

to specific requirements for lot release by the FDA. In comparison, drugs are approved under Section 505 of the FD & C Act (21 USC 301–392), where there is not lot release by the FDA except for insulin products. Solomon gives the example that insulin, growth hormone, and many other hormones have been treated as drugs, while erythropoietin (abbreviated EPO), which also fulfills the criteria of a hormone, was reviewed in the biologic division of the FDA. (Note that insulin is derived from a bacterial fermentation while erythropoietin is obtained from mammalian cell culture.) Hormones, for the most part, will continue to be reviewed as drugs.

The analysis of regulations and their potential application to new bioproducts is not the intent of this chapter. Government regulations change, and the countries in which a product is sold influence the regulations that the product is subject to. Rather, this brief mention of government regulations is an attempt to reflect on other issues that could influence the design of bioseparation unit operations on a process scale, and the constraints on process development that grow as a recovery and purification scheme approach, and then pass, the licensing stages for commercial manufacture.

INSULIN CASE STUDY

Biosynthetic human insulin is generated biologically through fermentation of a transformed microorganism, and then modified using chemical and biochemical methods. The term “biosynthetic” can denote a combination of biological, recombinant, chemical, and/or biochemical steps followed by, or interspersed with, purification of the target molecule (i.e., product) (Baker, 1996).

8.6 BIOSYNTHETIC HUMAN INSULIN IS THE FIRST RECOMBINANT POLYPEPTIDE FROM *E. COLI* LICENSED FOR HUMAN USE

Insulin is a polypeptide hormone that stimulates anabolic reactions for carbohydrates, proteins, and fats and thereby produces a lowered blood glucose level. Porcine and bovine insulins were used to treat diabetes prior to the availability of human insulin. All three types are similar in amino acid sequence (Figure 8.1), although the sequence variation (of amino acid residues) could lead to immunogenic responses. The desire to not be restricted to animal tissue sources for insulin production reportedly led to interest in manufacture of human insulin by fermentation. Eli Lilly’s human insulin was approved for testing in humans in 1980 by the FDA, and was placed on the market by 1982 (Bernton and Bodelle, 1991; Ladisch and Kohlmann, 1992). Since insulin is an endocrine hormone, it is regulated as a drug, whereas other types of hormones are likely to be viewed as biologics in the United States.

Human insulin was the first animal protein to be made in bacteria in a sequence identical to the human pancreatic peptide, and it resulted from the cooperation between Eli Lilly and Genentech. Expression of separate insulin A and B chains were achieved in *Escherichia coli* K-12 using genes for the insulin A and B chains synthesized at City of Hope National Medical Center, (Duarte, CA) and cloned in frame with the β -galactosidase gene of plasmid pBR322 (Chance et al., 1981a, 1981b). The product was a chimeric protein in an intracellular, granular form known as inclusion or refractile body. An example of a cell containing an inclusion body is shown in Figure 8.2. Insulin’s small size (21 amino acids for the A chain, $M_r = 2300$; and 30 for B chain, $M_r = 3400$), and absence of methionine (Met) and tryptophan (Trp) residues in the A and B chains were critical elements in the decision to undertake cloning of this peptide hormone, as well as in the rapid development of the manufacturing process. The Met and Trp residues in the chimeric precursor, produced as a consequence of engineering and expression of the gene in *E. coli*, are hydrolyzed by the reagents used during the recovery process. The presence

