PROTEIN PURIFICATION: MICRO TO MACRO

Richard Burgess, Organizer
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Plenary Sessions Page

Posters

Micropurification and Analysis

M 001 MICROPURIFICATION OF CYTOKINES, Bharat B. Aggarwal, Department of Molecular Biology and Biochemistry, Genentech Inc., 460 Point San Bruno Blvd., South San Francisco, CA 94080

Lymphokines and monokines are the cytokines produced in trace amounts by activated lymphocytes and monocytes respectively. Several investigators have attempted isolation of cytokines from normal peripheral blood leukocytes, but have achieved only minimal success due to the small amounts and heterogeneity of the secreted molecules. Tumor Necrosis Factors (TNFs) $-\alpha$ and $-\beta$ are two such cytokines which we have purified and characterized. Several human cell lines were screened for the suitable source of TNFs and it was found that the promyelocytic cell line HL-60 and the B-lymphoblastoid cell line RPMI-1788 are good sources for TNF-α and TNF-β respectively. These cell lines can be grown on a large scale to provide sufficient starting material for isolation of the cytokines.

Special procedures were developed to purify these trace proteins from large volumes of cell conditioned media. This was essential since the activity of these proteins is labile. Our purification procedures included removal of cells from hundreds of liters of media by filtration through Pall Sealkleen 3μM filter, followed by batch adsorption of the given cytokine activity to controlled pore glass beads. The binding to the beads could then be reversed by ethylene glycol. This step was followed by DEAE ion-exchange chromatography, MONO Q- and Chromatofocusing fast protein liquid chromatography and reverse phase high performance liquid chromatography. Preparative denaturing and non-denaturing polyacrylamide gel electrophoresis were also employed to purify these cytokines. These procedures have provided materials of high purity rapidly and reproducibly. The purity of cytokines was determined by amino acid sequence analysis.The physicochemical characteristics of these highly purified cytokines will be discussed.

M 002 INSTRUMENT AND SAMPLING OPTIMIZATION FOR MICROSAMPLE ANALYSIS, K.J. Wilson, Applied Biosystems, 850 Lincoln Centre Drive, Foster City, CA 94404.

The isolation and chemical analysis of biological samples are demanding procedures often complicated by limited sample availability. Conventional sample handling techniques add to the problems by exhibiting poor recoveries at the ug level and extensive dilution of the desired sample. The need for sample concentration, dialysis or lyophilization subsequently increases the risk of chemically modifying, contaminating and/or losing the sample. Each of these methods obviously limits the attainable sensitivity and quantitative value of the data.

Narrow and microbore HPLC has proven useful for analyzing, as well as isolating, at or below the μg level. Samples present in relatively large volumes (mLs) can be conveniently concentrated to <30 μL. In a similar manner they can be recovered in such volumes following any chemical modification which required denaturants, buffers, and/or reagents. The utility of reduced column diameters and lengths improves overall yields, especially for the more hydrophobic proteins and peptides.

Direct collection of samples from microbore HPLC separations onto appropriate surfaces circumvents many recovery problems. Glass fiber discs provide a physical support ideal for various micro-chemical characterization techniques. Such a collection system elminiates the need for sample transfer, greatly reducing the risk of sample loss or contamination.

The immobilized sample can be submitted for multiple analyses simply by dividing the disk appropriately. For example, one portion of a disc can be used for Edman sequencing while another is hydrolyzed and the concentrations of the amino acids determined. Similarly, a portion can be analyzed directly by SDS-PAGE or chemical reactions such as oxidations or cleavages can be performed directly on the disk. The utility of the discs, serving as support or containment surface, is multi-fold and with the appropriate cleanup, they are even reusable.

M 003 PURIFICATION OF SYNTHETIC PROTEINS, David D.L. Woo, Medicine, UCLA, CA, Ian Clarke-Lewis and Steven B.H. Kent, Division, CalTech, Pasadena CA.

Total chemical synthesis of small proteins (up to 20,000 daltons) and their analogs is a powerful and practical research approach to structure-function analysis of rare proteins. On rare occasions, synthetic proteins will spontaneously assume their active forms after release from the solid-phase synthesis resin. More often, they are inactive immediately after synthesis and initial isolation. They require deliberate refolding to produce the active forms. Refolding methods used will vary depending on properties of individual proteins, experimental needs and investigator's preferences. We have learned and applied modern principles of refolding, criteria for purity and methods of analysis from our recent success in obtaining sufficient active synthetic human transforming growth factor-alpha (TGF-α) for structure-activity analysis.

Human TGF-α was synthesized on a fully automated peptide synthesizer (Applied Biosystem, 430A) reprogrammed to use a double coupling protocol. Preformed symmetrical anhydride of protected amino acids (t-Boc AA's) in dimethylformamide was used in the first coupling, followed by in situ activation of t-Boc AA's in dichloromethane during the second coupling step. Using technique of quantitative Edman degradation (preview sequencing), the yield of full length target sequence was 84.5% , representing an average coupling yield of 99.65% per residue. The peptide was then cleaved and deprotected. This sample, when analyzed on HPLC (Vydac C4 column), showed a single major peak, representing 44% of the total product. The major peak was isolated using preparative HPLC. The purified peptide had no biological activity at this stage and was refolded under controlled oxidative conditions in guanidium hydrochloride. After refolding several forms of TGF- α were apparent. They were separated from each other by preparative HPLC and their activities measured. One peak was found to have activities indistinguishable from isolated natural murine EGF in receptor binding, mitogenic and soft agar colony formation assays. The purified product focussed as a single band at pI = 6.2 on Immobiline gels, and displayed a MW = 5,546.2 (Th. = 5546.3) by mass spectrometry. The locations of the three disulfide bonds in this active, synthetic human TGF-α were determined and were found to be analogus to those in epidermal growth factors.

Protecting Proteins During Purification and Storage

M 004 MICROPURIFICATION OF PROTEINS BY ELUTION FROM SDS POLYACRYLAMIDE GELS AND RENATURATION, Richard R. Burgess, McArdle Laboratory for Cancer Research, University of Wisconsin, Madison, Wisconsin 53706.

Several years ago we published a procedure for micropurification of proteins (1). This procedure utilizes the high resolution fractionation of polypeptides by SDS polyacrylamide gel electrophoresis. Individual bands containing a microgram or less of protein are eluted from the gel. The SDS is removed and the protein concentrated by acetone precipitation. The precipitated protein is then completely denatured with GuHCl and the enzyme activity restored by dilution. This procedure has been used successfully to isolate small quantities of a variety of enzymes. Improvements of this procedure and several recent examples of its use will be presented. Limitations and extensions of this procedure will be discussed.

1) Hager, D. A. and R. R. Burgess. Anal Biochem., 109: 76-86 (1980).

Bulk Precipitation and Phase Partitioning Methods

M 005 PROTEIN SEPARATIONS USING REVERSED MICELLES, T. Alan Hatton, Department of Chemical Engineering, Massachusetts Institute of Technology, Cambridge, MA 02139.

The recovery of biological products from dilute fermentation and cell-culture media poses a obstacle to the successful realisation of the promises afforded by the dramatic advances made in the "new" biotechnology in recent years. The traditional tools used by the biochemist, such as chromatography, are difficult to scale-up effectively, and are characteristically batch-type processes. Liquid extraction techniques, on the other hand, are readily scalable and can be operated in the continuous mode, although the lack of suitable, selective solvents for labile proteins has inhibited their wide-scale use in biopolymer separations.

We have been investigating a new class of solvents which show promise in the selective extraction of biologicals from dilute aqueous solution. These are based on the observation that certain surfactants form aggregates, called "reversed micelles," which can effectively solubilise significant quantities of water in organic media, thereby providing a benign environment for the solubilisation of proteins in otherwise inhospitable apolar solvents. Factors affecting the solubilisation of the proteins in organic solvents will be discussed, and rationalised based on electrostatic and hydrophobic interactions between the proteins and the charged, inner micelle walls. The ability of these reversed micelle solutions to resolve a ternary mixture of equi-sized proteins has been established, as has the fact that frequently the recovered biopolymers retain a significant degree of enzymatic activity. In addition, the selective recovery of low molecular weight biomolecules such as amino acids using reversed micelle solutions appears to be a fruitful area for exploitation.

M 006 THE USE OF POLYETHYLENEIMINE IN PROTEIN PURIFICATION, Jerry Jendrisak, Promega Biotec, Madison, WI 53711.

The use of polyethyleneimine (PEI or Polymin P^R) as a reagent for protein purification is discussed. PEI, which has the structure:

$$H_2N{\cdot}x(C_2H_4NH)C_2H_4NH_2,$$

and a molecular weight of 30-40,000 (x=700-900), is a molecule with a high density of linear positive charge in solutions at neutral pH values (the pKa value of the imino groups is 10-11). This property makes PEI a very effective agent for the precipitation of nucleic acids in crude cellular extracts (presumably due to cooperative electrostatic charge attraction). PEI-nucleic acid complexes are stable at high salt concentrations (e.g. 1.0 M sodium chloride).

PEI has been exploited as a selective protein fractionation agent. PEI is capable of precipitating a subset of cellular proteins along with nucleic acids under low ionic strength conditions. Proteins are extracted (eluted) and separated from the PEI-nucleic acid-protein complex (precipitate) with buffers of higher ionic strength followed by centrifugation. The method is applicable from the micro scale to very large scale purifications. PEI is inexpensive, is effective at low concentrations, and poses no waste disposal or reagent recovery problems. Fractionations are carried out at neutral pH values and enzymes are recovered with good yields and increases in specific activity, and are free of nucleic acid contamination. The PEI fractionation step can complement other fractional precipitation methods (where proteins are precipitated due to other chemical and physical properties) to afford efficient, inexpensive purification prior to the use of column chromatographic steps. Specific examples of enzyme purification with a PEI step will be presented. Finally, our understanding of the precipitation-elution process will be discussed with emphasis on suggestions for further experimentation and refinements in the use of PEI for protein purification.

