. An Etl Lilly Co. patent (Beckage and Ingolia, 1988) describes a process of aerobic culturing of yeast, followed by anaerobic and then back to aerobic conditions. This method is reported to result in a high expression of product (produced intracellularly in S. cerevisiae).

Analytical Separation of Insulin

Reversed-Phase High-Performance Liquid Chromatography. Reversed-phase high-performance liquid chromatography (RP-HPLC) over alkylsilane supports appears to be the method of choice in the analysis of insulin (Tables I and II) (Monch and Dehnen, 1976; Dangaard and Markussen, 1976; O'Hare and Nice, 1976; Terabe et al., 1979; Dinne and Lorenz, 1979; Krooff and Chance, 1982; Lloyd and Corran, 1982; River and McClintock, 1983; Parman and Rideout, 1983; McLeod and Wood, 1984; Knip, 1984; Kalant et al., 1985; Smith and Venahle, 1985; Vigh, 1987). The high selectivity and high resolving capabilities of RP-HPLC using a wide variety of stationary and mobile phases allow the separation of insulin species which differ by only one amino acid (Krooff et al., 1989). The mechanism for the separation of insulin in RP-HPLC systems is based on the hydrophobicities of insulin and related compounds. The tendency of some proteins (particularly large proteins) to bond so tightly to the stationary phase that they are difficult to elute appears to be the major limitation of the alkylsilane supports.

It is difficult to compare the analytical systems described in Tables I and II. Each particular system was designed and optimized for a specific series of separations. In general though, Smith et al. (1985) have summarized the conditions affecting the RP-HPLC of insulin and related compounds. Acetonitrile or methanol with various mixtures of aqueous buffers is usually used for the mobile phase in insulin analysis. Capacity factors of insulin decrease with increasing acetonitrile concentration up to 40% (Grego and Hearl, 1981). Since most polypeptides and proteins strongly bind to alkylsilica supports, chloride salt may be substituted for phosphate salt in the mobile phase. Most separations are carried out at ambient temperatures, with optimal detection of insulin being between 190 and 220 nm. All systems suffer, in varying degrees, from interferences that are due to preservatives or other insulin impurities (Smith et al., 1985).

With the advent of recombinant insulins, other analytical systems as well as modifications of the RP-HPLC system have been described for the analytical separation of insulin, proinsulin, C peptide, and insulin A and B chains from each other and from recombinant fusion proteins. De Gouvea (1985) isolated and quantitated the A and B chains of insulin with a RP-HPLC system (Waters Radial Pak with Bondapak C18 cartridges, Waters, Milford, MA) using ion pairing with trifluoroacetic acid (TFA) as the counterion for the A chain and formic acid for the B chain. Kalita et al. (1981) used gel chromatography (Bio-Gel P-30, Bio-Rad, Richmond, CA) to separate proinsulin from the C peptide with 1 M acetic acid as the eluting buffer. Welinder (1984) looked at the homogeneity of crystalline insulin using RP-HPLC (Nucleosil 10 C3) and high-performance gel permeation chromatography (HP-GPC) (2.15 column, Waters). Welinder and Lindes (1984) reported the separation of insulin and insulin derivatives using high-performance ion exchange chromatography (RP-IEC) and gave a comparison to RP-HPLC. Ion exchange chromatography using a 0.0-0.3 M NaCl salt gradient over a DEAE-derivatized polymeric stationary phase was recently reported by Lodisch et al. (1990). In this ion exchange based separation, insulin is separated from 3-galactosidase and from the insulin A chain.

Affinity Chromatography. Affinity chromatography may also be used in the purification of fusion proteins. A one-step affinity purification procedure which gives an overall yield of 85-95% of hybrid protein with low activity using TPEG-Sepharose under high-salt conditions has been described (Burgess, 1987). Nilsson et al. (1989) describe a general gene fusion approach to facilitate purification of recombinant proteins based on the fusion of a gene of interest to a gene encoding a protein with a strong affinity to a ligand (affinity fusion).

Smith et al. (1988) report the use of a technique called chelating peptide immobilized metal ion affinity chromatography (CP-IMAC) to purify recombinant proinsulin. In this method the proinsulin is expressed with a chelating peptide on the NH3 terminus. The proinsulin can then be affinity-purified with immobilized metal ions, and the chelating peptide is removed chemically or enzymatically.

Electrophoresis of Insulins. Electrophoresis is a common method for detecting contaminating proteins in a previously purified sample. However, unlike many other proteins, the insulin presents a special challenge. Conventional electrophoretic techniques separate proteins down to molecular weights of about 12 000
Table 1. Isoelectric Reversed-Phase Chromatography Systems for Insulin