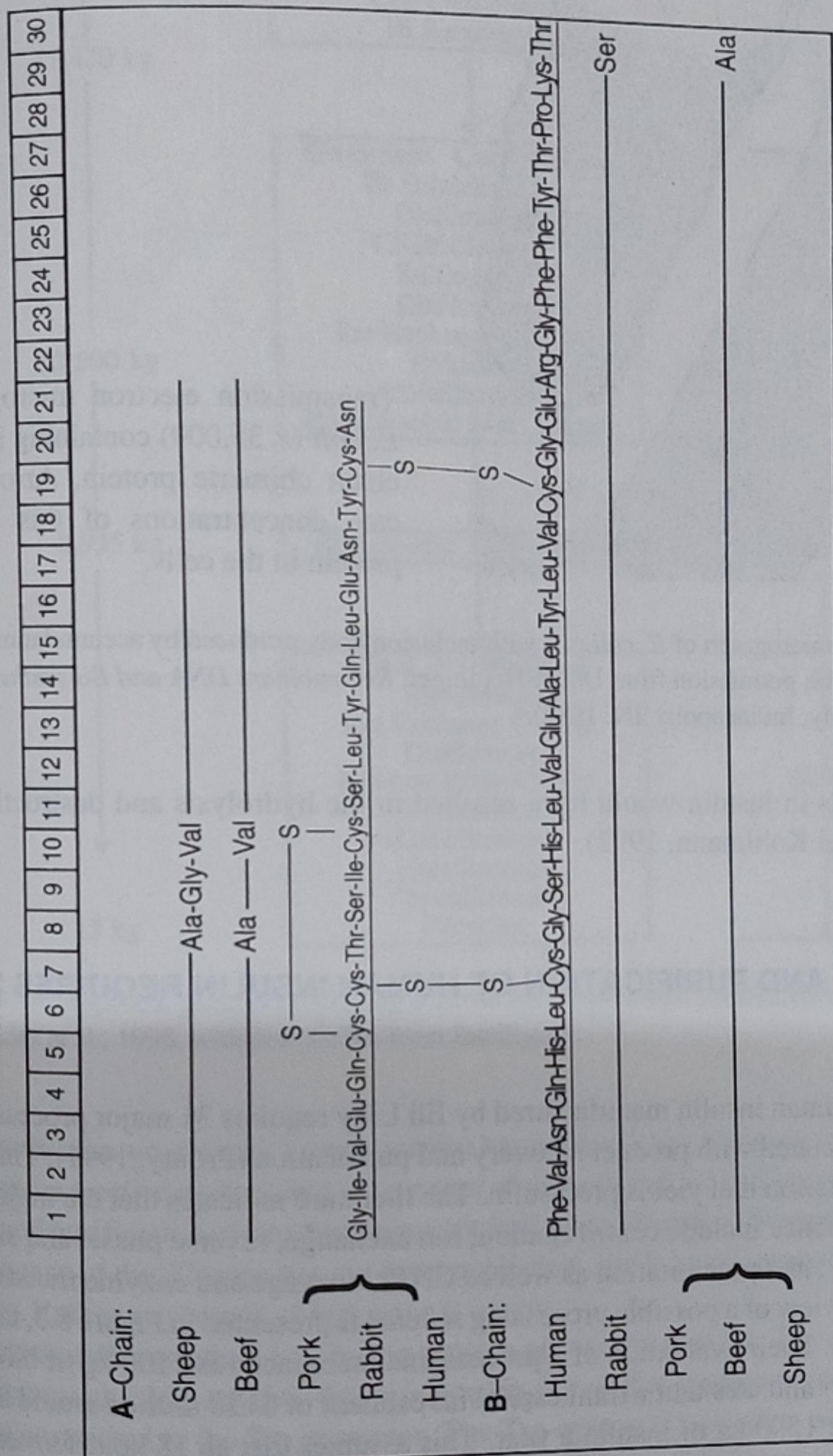
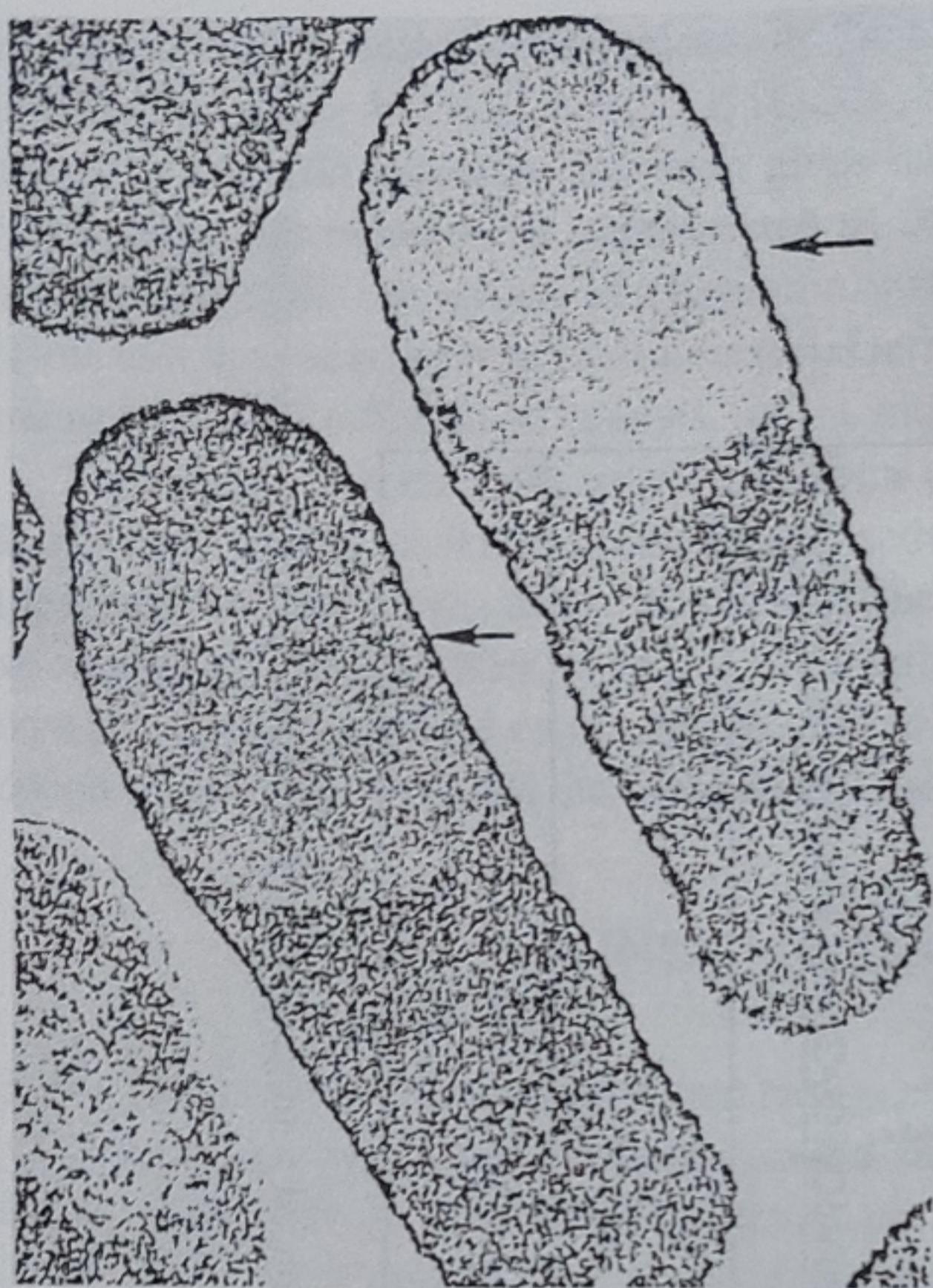


Figure 8.1 Amino acid sequence of A and B chains of insulin from different sources. (Reproduced with permission from Dolan-Heilinger, *Recombinant DNA and Biosynthetic Insulin: A Source Book*, Eli Lilly, Indianapolis, IN, 1982.)



Transmission electron micrograph of *E. coli* ($\times 35,000$) containing insulin A chain chimeric protein. Arrows indicate concentrations of this chimeric protein in the cells.

Figure 8.2 Electron micrograph of *E. coli* cell with inclusion body produced by accumulation of insulin protein (reproduced with permission from Dolan-Heitlinger, *Recombinant DNA and Biosynthetic Insulin: A Source Book*, Eli Lilly, Indianapolis, IN, 1982).

of these amino acids in insulin would have resulted in the hydrolysis and destruction of the product (Ladisch and Kohlmann, 1992).

8.7 RECOVERY AND PURIFICATION OF HUMAN INSULIN REQUIRES 27 STEPS

The production of human insulin manufactured by Eli Lilly requires 31 major processing steps of which 27 are associated with product recovery and purification (Prouty, 1991). This process is based on a fermentation that yields proinsulin. The literature indicates that the major steps in this purification sequence include centrifugation; ion exchange, reverse phase, and size exclusion chromatography; and precipitation as well as CNBr cleavage and enzyme transformation. A recent (1996) overview of a possible processing scheme is presented in Figure 8.3, taken from Petrides et al. (1996). Their evaluation of a process that manufactures 1500 kg of biosynthetic human insulin a year indicates that a total capital investment of \$130 million would be needed for a plant producing 1500 kg of insulin a year. This assumes that an 18 hour fermentation is required and gives a concentration of *E. coli* cells (dry weight basis) of 37.5 g cells per liter of fermentor volume, with 20% of the cell weight corresponding to intracellular inclusion bodies containing the proinsulin fusion protein. Twelve hours are allotted for turnaround of the fermentor, resulting in a total time of 30 hours. On this basis the unit production cost was estimated by Petrides et al. (1996) to be \$41/gram of insulin, with a selling price of \$110/gram. This is significantly lower than the estimate of \$375/g based on earlier data given in Table 8.1. This decrease likely reflects, in part, growth in use of recombinant forms of insulin, with its attendant economies of scale.