M 007 THREE PHASE PARTITIONING (TPP) VIA t-BUTANOL, AQUEOUS SALT SYSTEMS FOR ENZYMES ISOLATION. Rex Lovrien and Craig Goldensoph; Biochemistry Department, University of Minnesota, St. Paul, MN 55108.

Tertiary butanol is infinitely soluble in neat water but this alcohol forms a second phase with ca. 25% or more aqueous salts. If the beginning aqueous phase contains crude enzymes or proteins, frequently the desired enzyme or protein comes out of solution and forms a third phase on mixing all components, then allowing settlement. These third phases often are gels containing the desired enzyme which position themselves between an upper (t-butanol) layer and lower (aqueous salt) layer on low speed centrifugation. Unwanted pigments, proteins, and carbohydrates get shunted to the upper (t-butanol) or lower (aqueous) layer. Hence for numbers of enzymes, 3X to 10X increases in specific activity (S.A.) often were retrieved, and rather often comparable increases in total activity (T.A.) also were yielded. Cellulases, some α-amylases, a peroxidase, two proteases, a protein enzyme inhibitor (Bowman-Birk), invertase, β-galactosidase, β-glucosidase, amyloglucosidase have been isolated via TPP-t-butanol. The process works at room temperature, is readily scaled up and is rapid (a few minutes). Of 15 enzymes attempted, 12 came through with nearly complete retention of all T.A. TPP-t-butanol is analogous in some respects to well known (Albertsson) partitioning systems using polymers. However TPP-t-butanol has certain advantages, notably that the enzymes and proteins gotten back are not contaminated with PEG, dextran, etc. (t-butanol easily dialyzes out). The system is manouverable and adjustable (pH in the aqueous phase). Some of the outputs have been analyzed via slab gel electrophoresis, $E^{1\%}_{\lambda}$ values, comparison to column chromatographed enzymes, in addition to measurements of T.A., S.A. and neutral sugars. The process is not a hyperpurifying or a fully resolving separations method. However it likely will afford a useful means for dealing with crude enzymes on a large scale to rapidly give partial purification with, often, little loss.

References: K. Tan and R. Lovrien, J. Biol. Chem. 242, 3278-85 (1972); B. Odegaard, P.C. Anderson, R. Lovrien, J. Applied Biochem. 6, 156-183 (1984).

Chromatography

M 008 AFFINITY CHROMATOGRAPHY OF SEQUENCE-SPECIFIC DNA BINDING PROTEINS. Michael R. Briggs, James T. Kadonaga, and Robert T. Tjian. Biochemistry Department, University of California, Berkeley, CA 94720.

The rate of mRNA synthesis in the eukaryotic cell is dependent upon the interactions of one or more DNA binding proteins with promoter elements. Rapid and efficient purification of such transcription factors would overcome a major technical barrier in the effort to understand the role of these proteins in the transcription process. We have developed a method for affinity purification of sequence-specific DNA binding proteins that is fast and effective. A necessary prerequisite for this chromatographic procedure is the precise definition of the DNA recognition sequence for the protein of interest. Complementary chemically synthesized oligodeoxynucleotides that contain a recognition site are annealed and ligated to form concatemers. This DNA is then coupled to Sepharose CL-2B with cyanogen bromide to yield the affinity resin. A partially purified protein fraction is passed through the resin in the presence of nonspecific competitor DNAs. The desired sequence-specific DNA binding protein is retained at low salt and eluted with an linear salt gradient.

We have successfully applied this procedure to several eukaryotic DNA binding proteins including the human cellular transcription factor, Sp1. Initially starting with protein fractions that are up to two percent pure, we were able to purify Sp1 to apparent homogeneity. Improvements have allowed us to move the DNA affinity column step earlier in the purification scheme with consequent purification by the affinity resin of 500- to 1000-fold and overall 50,000 fold purification in two chromatographic steps after a nuclear extract.

Recently, the general applicability of the technique has been shown with the purification to near homogeneity of several other transcription factors including CTF (identical to Nuclear Factor I) which recognizes the CCAAT-box sequence, AP1 and AP2 which bind to the metallothionein IIA and SV40 enhancers, a Drosophila transcription factor Adf-1, involved in Adh gene expression, and a ribosomal gene transcription factor UBF-1. Precise copurification of the DNA binding and transcriptional activities with the polypeptides provides strong evidence that we have identified the active species, however final confirmation has been achieved by the excision of the polypeptides from the SDS acrylamide gels and subsequent renaturation of the specific DNA binding activity.

We anticipate that the identification and purification of these low abundance DNA binding proteins will substantially facilitate the study of transcriptional regulation of eukaryotic gene expression.

M 009 IMMOBILIZED METAL AFFINITY CHROMATOGRAPHY OF PROTEINS, Eugene Sulkowski, Roswell Park Memorial Institute, Buffalo, New York 14263.

Immobilized Metal Affinity Chromatography (IMAC) of proteins has been practised on micro and macro scales for the last twelve years (1). Usually, metals of the first transition series: Co^{2+}, Ni^{2+}, Cu^{2+} and Zn^{2+} (chelated to immobilized iminodiacetic acid (IDA), have been exploited for the purification of proteins. Recently, IDA-Fe^{3+} was reported to display selectivity in binding of phosphoproteins (2). Porath, in his seminal article, postulated that cysteinyl, histidyl, and tryptophyl residues, resident on protein surface, were most likely to form stable coordination bonds to chelated metal ions (IDA-Me^{2+}) at neutral pH (1). This postulate was subsequently confirmed by a systematic study of the chromatographic behavior of natural amino acids and dipeptides (3).

Binding of proteins to chelated Cu^{2+} and Zn^{2+} - the most widely used metals - can be significantly influenced by the equilibrating solvent: its pH, ionic strength, nature of salt ($NaCl/K_2SO_4$), detergent, etc. (4). Typically, a protein(s) is charged on an IDA-Me^{2+} sorbent at pH 7 or higher, at 1 molar NaCl (to quench electrostatic interactions), and is recovered by lowering the pH of the eluant (pH 7 to pH 4). If a protein of interest is labile at low pH, then it may be recovered by inclusion of imidazole (pK_a 6,95) in the eluant at neutral pH.

Typical and atypical cases of IMAC of proteins will be presented. The chromatographic properties of some commercially available chelating sorbents will be described.

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2. L. Andersson and J. Porath, Anal. Biochem. 154, 250 (1986).
3. E.S. Hemdan and J. Porath, J. Chromat. 323, 255 (1985).
4. J. Porath and B. Olin, Biochemistry 22, 1621 (1983).

M 010 FROM AFFINITY CHROMATOGRAPHY TO HIGH PERFORMANCE AFFINITY CHROMATOGRAPHY, *Meir Wilchek*, Department of Biophysics, The Weizmann Institute of Science, Rehovot, Israel

Over the years, affinity chromatography has proved an excellent tool for the purification of biologically active materials in research-laboratories. Thus many proteins (including antibodies, antigens, enzymes, receptors, lymphokinases) have been purified with high efficiency. In order to apply affinity chromatography for the large-scale purification of proteins as required by biotechnology-based industry, the columns necessary for such application should exhibit several important properties: a) the columns should be of high capacity; b) they should be efficient (as small as possible, exhibiting good flow rate, etc.); and c) they should be stable to application and elution conditions (no leakage of ligand bond). The first two requirement collectively mean economy (high yield, time and money) while the last could encompass a health requirement, particularly when mouse monoclonal antibody columns are used to purify products (e.g. prepared by genetic engineering) for human consumption. High capacity and high efficiency columns can be prepared by using high performance affinity chromatography, and the problem of leakage can be prevented or minimized by using the right chemistry. In this communication we will describe the introduction for high performance affinity chromatography of improved silica carriers which contain primary hydroxyl groups. Their activation with different reagents (e.g. cyanogen bromide chloroformates, tosylchloride and carbomyldiimidazole) and coupling of ligands (e.g. trypsin antibodies) to yield high capacity, stable and efficient affinity columns, will also be discussed. The reasons for instability or leakage of ligands from affinity columns, due to inherent problems or the type of chemical reactions used (e.g. cyanogen bromide, *N*-hydroxysuccinimide ester, etc.) for coupling, will be described, and alternative approaches will be suggested.

New Separation Concepts

M 011 GELS AS SIZE SELECTIVE EXTRACTION SOLVENTS, E.L. Cussler, Department of Chemical Engineering and Materials Science, University of Minnesota, Minneapolis, Minnesota 55455 We have successfully used cross-linked polymer gels to concentrate protein solutions and fermentation beers by as much as twenty times. The concentration is selective for solutes greater than 3 nanometers in diameter but not for solutes less than 1 nanometer in diameter. Solutes concentrated include E. coli, bovine serum, albumin, hemoglobin and vitamin B_{12}.

The separation works as follows. The unswollen gel is added to the polymer solution, where it swells to as much as eighty times its dry weight. In this swelling, it adsorbs water and low molecular weight solutes but not high molecular weight solutes. The remaining high molecular weight solution then can be withdrawn as product, leaving only the swollen gel.

The gel is regenerated by a slight warming or by adding a small amount of acid. For example, one gel we have made gives up eighty percent of the absorbed water when it is warmed from 30° C to 33° C. Another gel gives up eighty-five percent of its absorbed water over a pH change of one-half unit. The gel can then be cooled or the pH raised and then reused. It is this regeneration which makes these separations new.

