Production of Therapeutic Polypeptides through *Escherichia coli* : A Perspective

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INTRODUCTION

During the recent years, a large number of eukaryotic proteins have been reported to have been expressed in prokaryotic systems. Most of these expressions have been in *E. coli* (1). A protein hormone, Insulin produced by recombinant DNA(R-DNA) technology is available commercially during the last 5 years or so. Others like Somatotropin, Betaendorphin, Interleukin, Urokinase, Interferon etc. produced through R-DNA technology are expected to be available commerically in near futurc at reasonable prices. Thcse polypeptides having diverse cllnical use, are not availablc in large quantities from the natural sources. Production of these in large quantities is possible by gene cloning and cxpression in microorganisms like *E. coli*, yeast or *B. subtilis*. The eukaryotic polypeptides thus synthesised in prokaryotic systems are either located in cytoplasm of the microorganism or secreted out into the medium through the cell membrane. As per the current knowledge, most of the polypeptides are however expressed intracellularly (1). This apart, the proteins are also accumulated as insoluble aggregates (2), although many of the authentic eukaryotic proteins are soluble ones. The isolation process thus requires solublization and purification followed by refolding into their native states. Both the solubilization and the refolding steps are critical as inactivation possibilities during processing are very high.

As stated earlier, for the eukaryotic proteins that have been expressed in prokaryotic system, most of the work has been carried out on *E. coli*. Some of the eukaryotic proteins expressed in *E. coli* are secretory in nature. The process of secretion of proteins into the extracellular medium has not yet been well established. Moreover, in a few cases, where secretion has been possible, the extent of secretion has been low except in a few isolated instances e. g. betaendorphin (3) and A-alpha-fibrinogen (4).

As the mode of expression of eukaryotic polypeptide in *E. coli* is predominantly intracellular, the discussion in this paper has been limited to the outline of the expression, isolation and purification techniques of intracellular polypeptides synthesised in *E. coli*. It is clearly foreseen that the mainstay of production of eukaryotic therapeutic polypeptides would be through the R–DNA technology. The review would indicate the kind of multidisciplinary interactions required for converting the science into a technology.

Outline of R-DNA process :

It is perhaps necessary at this stage to outline the processes involved for eukaryotic