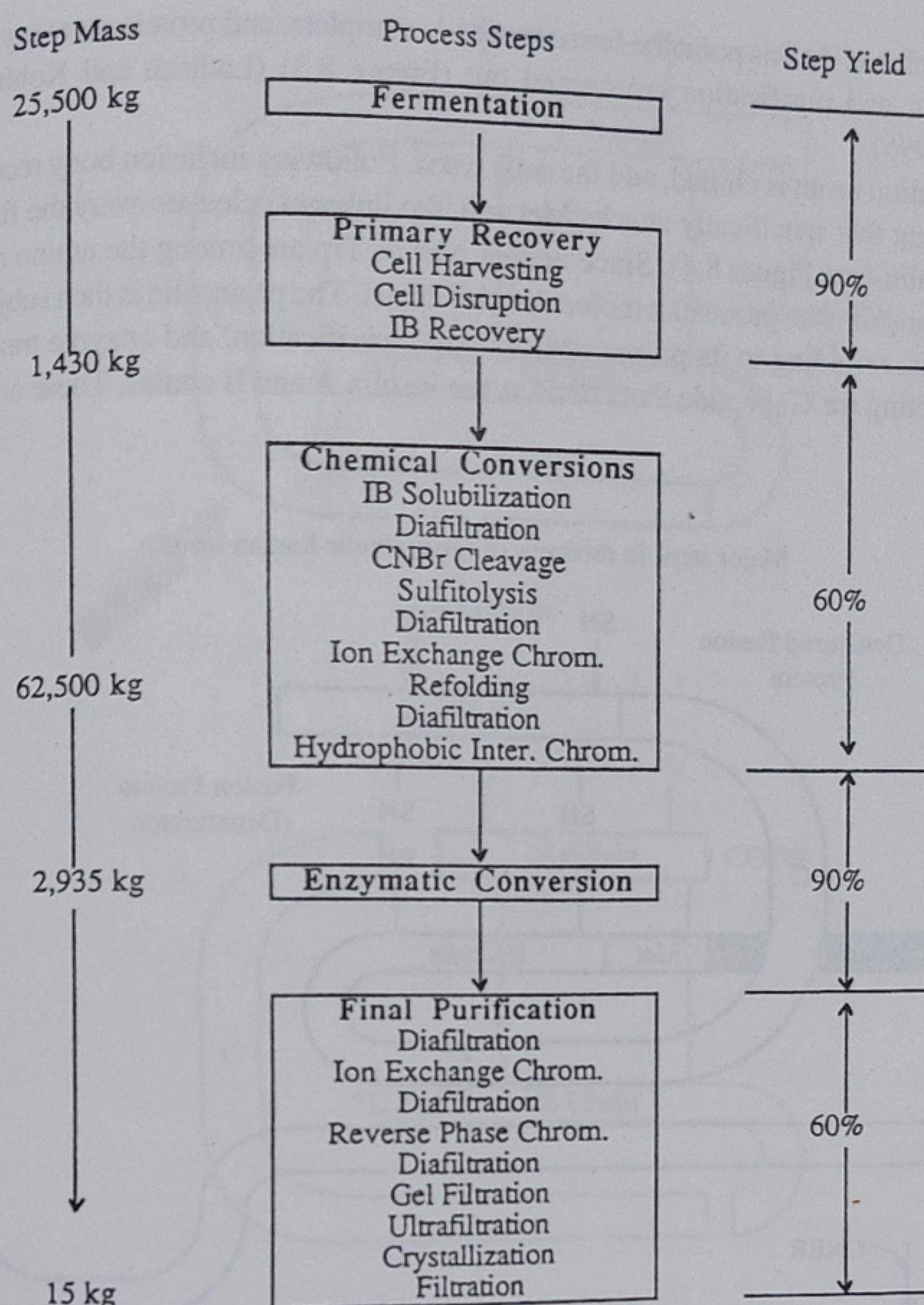


Figure 8.3 A proposed, general processing scheme for recombinant insulin. IB denotes inclusion body (from Petrides et al., 1996, with permission from Intelligen).

The production process for human insulin has a significant literature associated with it and provides an instructive case study on the range of unit operations that must be considered in the recovery and purification of a recombinant product from a bacterial fermentation. However, the exact sequence of the 27 steps has not been published, and hence it cannot be presented here in its entirety. Rather a synopsis of key steps is given.

The fermentation product is a fusion protein of a portion of the Trp enzyme connected to proinsulin through a Met residue. It is produced in *E. coli* that contains a plasmid with the proinsulin gene connected to the Trp promoter. The Trp operon is turned on when the fermentation media is allowed to become depleted of tryptophan.¹ This causes the production of a fusion protein of proinsulin to occur, which quickly accumulates intracellularly in the *E. coli*. An inclusion body (i.e., a large body of aggregated protein and nucleic acids) is formed that occupies about

¹The formation of inclusion bodies causes cell growth to stop. Since the proinsulin fusion protein is an intracellular product, productivity is proportional to cell mass (i.e., the number of cells). It is important to maximize the number of cells before formation of inclusion bodies. Premature formation of inclusion bodies would result in a lower productivity. Hence the "Trp switch" is an important practical tool in maximizing productivity.

half of the cell volume. At this point the fermentation is complete, and protein recovery, dissolution, protein refolding, and purification are carried out (Figure 8.3) (Ladisch and Kohlmann, 1992, Petrides et al., 1996).