The regeneration occurs because the gels are close to a phase transition. Near such a transition, their swelling is a violent function of process conditions like temperature and pH. As a result, the gels are like a supercritical solvent which can be held in the palm of the hand and which is suitable for biochemical separations.

M 012 RECYCLING ELECTROPHORETIC INSTRUMENTATION FOR PREPARATIVE SCALE PROTEIN PURIFICATION, Richard A. Mosher, Jeffery Sloan, Ned B. Egen, Wolfgang Thormann, and Milan Bier, Center for Separation Science, University of Arizona, Tucson, AZ. 85721. Two instruments for preparative scale protein purification by isoelectric focusing (IEF) have been designed and built at the Center for Separation Science. Each is based on the principle of continuously recycling the fluid until the separation is achieved. This mode of operation is possible because IEF achieves a final stationary steady state distribution of sample components and buffers. The devices are modular, with the process fluid recycled by a multi-channel pump between a heat exchange reservoir, which provides most of the fluid handling capacity, and the separation cell. With each pass the proteins migrate toward their equilibrium positions until the final distribution is attained. The modular design provides a means to easily vary the process volume, simply by using different heat exchange reservoirs. These operational characteristics offer the possibility of rerunning single fractions, thus improving resolution. The primary difference between these two devices is in the manner in which the flow is stabilized in the separation cell. In the first, monofilament nylon screens are used to divide the cell into ten flow-through subcompartments. The screens prevent bulk fluid flow between recycling channels but also impose a pH step-gradient. The fluid in each channel can be continuously monitored, under computer control, for UV absorbance and pH, providing real time data output and the potential for feedback control of the separation. This instrument can resolve proteins which differ in pI by 0.05 - 0.1 pH units. In the second device, the separation cell is a narrow gap (0.75 mm) between 2 flat plates. Fluid stability is achieved by rapid flow through this chamber. There is no internal compartmentation, thus the pH gradient is continuous. Although originally conceived as a recycling IEF device, this instrument offers the opportunity to determine if the recycling principle is applicable to isotachophoretic separations. In addition, both devices can be operated in a continuous mode called feed and bleed. This is essentially a recycling zone electrophoretic process which has the advantage of greatly increased throughput, but entailing a decrease in resolution.

M 013 THERMAL ELUTION HIGH PERFORMANCE AFFINITY CHROMATOGRAPHAY, Alan F. Bergold and Peter W. Carr, Department of Chemistry and Institute for Advanced Studies in Biological Process Technology, University of Minnesota, Minneapolis, MN 55455.
High-performance affinity chromatography can be defined as the hybrid technique of liquid chromatography which uses the biochemical selectivity of affinity ligands in conjunction with micro particulate, incompressible solids. Although chemical methods have been developed for the attachment and presentation of the binding activities of many types of biologically active ligands to silica supports, to date, there have been few reports of the very narrow peaks which characterize other modes of modern liquid chromatography. We and others have shown that the major broadening factor in affinity chromatography is the slow chemical desorption rate of the eluite from the surface bound ligand. In order to overcome this problem we have been exploring the possibility of temperature programmed desorption liquid chromatography which is an exact analog of temperature programmed gas chromatography. The basic concept is to utilize the often very large temperature coefficient of both the thermodynamic and kinetic aspects of biological binding processes to simultaneously cause elution (in a thermodynamic sense), as narrow peaks by increasing the desorption rate constants. Thus far we have been able to resolve ovalbumin and a number of peroxidases into subfractions by thermal elution chromatography using Con A on silica. Various aspects of the methodology including reproducibility, stability and the factors which control resolution will be presented.

Overproduction of Proteins in Bacteria

M 014 IMPROVING PRODUCT HOMOGENEITY THROUGH SITE-DIRECTED MUTAGENESIS. Kirston Koths*, Robert Halenbeck, Alice Wang, Shi-Da Lu, Albert Boosman, and David Mark, Cetus Corp., 1400 Fifty-Third Street, Emeryville, CA 94608

Clinical testing of recombinant protein pharmaceuticals has required the production of highly purified proteins. We have identified several sources of heterogeneity in preparations of recombinant human interleukin-2 and have essentially eliminated these unwanted forms by altering single amino acids through site-directed mutagenesis. The resulting protein, purified in large amounts from E. coli, is fully active and homogeneous as assayed by a number of biochemical criteria.

M 015 HIGH PERFORMANCE CHROMATOGRAPHY IN THE PREPARATION OF RECOMBINANT PROTEINS FOR HUMAN USE: ANALYTICAL AND PREPARATIVE APPLICATIONS, Ernst H. Rinderknecht, Genentech, Inc., South San Francisco, CA 94080

Combinations of both classical and high performance liquid chromatography methods have been traditionally used in the isolation of naturally occuring proteins and peptides in order to elucidate their primary structure and function. Peripheral blood lymphocyte derived natural human Interferon-gamma will be used to show, for example, the power of combined use of lectin and reversed phase HPLC columns in the determination of glycosylation sites in proteins. More recently, these methods have become indispensable tools in the characterization of recombinant proteins destined for parenteral use in humans. Combination of both HPLC and FPLC now often allow for quick and reliable detection of not only minor contaminants but also of minute amounts of N- or C-terminally processed protein chains, glycosylated peptides, modified amino acid residues etc. Recombinant Interferon-gamma and Tumor Necrosis Factor will be used as examples to show the high resolution power in the separation of many of their processed or otherwise modified species. Although initially developed as analytical tools both HPLC and FPLC might be turned into powerful production methods not only for small peptides but also for larger proteins and might help to achieve desired purity levels of 99.99% or higher even on larger scales (10-100g of protein or more). Quite a few problems will have to be solved, however, to achieve this goal (eg. support characteristics and stability, particle size versus resolution, changes to the product upon binding to the resin, equipment, regulatory issues and last but not least costs). A collection of recombinant lymphokines, used as model proteins, will be used to point out some advantages and limitations of the use of FPLC systems for large scale purification.

M 016 EFFICIENT EXPRESSION AND PURIFICATION OF RECOMBINANT GENE PRODUCTS, Dr. Martin Rosenberg, Vice President, Biopharmaceutical R&D, Smith Kline Beckman, Philadelphia, PA 19101; Professor, Department of Human Genetics, University of Pennsylvania.

There are numerous gene products of biological interest which cannot be obtained from natural sources in quantities sufficient for detailed biochemical and physical analysis. Moreover, the limited bioavailability of these molecules has made it impossible to consider their potential utilization as either pharmacological agents and/or targets. One solution to this problem has been the development of recombinant vector systems which are designed to achieve efficient of clone genes in a variety of biological systems. I will describe the development and application of a set of vectors which have been designed to achieve efficient expression of genes in E. coli. The system utilizes efficient phage transcriptional and translational regulatory signals to ensure efficient expression. In addition, host strains have been developed in order to help control, stabilize, and maximize expression of various cloned genes. The ability to carefully regulate and achieve rapid production of the gene product has proven particularly useful in expressing potentially lethal and/or unstable gene products. The system has now been used to express efficiently more than 100 different prokaryotic and eucaryotic gene products. Proteins of interest have been obtained at levels ranging from 1 to 40% of total cellular protein. Since the coding sequence of interest is fused directly to the translation initiation signal on the vectors authentic gene products, rather than gene fusion products, are obtained. Of course, the expression of gene fusions and gene deletions can also be obtained. Purification of the product can be effected in a variety of ways depending upon the nature of the particular protein and the solubility properties it exhibits in different host cells. Direct visualization antibody detection and functional assays have all been employed in monitoring both the synthesis and purification of these proteins. The application of the system to the expression, purification, and characterization of several different gene products of biological and biomedical interest will be described.

Overproduction in Non-Bacterial Hosts

M 017 THE USE OF BACULOVIRUS VECTORS TO PRODUCE FOREIGN PROTEINS IN INSECT CELLS, Lois K. Miller, Departments of Entomology and Genetics, University of Georgia, Athens, GA 30602

The production of biologically active proteins from cloned eukaryotic genes may require expression of the genes in a eukaryotic cell so that post-translational modifications are made and the natural three-dimensional structure of the protein is adopted. Baculoviruses were developed as helper-independent vectors for high-level expression of foreign genes in insect cells (1,2,3,4,5). The rapid adoption of these vectors, by University and industrial laboratories may be attributed to the ease, rapidity, and success in achieving substantial production of numerous biologically active foreign proteins. The most common baculovirus expression vectors are based on the replacement of a highly expressed but nonessential polyhedrin gene with the foreign gene to be expressed. Polyhedrin normally accounts for over 20% of the total protein of an infected cell very late after infection (24-70 hrs), following the synthesis of progeny virus particles which bud from the plasma membrane of the cell. Thus, foreign protein production does not interfere with extracellular virus production and high level expression can be achieved before cell death at very late times postinfection.

Insect cells are able to utilize mammalian signal sequences for the transport of attached peptides across cellular membranes and the mammalian signals are properly cleaved from the exported proteins (5,6). Insect cells also glycosylate proteins although the nature of the terminal glycosylation reactions differ from those occuring in mammalian cells. Insect cell cultures can be produced in large scale culture although much work remains to define the optimum conditions for growth. Alternatively, high level foreign gene expression may be achieved in insect larvae. For example, silkworms have been utilized for high level production of alpha-interferon (5). Isolation of pure proteins from insect larvae, however, may be a challenge.

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M 018 PRACTICAL CONSIDERATIONS IN GOING FROM MICRO TO MACRO PURIFICATION OF RECOMBINANT PROTEINS, Satish K. Sharma, David B. Evans, James C. Cornette, and Anne M. Furlong, Biotechnology, The Upjohn Company, Kalamazoo MI 49001.