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<th>separation</th>
<th>system</th>
<th>comments</th>
<th>reference</th>
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<tr>
<td>bovine and pork insulin from monodesamido derivatives</td>
<td>LiChrosorb RP-8®-8 elution with acenetonitrile in sulfuric acid buffer</td>
<td>higher molecular weight</td>
<td>proinsulin impurities retained until acenetonitrile concentration increased to 26-27%</td>
<td>Diner and Lorenz, 1979</td>
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<tr>
<td>bovine and pork insulin from monodesamido derivatives</td>
<td>Nucleosil ODS®-4 Ultrasphere ODS®-4, Hydrophil ODS®-4, LiChrosorb RP-18, Sphatago ODS®-4 and Zorbax TMS® elution with (1) tartaric or acetic acid with acenetonitrile and acetonitrile and (2) acenetonitrile in acetic acid phosphate buffer</td>
<td>suggests that HPLC techniques can also be used to determine potency of insulin crystals and formulations</td>
<td>Lloyd and Corran, 1982</td>
<td></td>
</tr>
<tr>
<td>bovine, porcine, and human insulin, monodesamido insulin and insulin diners</td>
<td>Zorbax TMS® elution with acenetonitrile in an acid phosphate buffer</td>
<td></td>
<td>all three columns and both buffers separated the insulin</td>
<td>Krooff and Chance, 1982</td>
</tr>
<tr>
<td>bovine, porcine, pancreatic human, chicken, ovine, rabbit, and rat insulins</td>
<td>Vyda® derivatized three ways: (1) C-16, (2) phenyl, (3) C-4, elution with (1) TFA in acenetonitrile and (2) triethylaminoum phosphate in acenitrile buffer</td>
<td></td>
<td>separated bovine, porcine, and human insulin with 22% acenetonitrile in the mobile phase; slight changes in pH or buffer concentration were not critical, but consistent acenetonitrile concentration was critical for retention times</td>
<td>Rivier and McClintock, 1983</td>
</tr>
<tr>
<td>hormones: (2) ACTH analogues, (3) LH-RH analogues, and (4) insulin</td>
<td>Nucleosil LC-18® elution with tertartate buffer-acenetonitrile containing sodium + butane sulfonate and sodium sulfate</td>
<td></td>
<td>separated bovine, porcine, and human insulin with 22% acenetonitrile in the mobile phase; slight changes in pH or buffer concentration were not critical, but consistent acenetonitrile concentration was critical for retention times</td>
<td>Torabe et al., 1979</td>
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<tr>
<td>bovine, human, porcine, mouse, turkey, rooster insulin, bovine proinsulin and a series of bovine insulin analogues</td>
<td>ODS Ultrasphere® isocratic and shallow gradient elution in acetic acid phosphate buffer, included chaotrope salts</td>
<td></td>
<td>use of insulin analogues allowed comparison of predictions</td>
<td>McLeod and Wood, 1984</td>
</tr>
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<td>bovine, porcine, recombinant human, pancreatic human insulin, oxidized bovine insulin A and B chains</td>
<td>LiChrosorb RP-8® and a Bondapac C-18® elution with three systems: (1) ammonium sulfate acnidanized with sulfuric acid and mixed with acenetonitrile, (2) tetramethylammonium hydroxide, orthophosphoric acid mixed with methanol, (3) ethanolamine, orthophosphoric acid mixed with acenetonitrile</td>
<td></td>
<td>better separation of human insulin was obtained with the µ Bondapac C-18 column</td>
<td>Kalant et al., 1985</td>
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<td>bovine and porcine insulin</td>
<td>Nucleosil C-8® displacement chromatography with methanol phosphate buffer and acenitrile displacer</td>
<td></td>
<td>a proinsulin contamination target of 100 ppm was achieved</td>
<td>Vigh et al., 1987</td>
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* ODS = octadecylamine. a Merck, Dormstadt, FRG. b Camlab, Great Britain. c Anaehem, Great Britain. d Shandon-Southern, Great Britain. e Phase Separations, Great Britain. f Hichin, Great Britain. g Separations Group, Hearpark, CA. h Alterx. i Waters, Milford, MA. j Macksey and Nagel, FRG.

Large-Scale Purification of Human Insulin

Purification of a protein that has been overproduced in E. coli requires a somewhat different methodology than that used in conventional protein purification or techniques used for analytical purposes (Welander and Lindie, 1984). An exceedingly high degree of purity is required as well as the freedom from contaminating solvents and toxins (from E. coli) and the nutrients, metabolites, catalyst, and host cell functional molecules which are present as a consequence of cell growth (Johnson, 1986). The recombinant protein must also be free of fusion protein components. Furthermore, processing conditions must be chosen to avoid proteolysis of hybrid proteins, especially the larger proteins which are subject to proteolytic degradation (Ullmann, 1984). In particular, insulin must be separated from other forms of insulin (proinsulin, desamido insulin, etc.) which might be immunogenic. A large-scale purification scheme must meet all these re-
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<th>separation system</th>
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<td>Insulin and calibration proteins</td>
<td>ODS Nucleosil 10-C18*</td>
<td>Monch and Dehnen, 1979</td>
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<tr>
<td>(cysteine C, bovine serum albumin, aldolase, catalase, chymotrypsinogen A, bovine pancreatic DNAase)</td>
<td>2-aminomethylpropanol</td>
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<td>bovine and porcine insulin, porcine insulin from porcine monodansamido insulin</td>
<td>2-aminomethylpropanol</td>
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<td>(32) polyptides and (9) proteins</td>
<td>2-aminomethylpropanol</td>
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<td>bovine, porcine, and human insulin, desamido forms of insulin</td>
<td>Hyperil ODS*</td>
<td>O'Hare and Niss, 1979</td>
</tr>
<tr>
<td>pancreatic peptides, insulin, and proinsulin</td>
<td>Sherlefs SS ODS*</td>
<td>Smith et al., 1985</td>
</tr>
</tbody>
</table>