The fermentation broth is chilled, and the cells lysed. Following inclusion body recovery, CNBr (a hydrolytic agent that specifically attacks Met and Trp linkages) cleaves away the fusion protein from the proinsulin (see Figure 8.4) (Ladisch and Kohlmann, 1992). Since neither Met or Trp are among the amino acid residues that make up proinsulin, the proinsulin molecule is left intact. The proinsulin is then subjected to oxidative sulfitolysis, refolding to its proper conformation, purification, and enzyme treatment to remove the connecting (or C) peptide that connects the insulin A and B chains. These are sometimes

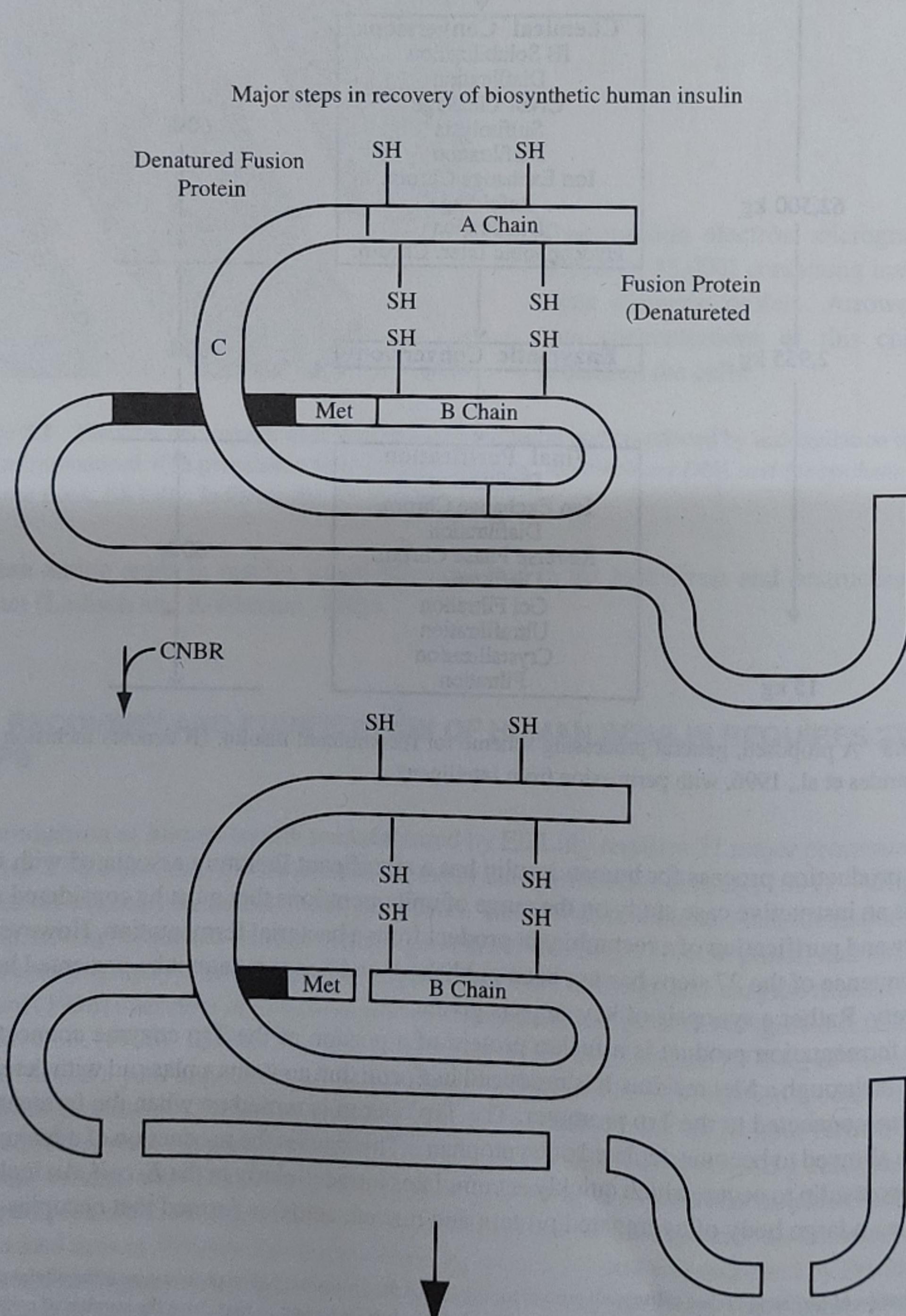


Figure 8.4 Schematic representation of major chemical and biochemical processing steps (continued).