Selection of nonbacterial systems for expressing heterologous proteins is generally made because correct folding or other post-translational modifications cannot be achieved in recombinant microorganisms such as *E. coli* (1). Cloning into yeasts may provide a way to obtain properly folded products although differences in glycosylation between yeasts and mammalian cells may pose serious problems. Animal cells, therefore, appear to be the optimal host to achieve post-translational modifications of proteins but many technical problems remain to be solved. Various practical considerations in the purification and activation of recombinant human prorenin secreted by two different mammalian expression systems will be described.

The scaling up of an analytical purification scheme developed for a given recombinant protein, whether produced by a bacterial or nonbacterial expression system, is a large and complex undertaking. Certain generalizations and decisions are made simply on the basis of projections and/or experience. Developing and manufacturing a recombinant DNA product, especially a pharmaceutical protein, demands many more steps after the cloning and expression. Fermentation or large scale cell culture, purification and characterization, pharmacology, formulation, pre-clinical and clinical testing must follow. This talk will address various critical issues related to downstream processing of recombinant proteins with emphasis on applications, problems and future potential. The impact of successful interaction between engineers, protein chemists, cell biologists, molecular biologists, and DNA chemists on the recovery of pure recombinant protein products will be discussed.

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Coping with Problems

M 019 SOLUBILISATION, REFOLDING AND PURIFICATION OF EUKARYOTIC PROTEINS EXPRESSED IN E. coli, Peter A. Lowe, Stephen K. Rhind, Richard Sugrue and Fiona A.O. Marston, Celltech Limited, 244-250 Bath Road, Slough, SL1 4DY, Berks. U.K.
The insolubility of many eukaryotic recombinant proteins expressed in E. coli (as N-terminal met alalogs or hybrid fusion proteins) presents both problems and opportunities for their purification. A review of solubilisation and refolding methods and major variables in selecting conditions to maximise yield will be presented. Techniques for analysis of the effectiveness of solubilisation and refolding procedures, including light scattering and fluorescence will be outlined. These general considerations will be illustrated by reference to the laboratory scale production of the human peptide hormone calcitonin (hCT).

hCT is a 32 amino acid hormone containing a 1-7 disulfide bridge and C-terminal proline amide residue. hCT was produced in E. coli as a precursor containing an additional C-terminal gly residue (hCT gly) fused to the C-terminal region of the E. coli enzyme chloramphenicol acetyl transferase (CAT) via a proteolytically (clostripain) sensitive Arg residue (CAT Arg hCT gly). The partially purified insoluble fusion protein was solubilised in 8M urea under reducing conditions and hCT gly liberated from the fusion by digestion with clostripain.hCT gly was partially purified by RP-HPLC and converted to hCT by enzymic amidation. Final purification of hCT was carried out by RP-HPLC.

M 020 NOVEL METHODS FOR SOLUBILIZATION OF INCLUSION BODIES, Dante J. Marciani, Chung-Ho Hung, and King-Lan Cheng, Cambridge BioScience Corp. 35 South Street Hopkinton, MA 01748. Recombinant proteins produced in E. coli, can be deposited inside the microorganism cells, as refractile inclusion bodies, i.e. clumps of insoluble protein. The inclusion bodies are separated from the host cell proteins through lysis of whole cells with lysozyme and non-ionic detergents treatment, followed by centrifugation. Inclusion bodies are solubilized in the presence of 6M guanidinium hydrochloride or 8M urea and 2-mercaptoethanol. The sulfhydryl groups of the protein(s) are protected by reversible or irreversible derivatization. If the sulfhydryl groups are not essential for maintaining the properties of the protein, iodoacetamide, or N-ethylmaleimide, can be used. If the sulfhydryl groups are needed, for formation of disulfide bonds or for preserving the properties of the protein, reversible modification using disulfide exchange or S-sulfonation are recommended. The recombinant protein is dissociated by converting cationic amino groups to anionic carboxylic groups. These highly negative charged proteins will repel one another, reducing the tendency of recombinant proteins to aggregate. Introduction of the carboxyl groups is achieved by reacting the free amino groups of the protein with selected cyclic dicarboxylic acid anhydrides. This reaction leads to the formation of N-acyl groups, which can be deacylated by exposure of the modified protein to acid conditions. The reversibility of the acylation depends on the presence of protonated carboxyl groups specially oriented to particpiate as intramolecular catalyst of the hydrolytic reaction (1). Following complete N-acylation, the modified proteins can be fractionated by use of conventional procedures, such as gel filtration, ion exchange chromatography, and others. The modified protein can be deblocked by exposing the protein to acid pH. The rate of this reaction can be controlled by changes in pH and/or temperature. Following the removal of the N-acyl groups, sulfhydryls can be regenerated and if needed induce the formation of disulfide bonds.

(1) Glazer, A.N.: R.J. DeLange and D.S. Sigman. "Chemical Modification of Proteins" in Work et al (eds). Laboratory Techniques in Biochemistry and Molecular Biology. Elsevier, Amsterdam (1976).

M 021 MULTIPLE PATHWAYS FOR PROTEIN BREAKDOWN IN MAMMALIAN CELLS, Lloyd Waxman, Merck Sharp and Dohme, West Point, PA 19486 and Julie M. Fagan, Department of Animal Sciences, Rutgers University, New Brunswick, NJ 08903.

Although lysosomes are a major site for degrading proteins in mammalian cells, recent studies indicate that cytosolic non-lysosomal pathways play an essential role in protein breakdown. In reticulocytes, which lack lysosomes, the degradation of abnormal proteins (e.g. those containing amino acid analogs) and proteins lost during maturation occurs by an energy-dependent process. Characterization of the major components of this system has led to the proposal that the ATP-dependent conjugation of ubiquitin (Ub) to potential substrates marks them for degradation. These conjugates are degraded by a protease which requires ATP hydrolysis. This ATP+Ub-dependent proteolytic system is also present in other mammalian tissues (e.g. liver and muscle). In addition, yeast contain many of the components required for ATP+Ub-dependent proteolysis. Genetic evidence for the importance of this degradative pathway comes from studies with fibroblasts having a mutation in an enzyme required for Ub conjugation which can no longer degrade abnormal proteins.

Erythroleukemia cells and possibly other cells have a cytoplasmic ATP-dependent proteolytic system which does not require Ub. Its function has not been established. Mammalian mitochondria contain an ATP-dependent protease like that from E.coli which may participate in the degradation of abnormal or short-lived proteins in this organelle. Although proteins in endoplasmic reticulum and other organelles also have half-lives which vary widely and are regulated by hormonal and other physiological influences, little is known about the processes which degrade them.

Red blood cells also rapidly degrade certain kinds of abnormal proteins, such as hemoglobin damaged by oxidants, by a process that does not require ATP. The properties of this degradative system are different from the ATP-dependent pathway, and the responsible proteases and other factors are unknown. Mammalian cells also contain several well-characterized proteases (Ca-requiring proteases and a multifunctional 670kDa protease) whose physiological function and regulation are not clear. The presence of endogenous inhibitors of lysosomal and cytosolic proteases may play an indirect role in regulating proteolysis.

Several recent studies have examined what features of a protein govern its site of degradation and half-life. It has been suggested that proteins may contain signal sequences which govern their uptake by the lysosome, and that short-lived proteins contain regions enriched in certain amino acids. Alternatively, the nature of the N-terminal amino acid may determine its susceptibility to degradation by the ATP+Ub-dependent pathway. Eliminating or altering small sequences in the primary structure may provide a new way to stabilize cloned gene products to obtain higher expression.

Poster Abstracts

M 100 PURIFICATION OF RECOMBINANT EPIDERMAL GROWTH FACTOR FROM A FUSION EXPRESSED IN ESCHERICHIA COLI, Geoffrey Allen and Cora A Henwood, Wellcome Biotech, Beckenham, Kent BR3 3BS, UK.

Epidermal growth factor (murine sequence) has been purified in gram quantities following chemical synthesis of the gene and expression in E. coli as a fusion protein with part of the Trp E gene product. The fusion protein was mainly present as an insoluble aggregate within the E. coli cells, and was pelleted by centrifugation after cell lysis. Following solubilization in 8M urea under reducing conditions, the EGF polypeptide was liberated by specific proteolysis and allowed to re-form disulphide bonds by controlled oxidation. EGF was purified by sequential chromatography on columns of reverse-phase (C18) silica gel, DEAE-cellulose and BioGel P-10. Chemical and biological properties were indistinguishable from those of the major component of EGF extracted from submaxillary glands.

M 101 A SIMPLE THREE STEP PURIFICATION OF *E. COLI* TRP APOREPRESSOR, Dennis M. Arvidson, Andrew A. Kumamoto, and Robert P. Gunsalus, Dept. of Microbiology and Molecular Biology Institute, University of California at Los Angeles, Los Angeles, CA 90024.

The *Escherichia coli* Trp aporepressor is a small dimeric (12.5-kilodalton/subunit) DNA-binding regulatory protein. The corepressor, L-tryptophan, binds to the aporepressor at two identical and independant sites forming the active repressor. Trp repressor negatively regulates initiation of transcription of three operons: *trpEDCBA*, *aroH*, and *trpR*. These operons have similar operator sequences which overlap their respective promoters and are specifically bound by Trp repressor. We have developed a simple three step procedure for large scale purification of the Trp aporepressor so that we can study these binding activities in detail. Aporepressor was produced via a pBR322 derivative in which the *trpR* gene was inserted downstream from the *lac* UV5 promoter and *lacZ* ribosome binding site such that approximately 0.75% of the total cellular protein was Trp aporepressor as determined by a radioimmunoassay. Cells were broken with a French pressure cell and the debris was removed by centrifugation. Following addition of Streptomycin sulfate (final concentration of 0.5%) the extract was heated to 85 degrees Centigrade for 10 min. Precipitate was removed by centrifugation and the supernatant was eluted from a heparin agarose column with 0.6 M KCl. Fractions were assayed for protein by the Bradford dye binding assay. Conductivity of pooled protein-containing fractions was adjusted by dilution to 0.05 M KCl and the material was eluted from an Affi-Gel Blue column with 0.6 M KCl. Fractions which contained protein were pooled, concentrated by Amicon filtration, dialyzed, and stored at -70 degrees Centigrade. This three step batch procedure routinely yielded 150 mg of aporepressor from 300 g of cell paste (approximately 50% yield). Aporepressor prepared by this method was >99% pure based on SDS polyacrylamide gel electrophoresis and HPLC size exclusion chromatograpy. Activity for the binding of tryptophan and operator was nearly 100% based on equilibrium dialysis and a restriction site protection assay, respectively. The protein is stable for at least one year under the defined storage conditions.