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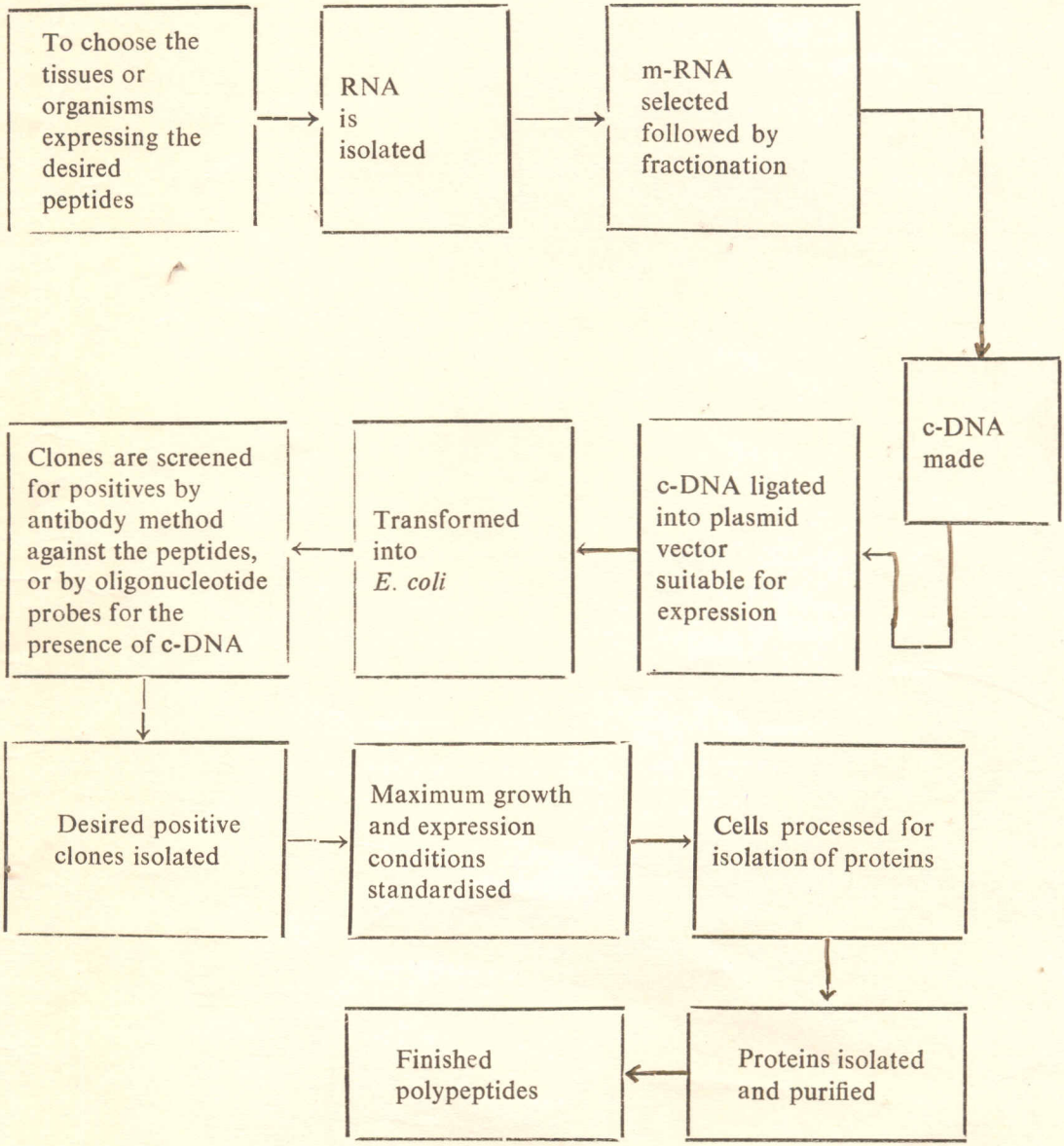
gene expression in *E. coli*. Eukaryotic genes are not easily expressed in *E. coli* for a number of reasons such as the inability of the protein chain to fold correctly in a foreign environment, presence of introns, susceptibility of the foreign polypeptides to the proteases present in *E. coli* which try to "chew up" the so called abnormal proteins etc. Each problem needs individual methods to tackle. In general the outline of the processes undergone for eukaryotic gene cloning and expression in *E. coli* involve the following steps:



Eukaryotic polypeptides expressed in *E. coli* : Eukaryotic polypeptides could be directly expressed in *E. coli* (direct expression proteins). However, the expression is often along with bacterial terminal peptides covalently linked to it (Fusion proteins). Eukaryotic polypeptides expressed in *E. coli* has been reported to get accumulated in the total cell proteins from small levels of about 0·05% for Somatostatin (5) to 20% each for Insulin A Chain (6) or Insulin B Chain (6) and as high as upto 25% for gamma Interferons (7). The factors for these low or higher levels of expression for various polypeptides are not yet fully understood. The following table indicates the few important eukaryotic polypeptides expressed in *E. coli*, % expression, location of the protein on cell-lysis and the number of cysteine residues.

Problems in handling recombinant polypeptides

Eukaryotic proteins which are foreign to the natural bacteria get sequestered into its inclusion bodies. Moreover the recombinant proteins are usually insoluble as well. Hence their isolation and purification procedures become extremely typical for each type of protein handled.

Direct expression proteins : Initially the proteins need to be solubiliscd. Several solubilizing agents have been used as indicated below (1)

Once the proteins are solubilized, they can be purified by solvent partitioning, high speed centrifugation or various chromatographic techniques. Specific purification steps have been found suitable for each kind of protein and there is no universal method applicable to all. Purified proteins are thereafter brought to appropriate conditions so as to enable them to refold to native states. Important factors that have been found suitable for adjustment of such conditions are dialysis, dilution and pH adjustments. Here also the extent of refolding as well as the loss by inactivation depends upon the protein being processed as well as the conditions it is subjected to.