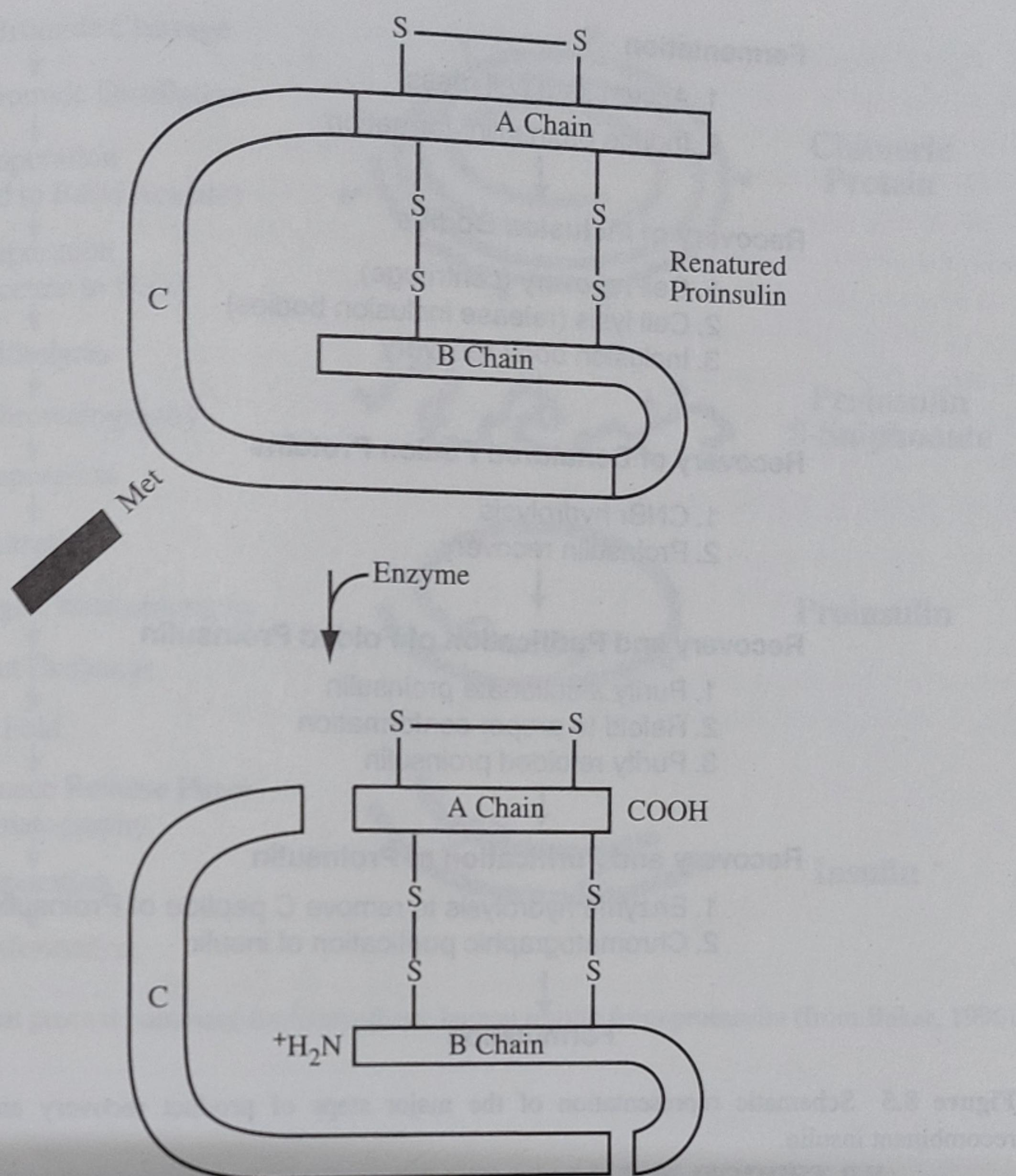


Figure 8.4 Continued.

referred to as front-end operations, since they are carried out early in the sequence of processing steps that leads to the final insulin product. Front-end operations generate, contain, and define process specific contaminants that must later be removed (Baker, 1996). The crude insulin consisting of A and B chains in their proper conformation are further purified using a sequence of ion exchange, reverse phase, and size exclusion chromatography steps as shown in Figure 8.5 (Kroeff et al., 1989; Wheelwright, 1991; Ladisch and Kohlmann, 1992). Another process summary is provided in Figure 8.6, which indicates locations of CNBr cleavage of the fusion tail of the chimeric protein, reduced disulfide bonds (proinsulin s-sulphonate), enzyme cleavage sites for proinsulin, and the refolded insulin as well as major purification steps.

Deamidation of asparagine or glutamine residues can occur readily in either acidic or neutral solutions, while disulfide exchange reactions causing the formation of isomeric monomers or aggregated forms (multimers) of the protein can occur at alkaline pH (Prouty, 1991). Deamidation products of insulin (also referred to as desamido insulin) can be formed during the processing of insulin. These variants of insulin require high-resolution chromatography techniques to remove. Therefore a multimodal sequence of chromatographic separations for the crude recombinant insulin is required and consists of the following:

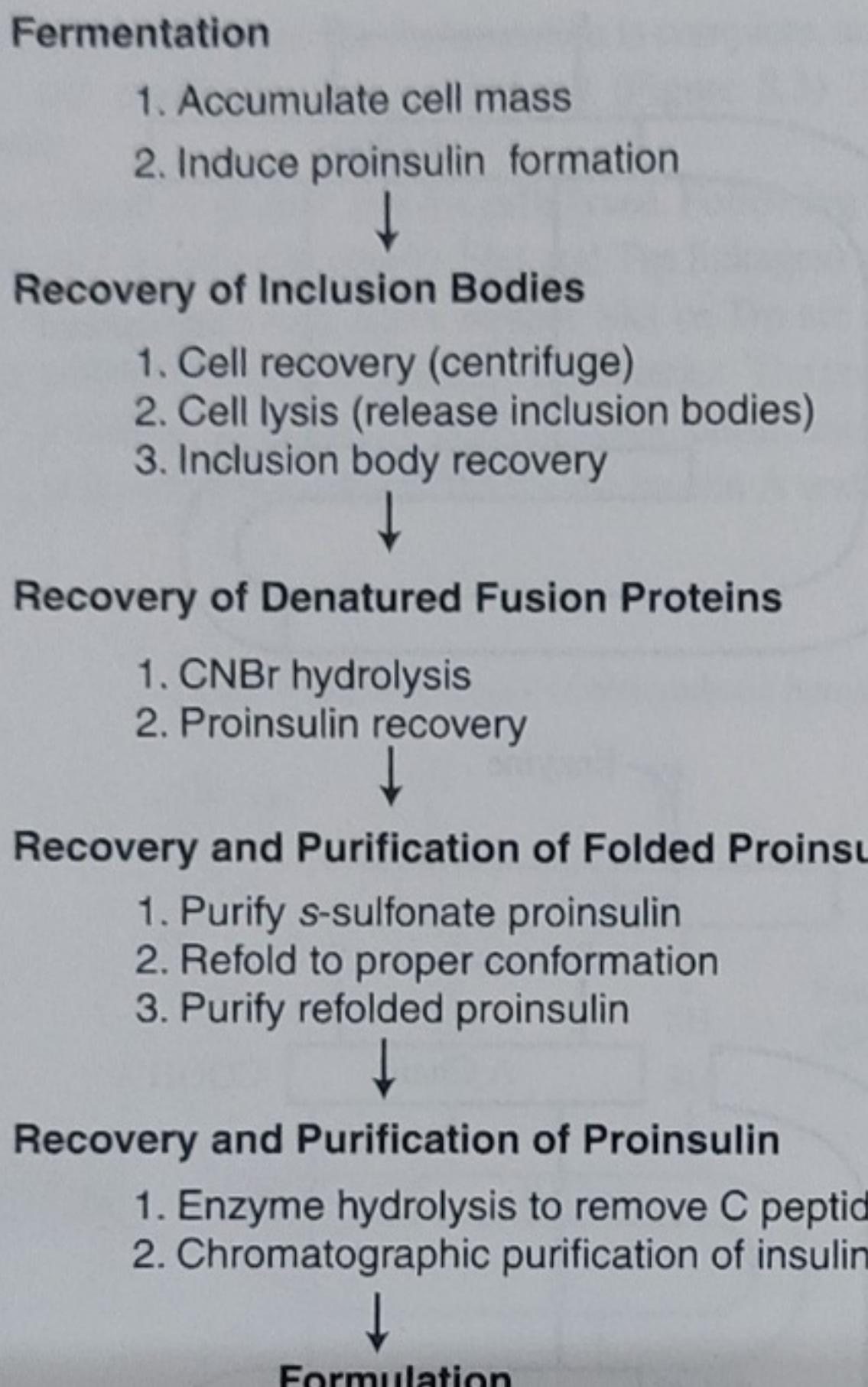


Figure 8.5 Schematic representation of the major steps of product recovery and purification for recombinant insulin.