M 102 MACROSORB KIESELGUHR-AGAROSE COMPOSITE ADSORBENTS
Maris G. Bite, Sterling Organics, Newcastle upon Tyne NE3 3TT, England

Macrosorb composite adsorbents consist of derivatised agarose gels trapped inside a macroporous, rigid granule which is prefabricated from purified Kieselguhr. These incompressible composites, whilst retaining all the desirable properties of traditional agarose-based media, offer configurational versatility and process economic advantages as a result of their physical properties: the ability to sustain usefully increased flow-rates, the exhibition of low non-specific adsorption levels and use in fluidised beds.
These factors are shown to be useful tools in the simplification of downstream processing protocols and in the furnishing of purer products. Thus, ability to rapidly cope with large volumes permits dilution to be used as an alternative to desalting, and is of value where product stability is a major consideration.
Use of the relatively dense composites in fluidised beds enables extraction directly from unclarified broths. The low non-specific adsorption levels have been shown to significantly decrease column scrubbing requirements in cyclic processes.
The economic advantages of increasing throughput by application of the above features to separations from complex feedsteams is demonstrated.

M 103 PURIFICATION OF RECOMBINANT HUMAN GRANULOCYTE-MACROPHAGE COLONY STIMULATING FACTOR FROM E. COLI, Tom Boone, Craig Crandall, Larry Tsai, Kris Zsebo, Amgen, Thousand Oaks, CA 91320
Recmbinant human granulocyte-macrophage colony stimulating factor (GM-CSF) has been purified to homogeneity from inclusion bodies formed in E. coli. The purified protein contains the potential for two disulfide bonds and has both in vitro and in vivo activity. The broken cell pellet from E. coli containing the GM-CSF was solubilized in 8M urea, diluted and allowed to oxidize. The GM-CSF was then purified by differential precipitation, hydrophobic chromatography, and anion exchange chromatography.

M 104 PARTIAL PURIFICATION OF HUMAN SPERM CHROMATIN DECODENSATION PROTEIN(S) ISOLATED FROM Xenopus laevis EGG EXTRACT, David B. Brown, W. Keith Miskimins and Frank H. Ruddle, Department of Biology, Yale University, New Haven CT 06511. The sperm nucleus of vertebrate organisms undergoes a series of well-characterized morphological and metabolic alterations during the process of fertilization, ultimately leading to the formation of the male pronucleus. We have previously shown that Xenopus laevis frog eff extract contains factor(s) that stimulate the early events of nuclear activation in lysolecithin permeabilized human sperm (Exp. Celi Res. 157, 409-418). These events include chromatin decondensation, nuclear swelling as a biological assay to detect the required protein(s). We have developed a protein purification scheme utilizing protamine affinity chromatography followed by Sephacryl S-300 gel filtration to obtain a 450 fold purification of the putative human sperm chromatin decondensation protein(s).

M 105 PURIFICATION OF TWO FORMS OF IGF-I BINDING PROTEIN IN HUMAN AMNIOTIC FLUID. W.H. Busby, Jr. & D.R. Clemmons, University of North Carolina, Chapel Hill, NC.

Insulin like growth factor I (IGF-I) is detectable in extracellular fluids bound to a binding protein. Since this protein may control the cellular response to IGF-I, we purified it from human amniotic fluid in order to determine its physicochemical and biologic properties. Purification to homogeneity was achieved by phenyl sepharose, DEAE cellulose, G-100 Sephadex chromatography and reverse phase HPLC. Binding protein activity was monitored by incubating ^{125}I-IGF-I with the chromatographic fractions and precipitating the bound IGF-I with polyethylene glycol. Following DEAE cellulose chromatography, the IGF-I binding activity was separated into two peaks eluting at 100 and 200 mM NaCl. Subsequently, these peaks (termed B and C) were purified as separate entities. Purity was confirmed by polyacrylamide gel electrophoresis and amino acid sequence determination. Peak B was purified 432-fold with 19% recovery while peak C was purified 398-fold and recovery was 30%. Peak B and C proteins appear to have similar primary structures. Both have molecular weights of 32,000 daltons, are acid and heat stable and have comparable amino acid compositions. Carbohydrate was absent or < 1% of the protein weight in both. Their affinities for IGF-I were 2.2×10^{10} and 1.7×10^{10} L/M respectively. These proteins have very different functional properties, however. While both bind IGF-I, peak B potentiates the replication of several cell types in response to human IGF-I. Peak C inhibits this response to IGF-I and can block the response to peak B plus IGF-I when added simultaneously. It appears that some as yet undetermined post translational modification accounts for this functional difference.

M 106 PURIFICATION OF RECOMBINANT HUMAN INTERLEUKIN 1ß PRODUCED FROM YEAST, M.C. Casagli, M.G. Borri, C. D'Ettorre, C. Baldari, C. Galeotti, P. Bossù, P. Ghiara and G. Antoni, Sclavo Research Centre, Via Fiorentina 1, 53100 Siena, Italy.

Recombinant human interleukin 1ß (IL-1ß) secreted from Saccharomyces cerevisiae into the culture supernatant medium has been purified. The culture supernatant was concentrated 10-fold by ultrafiltration and chromatographed on a DEAE-Sepharose column. IL-1ß which does not bind at pH 7.5 was collected in the flow through free from most of the contaminanting proteins. Hydroxylapatite chromatography was used to concentrate and further purify IL-1ß which was eluted by increasing the phosphate buffer concentration.

The reverse-phase HPLC on C-18 Vydac column was selected to complete the purification of IL-1ß; a linear gradient of acetonitrile in 0.1% TFA was used to elute the column.

The purified protein was electrophoresed on SDS-polyacrylamide gel and amino acid analysis was carried out. The different specific activity of native and recombinant human IL-1ß is discussed.

M 107 PURIFICATION AND CHARACTERIZATION OF HUMAN KALLISTATIN, A NEW TISSUE KALLIKREIN-BINDING PROTEIN. Julie Chao, Maoyin Wang and Lee Chao, Depts. of Pharmacology and Biochemistry, Medical University of South Carolina, Charleston, S.C. 29425.

We have recently identified a new and specific tissue kallikrein-binding protein in mammalian plasma and in the secreted culture media from several transformed cell lines (Chao et al., Biochem. J. 239: 325-331, 1986). We have designated this kallikrein-binding protein as "kallistatin". Kallistatin has been purified from human plasma using several chromatographic steps including DEAE-Sephadex, hydroxylapatite, Cibacron blue-Sepharose and preparative polyacrylamide gel electrophoresis. The purified kallistatin consists of a single polypeptide chain with an apparent Mr of ~ 54,000 as determined by SDS-PAGE under reducing conditions and by gel filtration. In two dimensional gel electrophoresis, kallistatin migrates as one 54,000 dalton protein band with pI of 5.4. Kallistatin was eluted as a single peak on reversed-phase HPLC. The Mr of the protein moiety is estimated to be 54,100 from amino acid composition anaylses. The purified kallistatin and tissue kallikrein forms a ~ 92,000 dalton SDS- and heat-stable complex. The complex formation is pH dependent with maximal binding at pH 8.5-9.0 in Tris-HCl buffer. The binding is inhibited by heparin, deoxycholate, SDS but not by Triton X-100, digitonin, Lubrol or CHAPS. Affinity-purified anti-kallistatin antibody inhibits the binding in a dose dependent manner. In Western blot analysis, a ~ 54,000 dalton protein was identified in the partially purified plasma proteins. This ~ 54,000 dalton protein was also visualized by autoradiography in its binding to ^{125}I-labeled-tissue kallikrein in ligand blotting. The role of kallistatin in regulating tissue kallikrein activity and metabolism may now be evaluated.

M 108 PRODUCTION OF HBsAg IN THE PERIPLASMIC SPACE OF E.coli, CHA Y. CHOI AND KEE S. LEE, Seoul National University, Seoul, 151, KOREA

A trucated HBsAg gene lacking the sequence encoding the NH_2-terminal hydrophobic region was put behind the E.coli lpp-lac double promoters and a successful expression could be achieved. The transformant harboring the newly constructed recombinant plasmid, pKSB292, did not inhibit growth and much of the produced antigen was present in the osmotic shock fluids of the E.coli host cells, indicating the transport of the gene product to the periplasmic space. There exists an optimal pH for the induction of gene expression with IPTG and this optimal pH for gene expression corresponded to the one for optimal cell growth implying the constancy of the gene product per unit cell mass. The growth rate of the induced recombinant cell was not much influenced in contrast to other similar study with trp promoter. An optimal time for the induction by IPTG also existed. The relative distribution of the gene product among culture broth, osmotic shock fluid, and cytoplasmic fraction remained approximately constant throughout the overall fermentation period of about 10 hours when the early induction was exercised.