Table 1

S. No.	Polypeptides	Mode of expression	Expression	Location on cell lysis	No. of cysteine residues
1.	AIDS peptide 121 (8)	Direct	5-10	Pellet	2
2.	A polipoprotein E (9)	—do—	1	—do—	1
3.	Calcitonin (10)	Fusion	17	—do—	2
4.	Calf prochymosin (11)	Direct	8	—do—	6
5.	Endorphin-beta (12)	Fusion	5	—do—	0
6.	Growth hormone-human (13)	—do—	5	—do—	2
7.	Growth hormone-bovine (14)	—do—	5	—do—	2
8.	Interferon-beta (15)	Direct	15	—do—	3
9.	Interferon-gamma (7)	—do—	25	Supernatant	2
10.	Insulin A-chain (6)	Fusion	20	Pellet	4
11.	Insulin B-chain (6)	—do—	20	—do—	2
12.	Interleukin-2 (16)	Direct	10	Supernatant	7
13.	Myoglobin (17)	Fusion	10	Pellet	0
14.	Urokinase (18)	Direct	Not estimated	—do—	24

Solubilizing agents	Concentration	Peptides that are solubilized
(a) Alkaline conditions	PH ⩾ 9	– Prochymosin
(b) Detergents	Varies	– Beta IFN IL–2
(c) Guanidinium chloride	8 Molar	– Bovine Growth hormone insulin A chain insulin B chain Urokinase
(d) Urea	6–8 Molar	– Gamma IFN – Light & Heavy Chains of IgG – Prochymosin – Salmon growth hormone

Fusion proteins : The success in the expression of comparatively low molecular weight eukaryotic proteins in *E. coli* is greatly prospected by combining specific eukaryotic genes with bacterial genes. Consequently this process produces expressions where eukaryotic proteins are fused with bacterial proteins as well. By this method was expressed somatostatin, insulin, betaendorphin etc. (1). However, the problems in these kinds of expression are that if the eukaryotic gene constitutes a small proportion of the prokaryotic gene, then the quantity of the eukaryotic protein would be a small fraction of the total protein produced. For low molecular weight eukaryotic proteins, this is unavoidable as direct expression of eukaryotic proteins of low molecular weight are probably recognised and degraded by the bacterial system. Fusion proteins are sometimes constructed by choice also. Certain bacterial genes producing specific polypeptides whose purification processes are already known, could be hooked to the eukaryotic genes for facilitating eukaryotic expression fused with known bacterial proteins. This is done in situations where the fusion proteins aid in the purification process.

Fusion proteins are first isolated from the rest of the cell debri and thereafter subjected to purification methods such as ultracentrtifugation, ion exchange, gel filtration, immuno purification etc. Detergents and denaturants are used in the buffer during the purification steps. The inclusion bodies are then separated by clevage using certain chemicals like cyanogen bromide, formic acid etc.,or enzymes like trypsin, collagenase, clostripain, factor Xa etc. The sequences recognised by various cleaving agents are indicated below

As examples of using a typical sequence for the production of eukaryotic proteins in *E. coli*. the production of human betaglobin and myoglobin have been effected with the sequence – Ile – Glu – Gly – Arg – Xaa – to a lambda c II protein fragment. The fusion proteins were solubilized in urea followed by purification by ion exchange chromotography and gel filtration (21, 17). The denaturants were then removed by dialysis whereafter clevage was effected with factor Xa followed by refolding of the protein.

Separation and purification methods : Recombinant proteins meant for human use

Cleaving Agents	Sequences recognised
Cyanogen Bromide (6)	– Met↓—X aa –
Strong Acid (19)	– Asp↓—Pro –
Trypsin (12)	– Arg↓Xaa –
	– Lys↓—Xaa –
Collagenase (20)	– Pro↓— Xaa – Gly – Pro – Yaa↓—
Clostripain (10)	– Arg↓— Xaa –
Factor Xa (21)	– Ile – Glu – Gly – Arg ↓ Xaa

Note : Arrow (↓) indicates the cleaving site.

must be produced at the highest level of purity. While the recombinant techniques could offer highest levels of prokaryotic expression, the purity requirements would probably demand more than 99% criterion. Consequently, the isolation methods would have stronger emphasis on trying to get rid of the last traces of the 1% contaminants.

To isolate the desired proteins from *E. coli* cells, the latter are concentrated by say filtration or centrifugation. The cells are then disintegrated and homogenised. Various methods such as bead-milling, vigorous sonication, grinding with inert abrassives, use of lysozomes etc. are practiced. Proteolytic inhibitors may be added into the soup. The half-life of the desired proteins could be very short from a few minutes to a few hours. Consequently the homogenates must be processed at the earliest. The homogenates may also be susceptable to temperature. Therefore, as far as possible, the processing may be carried out at low temperature.