1. Ion exchange (removes most of the impurities)
2. Reversed phase (separates insulin from structurally similar insulinlike components)
3. Size exclusion (removes polymers, residual proinsulin, salts and small molecules from the insulin)

The best pH range of the acetonitrile mobile phase for step 2 was reported as 3.0 to 4.0, since it is well below the isoelectric pH of 5.4, gives excellent resolution, and minimizes the deamidation of insulin to monodesamido insulin if the residence time in the reversed phase column is less than several hours (Kroeff et al., 1989; Ladisch and Kohlmann, 1992). The sequence of steps given by Kroeff et al. (1989)—ion exchange, reversed phase, and gel permeation (size exclusion) chromatography—follows the principle of orthogonality of the separation sequence, where each step is based on a different property, namely charge, solubility, and size, respectively (Willson and Ladisch, 1990). Near the end of the chromatography sequence, the insulin may be concentrated by precipitation to form insulin zinc crystals. The procedure consists of adjusting the insulin concentration to 2 g/L with 0.25 M acetic acid, adjusting the pH to 5.9 with ammonium hydroxide, and adding a 1.8 moles zinc chloride per mole biosynthetic human insulin. The zinc insulin crystals are then recovered by filtration.

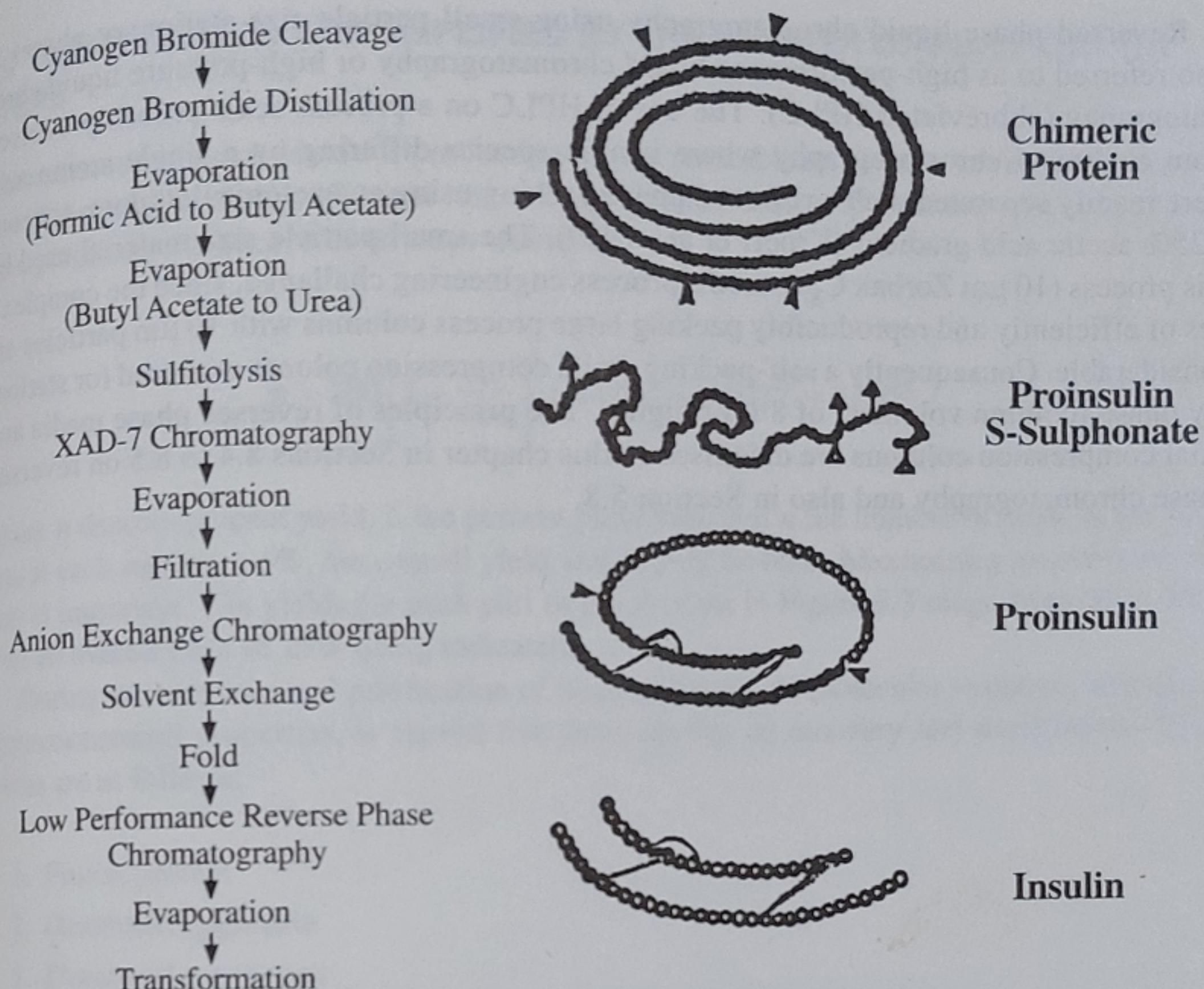


Figure 8.6 Recent process summary for biosynthetic human insulin from proinsulin (from Baker, 1996).

8.8 RECOVERY PROCESS EQUIPMENT VOLUMES ARE MODEST BY CHEMICAL INDUSTRY STANDARDS

The purification of insulin illustrates the many steps involved in product recovery and purification, and the unit operations required. These include cell lysis, centrifugation, refolding, buffer exchange, chromatography, precipitation, and filtration. Some of these steps are repeated. The volumes of the individual chromatography columns are estimated to range from 50 to 1000 L. While these volumes are small compared to other types of chemical recovery processes, they are large in the context of biotechnology manufacturing. An illustration of scale is provided by loadings reported for column sizes used in scale-up studies for the reversed phase insulin purification step illustrated in Figure 8.7, which is based on the data of Kroeff et al. (1989). The loading per unit column volume is about 15 g per liter of column volume, with the load increasing in direct proportion to the column volume. The mobile phase consumed was approximately 6 column volumes/run, or 536 L of mobile phase per kg of insulin loaded. This would correspond to 123 L acetonitrile if an average acetonitrile concentration of 23% is assumed. If the total amount of recombinant insulin produced annually on a worldwide basis is on the order of 7500 kg (Petrides et al., 1996), the chromatography column volume required would be on the order of 1500 L (assumes 1 run/day for 333 days/yr). The annual solvent usage would not be inconsequential with an estimated 804,000 L of mobile phase or 185,000 L of acetonitrile being consumed in a plant making 1500 kg/yr, assuming that the loadings per unit volume of stationary phase are the same at the commercial scale as was reported in Kroeff's scale-up studies.