M 109 PRODUCTION OF PEPTIDE HORMONES IN E. COLI VIA MULTIPLE JOINED GENES, Stephen Cockle, Michael Lennick and Shi-Hsiang Shen, Connaught Research Institute, Toronto, Canada.

Efficient overproduction of peptides and small proteins in E. coli has been achieved by generating them as fused polypeptides comprising a short bacterial leader and multiple copies of the required sequence, separated by a short removable linker. Thus over 90% of the precursor can consist of the desired product. Moreover, the precursor itself is large and unstructured, and can be simply isolated by homogenization and solid-liquid separation. The composition of the linker depends partly on the target sequence, but can also be adapted to purification requirements. The concept has been applied to the human hormones insulin (via proinsulin) and cardionatrin. Proinsulin was expressed as a four-copy precursor in which leader and linker sequences ended in methionine. Digestion with cyanogen bromide therefore released monomers of proinsulin "analogue" with the linker still attached at the C-terminus. Reversible formation of the cysteine S-sulfonate derivative facilitated purification by anion exchange chromatography and subsequent introduction of the three correct disulfide bonds. The linker was removed with trypsin and carboxypeptidase B, which concurrently converted proinsulin to mature insulin. Cardionatrin was expressed as an eight-copy precursor, also using a specific linker sequence. Monomers were obtained by sequential digestion with two proteolytic enzymes, and the single disulfide bond was then generated by mild oxidation with ferricyanide. Both hormones exhibited full biological activity.

M 110 A STRATEGY FOR THE ISOLATION AND CHARACTERIZATION OF NOVEL INSULINS. J.Michael Conlon and Lars Thim, Max-Planck-Gesellschaft, University of Göttingen, W-Germany and Novo Research Institute, Bagsvaerd, Denmark.

A general scheme has been developed for the purification and subsequent characterization of insulins from pancreatic extracts. The procedure involves (1) concentration of proteins/peptides on Sep-pak C18 cartridges (2) gel filtration on Sephadex G-50 (3) reverse-phase HPLC on a Vydac C18 column using a gradient of acetonitrile/aqueous trifluoroacetic acid for elution. The peak of insulin is identified from its characteristic retention time and ratio of absorbance at 280 nm and 214 nm. The insulin is purified to homogeneity using a Supelcosil LC-3DP phenyl column eluted with a gentle gradient of acetonitrile/aqueous trifluoroacetic acid. After reduction with dithiothreitol and derivatization with 4-vinylpyridine, the A- and B-chains are separated on a Vydac C18 column. Sequence analysis on 2-5 nmol peptide is accomplished using gas-phase automated Edman degradation. This strategy has been used to obtain the primary structures of insulins from Torpedo marmorata (an electric ray), Platichthys flesus (flounder), Hydrolagus colliei(Pacific ratfish) and Chimaera montrosa. (rabbit fish).

M 111 RAPID ISOLATION OF ACETYLCHOLINESTERASE (EC 3.1.1.7) FROM SNAKE VENON, Jeffrey R. Deschamps, Laboratory for the Structure of Matter, Naval Research Laboratory, Washington, D. C. 20375-5000.

Soluble forms of acetylcholinesterase (AChE) have been found in several elapid venoms and studies of their specificity have revealed many similarities, including substrate inhibition between elapid venom AChE and eel electroplax AChE, a true cholinesterase. As many as 15 different isozymes of AChE have been reported in one venom. The large numner of isozymes may be the result of proteolytic activity as proteases are also in the venom. It is therefore essential that the purification be carried out quickly so as to minimize proteolysis. Previously AChE has been isolated from cobra venom by a combination of chromatographic techniques; the final step is generally affinity chromatography. Recently a high performance affinity procedure for the isolation of acetylcholinesterase has been developed (Sam Morris, personal communication). This new affinity procedure was modified for batch chromatography and tested for use in isolating elapid venom acetylcholinesterase. Over 90% of the cholinesterase activity can be recovered using this procedure and there is a twelve-fold increase in purity in this batch extraction step. Using batch affinity chromatography, 0.2 mg of acetylcholinesterase were isolated in one afternoon. Thus the use of affinity chromatography as the first chromographic step allows rapid isolation of the enzyme and minimizes contact with proteases.

M 112 PURIFICATION OF MONOCLONAL ANTIBODIES FROM LARGE-SCALE MAMMALIAN CELL CULTURE PERFUSION SYSTEMS, Stephen A. Duffy, Bill J. Moellering, Christopher Prior, and Randy W. Scott, Invitron Corp. Clayton, Mo. 63105.

Monoclonal antibodies have become increasingly important to the biotechnology industry due to their immense potential as therapeutic and diagnostic pharmaceuticals. To produce gram quantities of monoclonal antibodies, 10-1000 liters of cell culture media is generally needed. Several ultrafiltration systems, including hollow fiber, plate and frame, and spiral cartridges, are effective in reducing the volume. The concentrated product can then be further purified by several methods including ammonium sulfate precipitation, ion exchange chromatography, protein A agarose, and size exclusion chromatography. Ammonium sulfate precipitaion removes a variety of contaminants including serum albumin. By optimizing pH and ionic strength, cation exchange chromatography alone can give up to a 200 fold purification, often eliminating the need for ammonium sulfate precipitation, or further chromatography steps such as anion exchange resins. As an alternative, protein A affinity chromatography will yield a very high purity product, but is not suitable for all subclasses of monoclonal antibodies. Finally, size exclusion chromatography is used to remove aggregates, process chemicals, and to exchange into the formulation buffer. By utilizing the above methods gram quantities of pharmaceutical quality monoclonal antibodies can be purified from mammalian cell culture media.

M 113 EXTRACTION AND PURIFICATION OF OVINE TRYPSIN

Ian T. Forrester, Otago University, Dunedin, New Zealand.

In New Zealand the processing of sheep and lambs for the export meat trade occurs via a highly developed and centralized slaughter house system. This procedure facilitates the efficient collection of large numbers of tissues suitable for the extraction of commercially important biochemicals. An integrated research programme has been initiated to examine the feasibility of using ovine pancreas as a source of proteolytic enzymes suitable for the leather industry.

Laboratory-based research was used to establish the general parameters required for the bioprocessing of pancreas. These procedures were then expanded into a pilot plant operation which processed 27 kg of ovine pancreas per batch. The pilot plant incorporated rotary vacuum filtration, ion-exchange chromatography, ultrafiltration and freeze drying. Each batch involved approximately 500 ℓ of protein extract and was operated on a 48 h cycle. The final freeze dried product referred to as Type T1, has a specific activity for trypsin of approximately 1,800 USP units per mg solid and for chymotrypsin, approximately 200 USP units per mg solid. The Type T1 material has now been successfully used in a series of tannery trials demonstrating its effectiveness as a bating agent and as an alternative to bovine or porcine derived pancreatic protease. Type T1 material has been subjected to further ion-exchange chromatography yielding a range of more purified trypsin including a fraction (Type T4), which is equivalent to crystalline grade (4,000 USP units per mg solid). Commercial production of ovine trypsin is now being considered by the New Zealand sheep processing industry.

M 114 PURIFICATION OF FLP RECOMBINASE USING SEQUENCE-SPECIFIC DNA AFFINITY CHROMATOGRAPHY. Cynthia A. Gates, Leslie Meyer-Leon, Janet M. Attwood, Elizabeth A. Wood, and Michael M. Cox. Univ. of Wisconsin, Madison WI 53706.

FLP recombinase mediates site-specific recombination between two 599 base pair (bp) inverted repeats of the 2 micron circle which is an autonomously replicating plasmid present in yeast. The gene encoding FLP recombinase has been cloned and expressed in E. coli. The recombination site is well defined and consists of three 13 bp repeats with the second and third separated by an 8 bp spacer. FLP-mediated cleavage occurs at the boundaries of the spacer. The recombinase not only binds to the two 13 bp repeats flanking the spacer, but also to the third 13 bp repeat. Using this information, a sequence-specific DNA agarose resin was synthesized for affinity chromatography of the recombinase. The immobilized ligand consists of a DNA polymer containing multiple 13 bp repeats ligated in "head-to-tail" orientation. After ammonium sulfate fractionation, cation exchange chromatography, and non-specific DNA agarose chromatography, FLP recombinase was purified to 95% purity using the sequence-specific DNA agarose affinity resin.

M 115 DEOXYINOSINE TRIPHOSPHATASE FROM E. COLI: TWO ENZYMATIC ACTIVITIES DIRECTED AGAINST DITP. Isaac Harosh and Joseph Sperling,
Department of Organic Chemistry, Weizmann Institute of Science, Rehovot, Israel

This study describes two distinguishable enzymatic activities present in E. coli protein extract that are capable of hydrolyzing dITP to dIDP and dIMP. Analysis of the enzymatic reaction products was carried out using a TLC system which allowed the separation between dITP, dIDP and dIMP. After 30 min incubation, dIDP and dIMP were produced in a molar ratio of 5:1, and the substrate was completely consumed. This ratio between dIDP and dIMP was maintained for up to 90 min of incubation, indicating the presence of two activities that hydrolyze dITP to the respective products. To confirm this result, we have shown that the enzymatic activity that directly produces dIMP from dITP could be selectively inhibited by dIMP, whereas the activity that hydrolyzes dITP to dIDP remained unaffected. Furthermore, the two activities were separated by DEAE-cellulose column chromatography. The enzymatic activity producing dIDP was eluted between 250-350 mM NaCl, while the activity producing dIMP was eluted between 50-100 mM NaCl. This fraction was contaminated with a minor activity of the enzyme producing dIDP. We have also shown that the separated activities have different temperature sensitivities. After 2.5 min incubation at $60^{o}C$, the activity producing dIDP + Pi remained unaffected, whereas the activity producing dIMP + PPi decreased to 50%. We thus designate the activities dITPase-P and dITPase-PP, respectively. Both activities have an apparent molecular weight of 68 kDa, as determined by gel filtration on Sephadex G-100. Neither enzymes require Mg^{++} for their activity nor are they inhibited by EDTA.