There are 5 principal ways of attaining separation of proteins which are precipitation, partitioning, adsorption, gel filtration and electrophoretic methods. Usually a combination of these would have to be chosen on a case by case basis for attaining the desired purity in the finished products. A brief description of each of these methods is given below (22) :

Precipitation : The protein molecules have hydrophobic as well as hydrophilic residues on their surface. These residues determine their solubilities in various solvents. Protein solutions are usually aqueous based. By altering the temperature, pH, ionic strength, addition of water miscible solvents, solutes and certain polymers, the solvent properties of water towards protein solubility could be manipulated. Of all the materials studied, the use of neutral salts have been most

extensive. Salts containing cations such as ammonium, potassium or sodium and anions like sulphates, phosphates and citrates have largely been employed, and ammonium sulphate has most extensively been used. Precipitation of proteins by use of ammonium sulphate also stabilizes the denaturation of protein to a great extent. Saturated ammonium sulphate solution at room temperature forms approximately 52% or 4 M solution (density = 1·235 gms per c c.). By use of solid ammonium sulphate or even its saturated solution, various salt concentration upto about 50% could be maintained in the protein solution aliquots and thus proteins could be fractionated to some extent. Each fraction of the precipitate, redissolved in water containing considerable quantity of ammonium sulphate could be subjected to dialysis or gel filtration for removing the salts and thereafter could be taken for further processing or fractionation using other methods.

Protein solution could also be fractionated or precipitated by using water miscible organic solvents like acetone, ethyl alcohol, methyl alcohol, propanol, dioxane, ether etc. Advantage of using organic solvents is that the operation could be carried out at sub zero temperatures. Solvent precipitation is an additional step of protein fractionation which could be used with advantage in certain situations. However, use of this step at room temperature and above runs the increased risk of denaturation of proteins.

Besides salt and organic solvent precipitation, several high molecular weight water soluble polymers have been used to precipitate proteins (23). Of these, polyethylene glycol (PEG) of molecular weight range from 4000 to 20000 have been entensively used. Purification of PEG precipitated proteins by chromatography or ion exchange or gel filtration eliminates PEG from the protein and it does not interfere. Polyelectrolytes (24, 25), cross linked poly-acrylates (25) and salts of caprylic acid (24) have also been used for various protein fractionation and purification.

Partitioning: Aqueous solutions containing pairs of organic polymers in certain minimum concentration separate into two phases (26). Aqueous solution containing one organic polymer and a salt also separate into two phases. Various such systems are already reported (27). Typical systems widely studied are dextran (Dx) – PEG – water system and PEG–inorganic salt–water system. Proteins when contacted with such system get distributed into phases depending upon their solubility and partitioning characteristics. The pH of the system greatly affects the partition coefficients of the proteins and therefore water distribution. In Dx-PEG-water system, the Dx-water phase settling at the bottom aggregate the cell debris and most of the proteins. In such system when a portion of the PEG terminals containing the 2 hydroxy groups are modified by attaching protein specific ligands such as p-aminobenzamidine, palmitate, estradiol, dinitrophenol and various dyes, the specific proteins get concentrated into the upper PEG-water phase along with the modified PEG-molecules. By choosing proper conditions and concentrations of the modified PEG molecules, this method has offered separation of a large number of specific proteins from the debris (28).

Phase partitioning technique offers gentler treatment of the protein molecules in liquid phase, is very fast in operation and can be used on large quantity of materials. The system can be operated on continous system by proper adjustments. Engineering, utilizing continous phase partitioning, is well developed. Consequently this single method offers a great prospect of being used on commercial scale.

Adsorption :— Adsorption techniques commonly known as chromatographic technique using a variety of solid phases have been used for purifying proteins. The adsorb ing solid phase in the form of beads is packed in columns through which the protein solution is made to elute. Materials used as adsorbants are ion-exchangers, calcium phosphate gel and ligands attached to natural or synthetic polymers. In the presence of a flowing phase, the mobilities of the solute including the protein molecules are altered. Such alteration occur due to varying partition coefficients of various molecules between the solvent and the stationary solid phase. During travel over the solid phase, the flowing molecules equilibrate with the solid stationary phase and travel at the same relative average speed. Thus in an oversimplified presentation, bands of similar molecules get separated from others over a period of time and either remain adsorbed on the column or get eluted depending upon the condition used, the proteins to be separated, the types of adsorbents used, kinds of ligands attached etc. Usually the proteins get adsorbed and are eluted out of the eolumn by changing the pH of the eluting buffer or by changing its ionic strength. The latter method is more commonly applied for getting the desired product recovery.