Reversed phase liquid chromatography using small particle size stationary phases is also referred to as high-performance liquid chromatography or high-pressure liquid chromatography (abbreviated HPLC). The use of HPLC on a process scale probably evolved from analytical chromatography where insulin species differing by a single amino acid were readily separated with a reversed phase packing using an acetonitrile/dilute, aqueous 0.25% acetic acid gradient (Kroeff et al., 1989). The small particle size material used by this process (10 μm Zorbax C₈) posed a process engineering challenge, since the complexities of efficiently and reproducibly packing large process columns with 10 μm particles are considerable. Consequently a self-packing, axial compression column was used for stationary phase (column volumes) of 8 L or higher. The principles of reversed phase media and axial compression columns are discussed in this chapter in Sections 8.4 to 8.5 on reversed phase chromatography and also in Section 5.8.

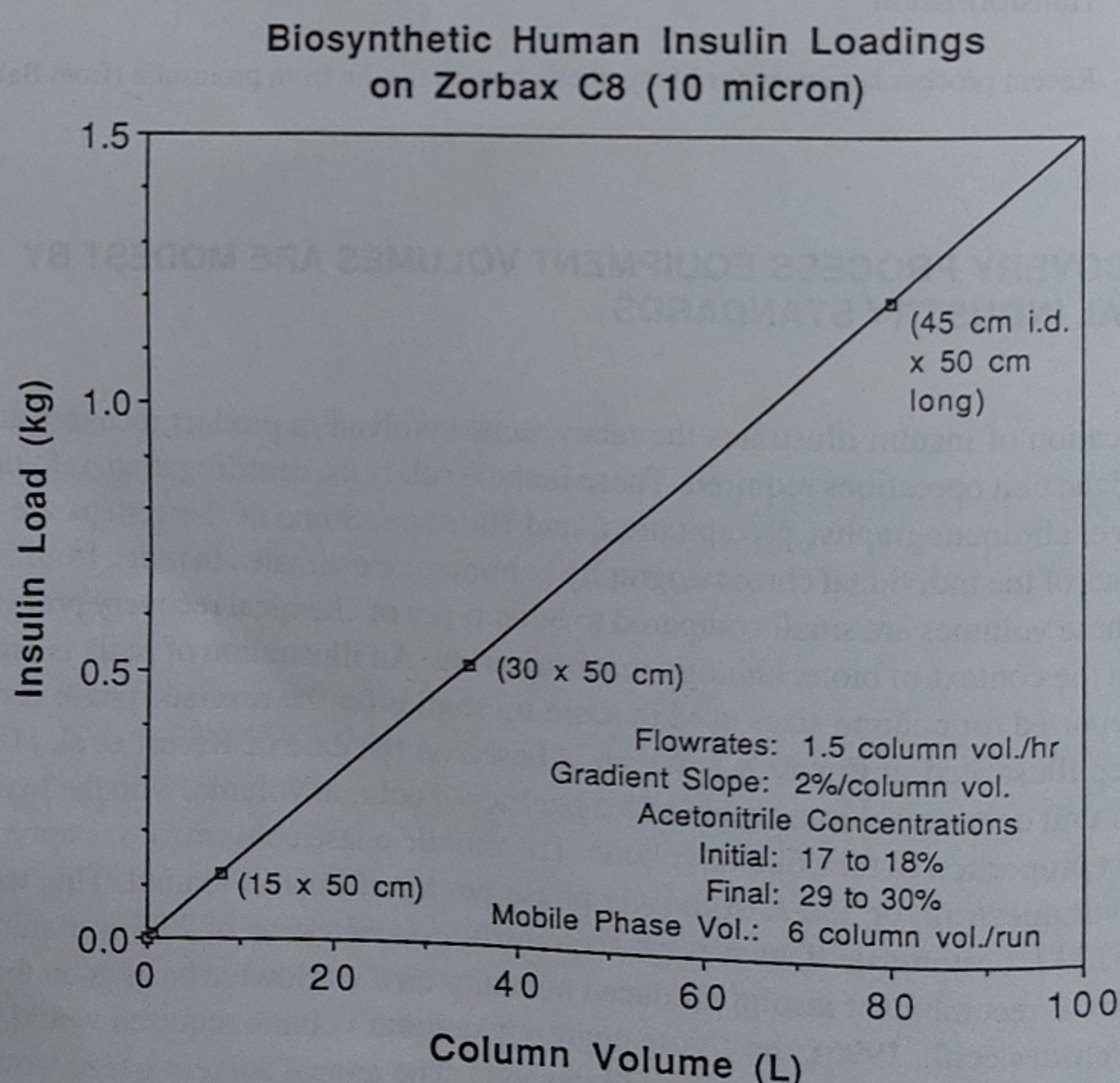


Figure 8.7 Insulin load as a function of column volume for reversed phase chromatography of biosynthetic human insulin over 10 micron diameter, Zorbax, process grade, C₈ reversed phase material. Column dimensions given in parenthesis (data from Kroeff et al., 1989).

8.9 YIELD LOSSES ARE AMPLIFIED BY THE NUMBER OF PURIFICATION STEPS

The numerous steps required for production of this recombinant protein incur a built-in yield loss. For example, if only 2% yield loss were to be associated with each step, the overall yield for a purification sequence of 10 steps would be

$$\eta = 100 \left(1 - \frac{L}{100}\right)^n = 100 (1 - 0.02)^{10} = 81.7\% \quad (8.1)$$

where η denotes percent yield, L the percent yield loss, and n the number of steps. If the yield loss at each step were 5%, the overall yield would only be 60%. Maximizing recovery at each step is important. The yields for each part of the process in Figure 8.3 range from 60 to 90%, with an overall yield of 29% being indicated.