M 116 PURIFICATION AND PARTIAL CHARACTERIZATION OF HUMAN HEPATIC ASPARTYL-GLUCOSAMINIDASE, Nisse Kalkkinen, Leena Peltonen and Marc Baumann,
Recombinant DNA-Laboratory, University of Helsinki, 00380 Helsinki, Finland.

The human aspartylglucosaminidase (EC 3.5.1.26) was purified from human liver to obvious homogeneity by using affinity chromatography on Concanavalin A, gel permeation chromatography on Bio Gel P-100, chromato-focusing, high performance cation and anion exchange and reverse phase chromatography. The enzyme seems to consist of three non-identical subunits with apparent molecular weights of 24000, 18000 and 17000. About 200pmol of each subunit was obtained from 500g of liver. The purified enzyme was partially characterized by measuring its K_m, pI, specific activity and thermostability. Edman degradation of the subunits in a gas-phase sequencer gave no result suggesting that they all have a blocked N-terminus. To get sequence information for oligonucleotide synthesis, the subunits were digested with trypsin or Achromobacter lysyl endopeptidase. Resulting peptides were isolated by wide pore reverse phase HPLC and some of them sequenced.

M 117 THE USE OF RADIAL FLOW COLUMNS FOR RAPID SEPARATIONS WITH SOFT GELS, R. T. Kawahata and W. C. McGregor, XOMA Corporation, Berkeley CA 94710.

The throughput (or linear flow rate) used in conventional chromatography is limited by a combination of column cross sectional area, bed height, and compressibility of the chromatographic media. Because of these factors, utilization of agarose or cellulose based "soft" gels in a conventional column configuration may result in extended processing times and/or oversized column dimensions. Radial flow columns offer advantages to conventional column configurations by increasing the apparent cross sectional area and thereby increasing throughput and decreasing total column volume. This is of considerable importance when high value affinity supports are utilized or where large volumes are processed over high capacity resins. Comparison data is reported regarding the flow dynamics of radial flow versus conventional chromatography columns utilizing agarose based soft gels. Results from radial flow affinity separations are reported and specific applications of the radial flow column is discussed.

M 118 ISOLATION OF E. COLI-DERIVED MURINE INTERLEUKIN-2 FROM INTRACELLULAR AND SECRETORY EXPRESSION SYSTEMS, H. V. Le, R. Syto, C. Mays, P. Reichert, S. Narula, K. Gewain, R. Greenberg, R. Kastelein, A. Van Kimmenade, T.L. Nagabhushan and P.P. Trotta. Schering Corp., Bloomfield, New Jersey 07003, USA

The mature form of recombinant murine interleukin-2 (muIL-2) (Yokota, T. et al (1985) Proc. Natl. Acad. Sci. 82, 68-72) expressed in E. coli with a secretory vector and extracted by osmotic shock was purified to homogeneity by anion exchange chromatography and gel filtration. The purified protein exhibited a specific activity of ca 3 x 10^7 units/mg in an HT-2 cell proliferation assay. Its apparent molecular weight estimated by SDS-PAGE and gel filtration chromatography was 19 kd and 30 kd, respectively. These data suggest that the secreted form of muIL-2 is a dimer, as previously described for native muIL-2 derived from T-cell lines. MuIL-2 was also isolated from an anionic detergent extract of E. coli engineered with an intracellular expression system. Removal of detergent from a partially purified preparation resulted in extensive aggregation associated with disulfide bond formation. Significant loss of biological activity occurred upon aggregation. The data indicate a difference in protein processing in the intracellular and periplasmic compartments of E. coli and suggest an advantage for the secretory expression system for the isolation of native muIL-2.

M 119 PREPARATIVE PURIFICATION OF VIRAL POLYPEPTIDES FROM MOUSE MAMMARY TUMOR VIRUS

Joseph K.K. Li,[1,2] Thomas Mercolino,[2] and Jeffrey Bruton[2]. [1]Department of Biology, Utah State University, Logan, Utah 84322 [2]Becton-Dickinson Research Center, Research Triangle Park, North Carolina 27709.

After the gradient-purified mouse mammary tumor virus (MMTV), a B-type retrovirus, was first disrupted with 1% Triton X-100 in the presence of high salt and TE buffer (pH 9.2), all the viral structural polypeptides have been purified preparatively to near homogeneity and with good yield by sequential affinity and ionic exchanges column chromatography. The smaller molecular weight viral proteins were targetd for initial purification to significantly reduce the loss of these minor structural components. All MMTV polypeptides, GP52, GP36, P28, P14, P12 and P10 have been isolated from the same batch of viruses to near homogeneity as determined by silver staining and autoradiography of SDS-PAGE. High titer monspecific polyclonal antibodies were produced against each of the isolated MMTV polypeptides except P12 and P10 and these antiserum showed little or no cross-reactivity among all the isolated MMTV proteins in RIA. With minor modifications, the same procedure has been used and proved successfully in the preparative purification of viral proteins from other C-type retroviruses such as ASV, RLV, GLV, MLV, GaLV, SSV-1 and FeLV.

M 120 PURIFICATION OF NATIVE AND RECOMBINANT TUMOR NECROSIS FACTOR, Leo S. Lin and Ralph Yamamoto, Deparment of Protein Chemistry, Cetus Coporation, Emeryville, CA 94608.

Tumor Necrosis Factor (TNF) is a cytolytic protein found in serum of lipopolysaccharide-stimulated mice and rabbits. A similar factor is produced by tumor cell lines when treated with carcinogens. This protein was purified from tissue culture supernatants and its amino acid sequence determined. Using recombinant DNA technology the TNF gene was cloned and the recombinant protein expressed in E. coli. TNF is a 17,000 dalton nonglycosylated protein, contains a single disulfide bond and exists soluble in aqueous buffers as a homodimer. The E. coli produced molecule is similar to the native protein in many aspects, most importantly, it is bioactive with a similar specific activity as the native counterpart. Additionally, it contains a single disulfide bond, and exists soluble in aqueous buffers as a homodimer. The similarity between the native and recombinant molecules, allows us to apply a similar purification scheme consisting of ion exchange and hydrophobic interaction chromatography to obtain highly purified TNF protein from either source.

M 121 CHOICE OF SALT AND FLOW RATE CAN AFFECT RECOVERY OF BIOMOLECULES IN ION EXCHANGE CHROMATOGRAPHY, David G. Maskalick, Marie T. Anderson and Kelly J. Hoke, Eli Lilly and Company, Indianapolis, IN 46285

Ion exchange chromatography of proteins usually involves losses due to either irreversible binding to the resin or inability to achieve the desired product purity and throughput without sacrificing yield. The salts present in the elution buffers may have an impact upon the product recovery analogous to the effect that different organics have upon performance in reversed phase chromatography. The utilization of alternate salts may lead to modified retention times and/or recoveries analogous to the use of propanol versus acetonitrile or the presence versus absence of triethylamine in a RP-HPLC buffer system. The relationship between binding kinetics and flow rates also may be exploited to effect better separations. Examples and explanations of these effects will be presented.
The impact of both the salt and the flow rate selection is primarily due to the ability of the biomolecules to interact with a single functional group in a multidentate fashion. The ramifications of this concept in the field of affinity chromatography is discussed.

M 122 PURIFICATION OF BETAINE ALDEHYDE DEHYDROGENASE BY HYDROPHOBIC INTERACTION AND AFFINITY COLUMN CHROMATOGRAPHY. Joern Dalaard Mikkelsen, A/S De Danske Sukkerfabrikker, Biotechnology Section, Langebrogade 1, DK-1001 Copenhagen K.

Cellular adaption to osmotic stress is an important biological process which protects plants and microorganisms against the lethal effects of dehydration. When plants are exposed to environmental draugt or salt stress, the enhanced osmotic pressure in the cells may be adjusted by the production of quaternary ammonium compounds such as betaine. Betaine is produced in the leaves of the plants by two enzymes. In the first reaction catalyzed by choline oxidase, choline is converted to betain aldehyde. In the second biosynthetic step betaine aldehyde is oxidised to betaine by the enzyme betaine aldehyde dehydrogenase.
Betaine aldehyde dehydrogenase from sugar beet leaves has been purified 6300 fold by ion exchange, hydrophobic interaction and affinity column chromatography. The characterisation of betain aldehyde-dehydrogenase will be reported.

M 123 HIGH-YIELD NON-DENATURING PROCEDURE FOR THE PURIFICATION OF CARCINOEMBRYONIC ANTIGEN (CEA), Cristina Mottola, Raffaella Conti and Costante Ceccarini, Sclavo Research Center, Siena, Italy.

Several purification procedures have been described for the oncofetal antigen CEA. The microheterogeneity of CEA, a membrane-associated glycoprotein, and its immunological cross-reactivity with a number of probably related proteins may play a role in the sometimes contradictory nature of the published data. Here we present results obtained with a purification method that allows a high recovery of CEA in its native molecular form (180 kdalton). A portion of human hepatic metastasis (30 g wet weight) was minced and solubilized in 2% deoxycholate. After centrifugation and dialysis, the amount of CEA recovered, measured immunologically (Abbott), was 35 mg. For comparison, the processing of a complete liver metastasis (more than 70% tumor) according to the perchloric acid procedure has yielded in a previous experiment less than twice this amount of CEA. The glycoprotein fraction of the extract was enriched on ConA-Sepharose. CEA was further purified by immunoadsorption; for this purpose a polyclonal antibody raised against purified CEA was conjugated to sepharose. The last few low molecular weight contaminants left in the preparation were separated from the 180 kdalton protein by molecular sieving. Analysis by immunoblotting reveals only one band in our preparation, while three major bands, not evident by staining of the acrylamide gel, are detected in the perchloric acid-extracted material.