Various types of ion exchangers have been in commercial use. Diethylamino ethyl (DEAE) group has been attached to varlous polymers like agarose, dextran, cellulose and other synthetic polymers to get anion exchangers. Similary by attaching carboxymethyl (CM) group, cation exchangers have been made. Cross linking has been effected in the polymer backbone to get more stable materials. Both cation and anion exchanger beads based on eellulose, dextran, agarose and trisacryl are being marketed by a large number ef companies like Pharmacia, LKB, Whatman, Bio-Rad Laboratories etc.

Several bio-affinity properties between pairs of biomaterials like antigen–antibody, harmone receptor, enzyme-substrate etc. are very specific and strong. immuno affinity chromatography using monoclonal antibodies has been used for the purification of recombinant human interferon and the results are very interesting as indicated below (29).

It could be seen that affinity column has achieved a purification of 1150 times over ammonium sulphate fraction with a loss of only 5% active material.

High performance liquid chromatography (HPLC) using various strong small cross-linked beads has been applied to protein separation on preparatory scale. However this method is very expensive and is limited to use when the final product requirements are less than say 5 mgs. The columns used are packed with very small beads which offer high resistance to the flow of liquids;

Purification of Recombinant Interferon

Fractionation step	Total protein	Total activity (units)	Specific activity (units/mg)	Recovery (%)
1. Ammonium Sulphate	37·1 gms	$7{\cdot}4\times10^{9}$	$2{\cdot}0\times10^{5}$	100 (Assumed)
2. Antibody Column	30 mg	$7{\cdot}0\times10^{9}$	$2{\cdot}3\times10^{8}$	95
3. CM 52	20 mg	$6{\cdot}0\times10^{9}$	$3{\cdot}0\times10^{8}$	85

consequently the system has to be operated under very high pressure. The operations are however quick and do not require equilibrium conditions to be maintained between the flowing molecules and the adsorbants. The method is more useful as an analytical tool than as a system for preparative scale.

Gel Filtration : Gels are cross linked three dimensional net work of various natural or synthetic polymers like cellulose, dextran, agarose, polyacrylamide, polytrisacrylamide etc. in the form of beads. The pores within the beads are of various dimensions. All small molecules can penetrate and pass; however any specific grade of material has certain range of pores which are accessible to particular size range of macromolecules. Therefore, in a pool of protein molecules of varying molecular weights and therefore sizes, all are not able to penetrate and pass through the pores. However the gel materials available are such that gradation could be made of molecules of size range 4 to 6 times greater or smaller. Gradation of molecules lower than these size distribution may not be sharp.

While applying separation by gel filtration, the column height and column diameter are of great importance. Usually the length is 20 to 50 times the diameter of the column. Gel filtration offers a useful but lengthy and slow process for protein purification on a preparative scale.

Electrophoretic methods : All protein molecules have some net average charge at all pH except at isoelectric pH. Utilizing this charge, a protein molecule can be made to move in an applied electrical field. Electrophoretic methods of protein separation are based on this basic concept. These methods cannot be routinely employed for

A large number of gel beads are commercially available which are suitable for certain ranges of globular protein separations. Some of these are given below :

Types of Gel material	Company marketing the Gels Name	Gel code	Useful molecular weight range of globular proteius	
Agarose	Biogels	A–0·5m	1×10^3	-5×10^5
		A–1·5m	2×10^3	$-1\cdot5\times10^6$
		A–5·0m	4×10^3	-5×10^6
Agarose-Crosslinked	Pharmacia	6B.CL–6B	1×10^4	-4×10^6
Agarose-Polyacrylamide	LKB	Ac A 54	5×10^3	-7×10^4
		Ac A 44	1×10^4	-7×10^5
		Ac A 34	2×10^4	$-3\cdot5\times10^5$
		Ac A 22	1×10^5	$-1\cdot2\times10^6$
Cellulose	Amicon	GC–200	1×10^3	-3×10^4
Dextran	Pharmacia Sephadexes	G–50	$1\cdot5\times10^3$	-3×10^4
		G–75	3×10^3	-7×10^4
		G–100	4×10^3	-1.5×10^5
		G–200	1×10^4	-6×10^5
Polyacrylamide	Biogels	P–60	3×10^3	-6×10^4
		P–150	$1\cdot5\times10^4$	$-1\cdot5\times10^5$
		P–300	6×10^4	-4×10^5

protein separation on preparative scale, but could be used preferably as analytical tools. However, before closing this paragraph, a mention is made of a few important electrophoretic methods such as affinity electrophoresis, isoelectric focussing and isotachophoresis methods which have been used to some cases of protein separation.