* ODS = octadecylsilane. ** Macherly and Nagel, FRG. *** Waters, Milford, MA. 4 Schaffner, Bernm, Switzerland. 5 Phase Separations, Queenbeery, Great Britain. 6 Merck, Darmstadt, FRG. 7 Du Pont, Natick, Great Britain. 8 Du Pont Instruments, Wilmington, DE.

Requirements, and in doing so, it has been estimated that more than half of the processing costs are in the downstream purification relative to the actual fermentation (Ullmaa, 1984).

Inclusion bodies are formed in more than 80% of the cases where proteins have been overproduced in E. coli (Weidler and Linden, 1984). The E. coli fusion protein β-galactosidase–insulin is no exception. Burgess (1987) gives a procedure to release an insoluble full-length β-galactosidase–insulin protein involving lysis, centrifugation, denaturation, renaturation, precipitation, and ion exchange chromatography steps. Many proteins have been purified from insoluble inclusion bodies by these methods of denaturation and renaturation. More current purification procedures can be followed for a secreted fusion protein such as β-lactamase–insulin.

There are few detailed, published procedures for a complete large-scale purification sequence for recombinant insulin from E. coli. However, Krooff et al. (1989) describe a multimodal chromatography preparative-scale system with an integral RP-HPLC step developed to purify and analyze recombinant human insulin produced from E. coli. This RP-HPLC system is placed fairly late in the insulin purification system since the majority of the impurities (mainly from E. coli) are removed prior to this step by an ion exchange step. A size exclusion separation follows the reversed-phase step. The reversed-phase systems were based on a stationary phase of 10 μm Zorbax (Du Pont Instruments, Wilmington, DE) process-grade C8 for the production-scale columns and 5-μm particles for the analytical scale columns. Partially purified human insulin zinc crystals prepared at Eli Lilly and Co. were the starting material for this part of the purification sequence. The insulin was applied in a water-rich mobile phase and then eluted in a linear gradient of 0-25% acetic acid (eluent A) to 60% aqueous acetonitrile (eluent B). An acidic mobile phase is recommended since it provides excellent resolution of insulin from structurally similar insulin-like components while maintaining insulin solubility. The ideal pH is thought to be in the region of 3.0-4.0, which is well below the isoelectric pH of 5.4. Under mildly acidic conditions insulin may deamidate to monodansamido insulin, but if the RP-HPLC is done fairly rapidly (within a matter of hours) the deamidation can be minimized. This RP-HPLC system was successfully used in a production-scale system to purify recombinant insulin with a biological potency equal to that obtained from the conventional purification system, which employs ion exchange and size exclusion chromatography (Krooff et al., 1989).

Weidler and Linden (1984) investigated high-performance ion exchange chromatography (HP-IEC) for insulin purification and compared this method to RP-HPLC for insulin separation. HP-IEC allowed rapid fractionation of crystalline insulin with no salt gradient. They found HP-IEC gave good recovery and had fewer organic contaminants than RP-HPLC, but needed to be performed under dissociating conditions (7 M urea).

Conclusion

Insulin is a therapeutic protein which now has a history of production using recombinant DNA methods. Improvements in genetic engineering methods have facilitated both the production and the recovery of this high-value peptide. The standard method for analysis of insulin and insulin derivatives is RP-HPLC. Standard methods for the purification of insulin produced by recombinant methods include ion exchange, gel permeation, and reversed-phase chromatography. While the current practice of these methods yields highly purified insulin, further refinement in separation is an area of continuing research. This reflects the increasing demand for therapeutic proteins with minimal contamination and the need to reduce the high cost of purification.

Notation

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>HCl</td>
<td>hydrochloric acid</td>
</tr>
<tr>
<td>CNBr</td>
<td>cyanogen bromide</td>
</tr>
<tr>
<td>RP</td>
<td>reversed-phase high-performance liquid chromatography</td>
</tr>
<tr>
<td>Met</td>
<td>methionine</td>
</tr>
<tr>
<td>TFA</td>
<td>trifluoroacetic acid</td>
</tr>
<tr>
<td>HP</td>
<td>high-performance gel permeation chromatography</td>
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<tr>
<td>GPC</td>
<td>gel permeation chromatography</td>
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</table>


Johnson, J. S. In Biotechnology and Biological Frontiers; The American Association for the Advancement of Science Publishers: Washington, DC, 1984; pp 46-56.


Dolan-Hastiger, J. In Recombinant DNA and Biosynthetic Human Insulin, A Source Book; Ell Liiy and Co.: Indianapolis, IN, 1985.


Johnson, J. S. In Biotechnology and Biological Frontiers; The American Association for the Advancement of Science Publishers: Washington, DC, 1984; pp 46-56.


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