During the recovery and purification of human insulin, its molecular structure, and hence, physicochemical properties, is altered five times during its recovery and purification. These forms are as follows:

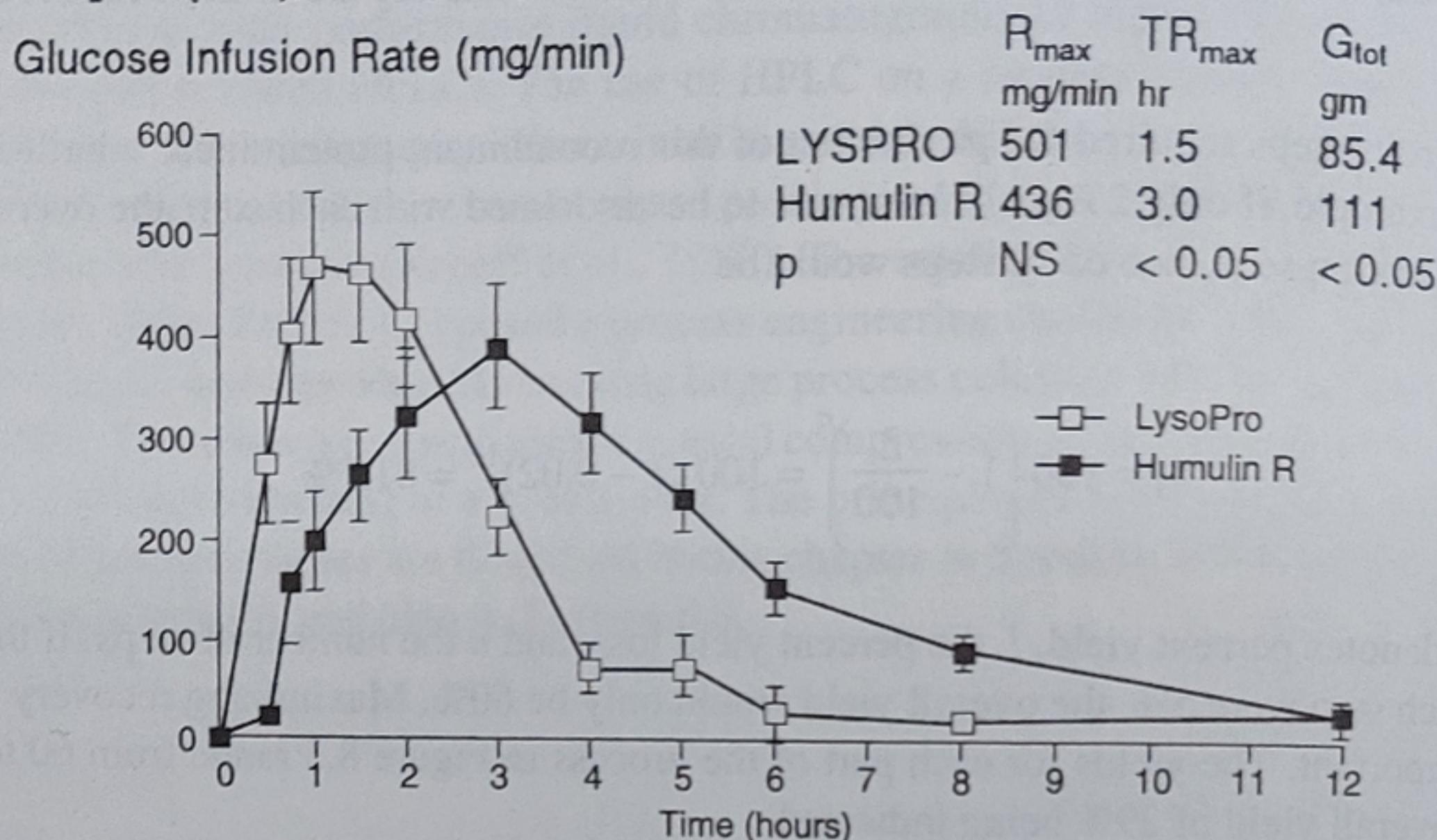
1. Fusion protein
2. Denatured aggregate
3. Denatured monomers
4. Properly folded proinsulin
5. Insulin

Prouty (of the Lilly research laboratories) points out that "the changes in properties, such as pI, size, and hydrophobicity, present an opportunity to use various purification procedures repeatedly in the process, and expect them to purify away different contaminants at each stage. Although an apparent disadvantage because it introduces more steps in the process, the multiple forms of insulin generated in the process maximize the chance of removing contaminants that are not likely to change chemically in the same way as the insulin molecule. The final purification after transformation of proinsulin to insulin utilizes procedures that rely on multiple properties of the insulin, such as size, hydrophobicity, ionic charge, and crystallizability, further assuring the removal of contaminants, even below the parts per million range" (from Prouty, 1991, p. 237). The final purity level is reported to be > 99.99% (Builder et al., 1989).

8.10 LYS(B28), PRO(B29) BIOSYNTHETIC HUMAN INSULIN IS A HUMAN INSULIN ANALOG

Another recombinant protein has been developed and has recently entered the insulin market. This analogue of insulin is chemically identical to human insulin except that its sequence on the B chain has been altered so that the order of the amino acids in the B28 (i.e., insulin B chain, residue 28) and B29 positions are reversed to give a Lys-Pro instead of a Pro-Lys sequence. This insulin analogue has a more rapid onset of activity and rapid clearance than human insulin (Humulin R®), as shown in Figure 8.8, and it will enable insulin, taken 10 to 20 minutes before a meal, to be effective in controlling glucose. The lag time

[LYS(B28), PRO(B29)] - Human Insulin Analog: Study IMAA



D.C. Howey, M.D., et al.
Lilly Research Laboratories

Figure 8.8 LysPro insulin's action is more rapid than human insulin (from Baker, 1996).

for LysPro insulin is less, and it translates into potentially better control of blood sugar levels in a diabetic (Baker, 1996).

8.11 THE FRONT-END STRATEGY FOR MANUFACTURE OF LYSPRO INSULIN IS DIFFERENT FROM BIOSYNTHETIC HUMAN INSULIN

LysPro insulin is also produced through fermentation of recombinant *E. coli*. However, its manufacture eliminates the need for CNBr. The removal of the *N*-terminal extension (abbreviated as MX in Figure 8.9) is achieved using a diamino peptidase from *Dyctiostelium discoidium* (Atkinson et al., 1995). The molecular weight of the fusion protein is lower, and the process of transforming the target molecule—from granule solubilization to enzymatic transformation—to an active insulin analog molecule is somewhat simplified (Figure 8.10). The purification steps for the insulin analogue that follow enzymatic transformation are likely to be similar to other insulin purification sequences.

TISSUE PLASMINOGEN ACTIVATOR

This example is also taken from a product developed during the early days of the industry. It illustrates how a biochemically complex molecule, much larger than insulin, was produced and tested. This case study also illustrates the effect of regulations on the product and process development. The intent is to introduce the student to effects of regulations, rather than the regulations themselves, which have changed over the last 15 years.