In affinity electrophoresis, ligands specific to proteins are introduced either into the buffer to alter the mobility of the protein e. g. separation of trypsin (30) or immobilizing the ligand into electrophoretic gel. The ligands interfere with movement of the protein. Methods using this principle have been used for separating glycoproteins (31). A review on the subject could be of interest to know the future prospects of the method (32).

Isoelectric focussing technique uses a pH gradient in a constant electric field. The proteins are made to move under the influence of the potential difference across thc electrodes. The proteins move till they reach their isoelectric pH zones. The pH gradient is established by use of polymeric buffer compounds called ampholytes. Under the influence of voltage difference between the cathode and the anode of the focussing column or slab, the ampholytes move and form a pH gradient bed which is subsequently used for protein resolution. The method is used for purification of proteins on preparative scale. However the method is extremely expensive. The ampholytes are proprietory and supplied by a few manufacturers like Pharmacia (Pharmalites) and LKB (Ampholines).

In isotechnophoresis method, proteins are made to move at the same speed over a pH gradient column or slab through an electrical field of varying strength at the location of each component. The separation is on tbe basis of mobility per unit of electric field applied. Voltage gradients are not required to be applied at various points but they get developed automatically due to the nature of the charges on the protein molecules. The method has been used with advantage in the separation and isolation of human growth hormones (33).

Concluding Remarks : Low natural abundance of many proteins required in the therapy of human like insulin, interferons, interleukins etc. would call for production of these through R-DNA technology in prokaryotic system for meeting the growing demands. Genetically engineered high expression commercial strains of *E. coli* would be produced and made available as a commercial commodity in years to come. While systems would be developed for extracellular expression, for the time being the processes would concentrate upon processing of intracellular proteins from *E. coli*. Such issues as the proper storage and handl ng of R-DNA cells would get adequately solved and special working environments will get standardised with more technological inputs. More inputs would pour in, considering the large derivable potential benefits compared to the anticipated risks.

Industrial processes of isolation and purification would harbour around techniques and processes which are reproducible, simple and as far as possible could be operated upon smoothly. Laboratory processes which would involve some possible chains of steps like salt precipitation followed by redissolution, gel filtration and ion exchange, or solvent precipitation followed by redissolution and ion exchange (or affinity absorption) followed by salt precipitation further followed by dissolution, gel filtration and isolation etc. may be meant for handling small quantities of proteins. Industrial scale production may involve direct concentration by partitioning using say PEG (with ligand modified PEG)–salt-water system on the disintegratcd cell soup.

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The concentrate after cleaning could be hooked up to continous column chromatography using appropriate system. Besides handling large quantities, the system would also require very precise resolution so as to get rid of the last 1% of the contaminating materials. Disintegrated *E. coli* cell soup containing pyrogenic lipopolysaccharides which may remain in the final product must be got rid of by chromatography using polymixin B-sepharose, DEAE sepharose (1) etc. Finally the isolated protein must be made to properly refold for maintaining its biological activity.

It is clear from the above that technology development for production of eukaryotic proteins in prokaryotic system would involve active inter-disciplinary interaction among biologists, chemical engineers and instrumentation specialists in particular. It is probably impossible to strive for a process development with only one group working in isolation of the other. Since such inter-disciplinary interactions would require considerable money, efforts and coordination, only resourceful enterprises with determined intentions of product development in these areas may succeed in their venture in the shortest possible time.

ABSTRACT

During the recent years a large number of eukaryotic proteins have been expressed in prokaryotic system. This method offers the possibility of availability of these proteins in unlimited quantities. The paper reviews the literature on the various published methods of expression of eukaryotic polypeptides in *E. coli*. It also outlines the methods of isolation and separation of proteins from a complex mixture of cell debri. The paper concludes that for converting the science of gene cloning, expression and isolation of proteins requires intimate inter-action among microbiologists, chemical engineers and instrumentation specialists for converting the science into a technology. Thirty three references have been cited.

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