M 124 THE BIOLOGICAL ACTIVITY OF DIFFERENT PREPARATIONS OF RECOMBINANT PORCINE GROWTH HORMONE, Joseph J. Patroni, Malcolm R. Brandon, and Michael J. Morey, Melbourne University, Parkville, Victoria, Australia 3052.

A method has been employed to assess the biological activity of recombinant porcine growth hormone prepared by a variety of techniques from inclusion bodies isolated from bacteria.

The method routinely utilises hypophysectomised female rats and only very small amounts of growth hormone. Growth hormone administered in the range 20 - 85μg/rat/day over four days is sufficient to promote measurable growth of the proximal tibial epiphysis. Measured against a highly purified natural growth hormone as a positive control, administration of equivalent, accurately quantified doses of the recombinant preparations have been found to give variable reponses in tibial width. This apparent variation in biological activity is to be expected in view of the refolding procedures used in the preparation of the recombinant protein - procedures in which the protein is exposed to a variety of largely unnatural chemical environments.

These observations can be most easily reconciled with the presence of substantial populations of inactive or partially active protein conformers in the recombinant preparations.

M 125 SEPARATION, ISOLATION, AND SEQUENCING OF POLYPEPTIDES FROM RAT PITUITARY BY TWO-DIMENSIONAL GEL ELECTROPHORESIS COUPLED WITH PRESSURE EXTRACTION, James D. Pearson and Daryll B. DeWald, Biotechnology Research, The Upjohn Company, 301 Henrietta St., Kalamazoo MI 49001

A procedure for resolving polypeptides on non-urea two-dimensional polyacrylamide gels (1) has been coupled with short high-recovery HPLC columns (2) to allow direct sequencing of various polypeptides and proteins (3). The present study extends these methods to the two-dimensional mapping of rat brain polypeptides. The advantage of this technique is the identification of proteinaceous components from crude extract without utilizing time consuming column chromatography procedures. The method is ideal for micro-scale preparation mapping where minimal sample is available for methods development.

1) D.B. DeWald, L.D. Adams, and J.D. Pearson (1986) Anal. Biochem., **154**, 502-508.
2) J.D. Pearson (1986) Anal. Biochem., **152**, 189-198.
3) J.D. Pearson, D.B. DeWald, H.A. Zurcher-Neely, R.L. Heinrikson, and R.A. Poorman, in Proceeding of the 6th Int'l. Conf. on Methods in Proteins Sequence Analysis, in press.

M 126 BACTERIOCINS FROM HALOBACTERIA, Ursula Rdest, Margarete Sturm and Werner Goebel Institut für Genetik und Mikrobiologie, Würzburg, W.-Germany

Some halobacteria strains secrete bacteriocinogenic agents (halocins) into the medium. Hal 1, the halocin from strain Halobacterium spec. GN101 is strictly specific for halobacteria (not for eubacteria or eukaryotes) and exhibits a bacteriostatic and a bacteriolytic effect on growing cells. Hal 1 is sensitive to proteinases but is relatively resistant to heat (only 50 % inactivation by boiling for 1 h). Analysis on gel-filtration columns (Sephadex or Sepharose) shows the halocin activity in the bulk protein fractions. This seems to be due to formation of large aggregates since in chromatography upon silica thinlayer in a basic eluent halocin activity migrates shortly below the front. It can be eluted from a HPLC RPSC Ultrapore column with 0.155M NaCl pH 2.1 and 0-40 % n-Propanol. At a n-Propanol concentration of 36 % the peak of halocin activity can be monitored without concomittant contamination of larger proteins. The present data indicate that Hal 1 is a peptide possibly complexed with a larger protein. Hal 1 may thus be comparable to peptide antibiotics from eubacteria.

M 127 AFFINITY IMMUNOELECTROPHORESIS AND CHROMATOGRAPHY FOR ISOLATION AND CHARACTERIZATION OF PLACENTAL INHIBITOR OF GRANULOCYTE ELASTASE. Michael J. Sinosich, Michael D. Bonifacio and Gary D. Hodgen. Eastern Virginia Medical School, Norfolk Va. 23507, Royal North Shore Hospital, St. Leonards N.S.W. 2065, Australia.

Pregnancy-associated plasma protein-A (PAPP-A), an inhibitor of granulocyte elastase, has many physicochemical similarities to α2 macroglobulin (α2M), frequently occurring as a contaminant in PAPP-A preparations. Similar interactions were demonstrated for both proteins by lectin affinity immunoelectrophoresis (AIE) and metal chelate chromatography. Crossed immunoelectrophoretic analysis after incubation with chondroitinase or B-glucuronidase demonstrated changes only for PAPP-A, indicating the presence of glucuronic acid in the carbohydrate moiety. Heparin AIE demonstrated an interaction with PAPP-A, whereas the electrophoretic mobility of α2M remained constant. Although the heparin-PAPP-A interaction was heterogeneous, this proteoglycan (PAPP-A) was completely recovered after chromatography on heparin-Sepharose. Under these conditions, 99% of applied serum proteins (including α2M) did not bind heparin and were eluted in column void volume. Heparin-binding proteins (ATIII and ATIII-protease complexes) which coeluted with PAPP-A were removed by size fractionation and negative immunoaffinity chromatography. This three step purification schedule yields pure PAPP-A with a 22% recovery.

M 128 MEASUREMENT OF PROTEIN INTERFACIAL MASS TRANSFER COEFFICIENTS IN AQUEOUS TWO-PHASE SYSTEMS, G.F. Slaff, National Bureau of Standards, Center for Chemical Engineering, Boulder, CO 80303.

Aqueous two-phase extraction systems have shown great potential for use in the separation and purification of proteins produced by recombinant DNA technology. Proper design, scale-up and optimization of these processes will greatly benefit from knowledge of the fundamental engineering parameters such as rates of mass transfer, coalescence kinetics and protein partition thermodynamics. This type of information is, for the most part, still unavailable.

In this paper a novel technique for measuring the rate of protein transport from one phase to another in a PEG/Dextran system will be described. The basis of this technique is to monitor the instantaneous protein concentration in the dextran phase by measuring the interaction between the protein and a substrate isolated in this phase. By knowing the protein concentration in the dextran phase as a function of time it is possible to determine ka, the product of the transport coefficient and the interfacial area. Data which correlates ka to the mixing power input will be presented. This information could be used for the rational design and scale-up of aqueous two-phase extraction processes.

M 129 COMPUTER SIMULATION AND ANALYTICAL ISOTACHOPHORESIS AS PREDICTORS OF PROPER ELECTROLYTES FOR PREPARATIVE RECYCLING ISOTACHOPHORESIS, Jeffrey E. Sloan, Richard Mosher, Wolfgang Thormann, Millicent Firestone, Milan Bier. Center for Separation Science, University of Arizona, Tucson, Arizona 85721.

Isotachophoresis (ITP) is a powerful method of analytical electrophoresis. Among the preparative electrophoretic methodologies however, ITP is the least explored. The process produces a steady state system which lends itself to automation and therefore deserves more attention. It is important to be able to predict the performance of electrolyte systems prior to undertaking a preparative separation since preparative ITP runs are both costly and time consuming. We have used both mathematical models and analytical instrumentation (LKB 2127 Tachophor) to determine the proper choice of electrolytes and spacer molecules which optimize the separation of protein samples. Simulation data, capillary data, and an example of a preparative separation for a system of model proteins will be presented.

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M 130 ENGINEERING PROTEIN EXPORT IN *ESCHERICHIA COLI*: EXPRESSION AND EXCRETION OF MUTANT CLOACIN MOLECULES, Arnold J. van Putten, Hans P. Thijs, Joen Luirink, Freek Stegehuis, Frits K. de Graaf and Bauke Oudega, Vrije Universiteit, Amsterdam, The Netherlands

The bacteriocin cloacin DF13 is one of the few proteins that are excreted efficiently into the culture medium of the gram-negative bacterium *Escherichia coli*. The bacteriocin is released from the host cells as a complex of two polypeptides, namely cloacin (M_r 59, 293) and its immunity protein (M_r 9, 974). The immunity protein protects bacteriocin producing cells against the RNA degrading activity of cloacin. The export process requires the presence of a third protein, namely the bacteriocin release protein (BRP; M_r 5,866). The aim of our research is to develop an efficient host/vector system for the high expression and excretion of homologous and heterologous proteins by *E. coli*.. To obtain more information about the structure/function relationship of cloacin molecules with respect to high expression and efficient excretion, we studied the expression/excretion of the cloacin part of the bacteriocin complex. For that purpose we made use of cloacin molecules lacking their RNA degrading activity at the carboxyl-terminus. We observed that the expression of these polypeptides was drastically reduced as compared to cloacin present in the complex. Similar results were found when N-terminal cloacin fragments, obtained after Tn*901* transposon mutagenesis, were analyzed. We concluded from these experiments that either the C-terminal part of the cloacin molecule, involved in binding of the immunity protein and possessing RNase activity, or the immunity protein itself or both are required for a high level expression of the protein. Furthermore, our data suggest that those regions are not indispensable for an efficient excretion of the protein. To further investigate the location of possible topogenic sequences within the primary structure of cloacin which are required for export, we have constructed a plasmid vector which allows the study of the expression/excretion of cloacin molecules having N-terminal or internal deletions, as well as the study of fusions of cloacin sequences and sequences encoding homologous or heterologous proteins. The results will be discussed and a model is presented.