PROTEIN PURIFICATION AND BIOCHEMICAL ENGINEERING

Organizers: William Drohan, Edward Cussler and William Velander

March 19-25, 1990

Plenary Sessions Page

March 20:

Source Materials for Important Proteins....16

Initial Crude Harvest Steps-I....17

March 21:

Initial Crude Harvest Steps-II....19

Volume Reduction and Prepurification Technology-I....19

Volume Reduction and Prepurification Technology-II....20

March 22:

High Resolution Purification....22

Immunoaffinity Purification....24

March 23:

Delivery Systems and Formulation....24

Product Production and Characterization....26

March 24:

Success Stories....28

Poster Sessions

March 20:

Solids and Prepurification Processing (K 100-110)....30

March 21:

High Resolution Methods (K 200-209)....33

March 22:

Large-Scale Processes (K 300-309)....37

Protein Purification and Biochemical Engineering

Source Materials for Important Proteins

K 001 EXPRESSION OF FOREIGN PROTEINS IN THE MILK OF TRANSGENIC ANIMALS, Lothar Hennighausen[1], Christoph Pittius[1], Robert McKnight[1], Avi Shamay[1], Vernon Pursel[2], and Robert Wall[2], [1]Laboratory of Biochemistry and Metabolism, National Institutes of Health, Bldg 10, Rm 9N113, Bethesda, MD 20892. [2]Reproduction Laboratory, Agricultural Research Service, USDA, Beltsville, MD 20705.

Milk protein genes are expressed in the mammary glands of lactating animals and the corresponding proteins are secreted in large quantities into milk. By combining regulatory elements from a milk protein gene with the protein coding sequence from another gene it is now possible to direct the synthesis of non-milk proteins to the mammary gland and milk of transgenic animals (BIO/TECHNOLOGY 5:1183, 1987) Consequently, transgenic livestock may prove useful for the production in milk of valuable proteins in large quantities. In addition, it should be possible to alter milk composition which could result in higher nutritional value, lower antigenicity or higher cheese yield per unit volume of milk.

In an attempt to alter milk composition in livestock we introduced the gene for a major mouse milk protein, the whey acidic protein (WAP), into the genome of swine which does not contain a homologous gene. Fourteen transgenic pigs carrying the mouse WAP gene were obtained. Based on experiments with these pigs we will discuss the ability of milk protein gene regulatory elements to be accurately utilized across species boundaries, the possibility to quantitatively and qualitatively alter milk protein composition and the purification of foreign proteins from milk.

K 002 OVERVIEW OF INDUSTRIAL PLASMA PROTEIN SEPARATIONS: SCIENCE AND TECHNOLOGIES, Fred Rothstein, Bio-Separations Consultants, Long Beach, CA 90807.

A review of the methods and technologies employed by industry in the preparation of therapeutic plasma proteins, as well as recent additions to the traditional procedures, will be presented. Some technological problems associated with industrial scale manufacturing of plasma products will be discussed, with emphasis on problems associated with the large scale implimentation of laboratory procedures on the manufacturing floor. Reference will be made to developments in the "Bio-Tech" industry. A cursory overview of the history and problems associated with plasma product development will be given.

K 003 PRODUCTION OF PHARMACEUTICAL PRODUCTS FROM MILK, L.P. Ruiz, Jr., S.B. Binion, D.R. Clark, M.E. Cady, Procor Technologies, Inc., P.O. Box 116, Minneapolis, MN 55440 Bovine milk is anextremely complex biological secretion containing well over 50 discrete protein components in addition to lipids, minerals, and lactose. Based on dairy industry experience and actual commercial scale production of a licensed biologic from milk, a computer simulation model has been developed to assess the potential recovery and economics for proteins derived from the milk of transgenic bovines. The recovery processes can be modified to accommodate a variety of possible end products and can consist of a series of fractionation steps utilizing centrifrigation, precipitation, membrane fractionation, ion exchange, and affinity chromatography. Results indicate that for certain protein products, production and recovery from the milk of a transgenic bovine would be cost effective.

Initial Crude Harvest Steps-I

K 004 FILTRATION PROCESSES, James L. Dwyer, Ventec, Inc, 33 Locke Drive, Marlborough, MA, 01752.

Solid-liquid separation is a common unit operation for preliminary processing of many protein based products. There are a variety of centrifugal and filtration operations that can be utilized, and selection is dependent on separation specifications as well as the physical/chemical properties of the crude starting material.

Many protein products are susceptible to microbial/viral contamination in-process, such that a filtration step must satisfy the requirements of sterile filtration, or at least considerable viable-count reduction.

Although filtration operations have been the subject of extensive theoretical analysis. The complex interactions of particles and suspensoids have to date prevented effective "dry" modeling of the operation. There are several off-line techniques that are useful in predicting filter performance and scaling of the process.

More recently, membranes have supplanted traditional filter media in many of these applications. Because of the high suspended-solids content of many crude protein products, these membrane techniques employ dynamic flow methods which maintain membrane flux.

K 005 ULTRAFILTRATION AND MICROFILTRATION IN PROTEIN RECOVERY William Eykamp, College of Engineering, Bechtel Engineering Center, University of California, Berkeley, 94720.
Membrane processes practiced on an industrial scale recover tons of protein every day. They do so at low cost, and with high yields. Ultrafilters are very efficient in isolating true proteins from microsolutes and solvents, but they are poor in separating proteins from each other. Microfilters perform similarly with respect to separating colloids and microorganisms from proteins in solution. The desired separation in protein purification is often more than either process can accomplish by itself, but the job these membrane processes do is accomplished with high efficiency and low cost. Examples of successful operation and limits of the technology are included. Estimates for capital and operating costs are given for large and mid-sized cases.

K 006 THE USE OF BIOPROCESSING AIDS IN THE CLARIFICATION PHASE OF PROTEIN PURIFICATION, Ian T. Forrester, Anthony C. Grabski and W. Nick Strickland, Protein Purification Facility, University of Wisconsin Biotechnology Center, Madison, WI 53705.
Protein purification usually requires three fundamental phases, involving (1) the harvesting and clarification of the original extract, (2) concentration of the clarified stream and (3) purification of the target protein. The type and effectiveness of the procedures adopted in Phase 1 will have a direct influence on the efficacy and processing options which can be considered for the Phases 2 and 3. Although every biological system presents the protein purifier with a different set of contaminants, which ideally should be removed in the Phase 1 process, there is some universality to these interfering components. For example cellular debris, nucleic acids, lipid, oligosaccharides and pigmented factors are, to varying degrees, commonly occurring factors. Incomplete removal of these factors may not restrict the completion of protein purification, when conducted at an analytical level. However removal of contaminating factors in an effective and economical manner, assumes greater importance when a process is transformed into a production-level process. We have been investigating the impact of different chemically-based clarification strategies on these broader issues of protein purification. In our program to-date a wide variety of different proteins from quite diverse biological systems, including plant, animal, fungal and bacterial, have been examined. In these studies we have assessed several different chemical options including a range of new materials, the Biocryls -- also referred as bioprocessing aids(BPAs) -- as well as more established chemical agents such as polyethyleneimine and chitosan. These chemically-based, Phase 1, clarification systems have been incorporated successfully into protocols to purify proteins such as: the Mn-dependent lignin peroxidase from *Lentinula edodes* (the shiitake mushroom) when cultured on a commercial wood substrate; trypsin from bovine pancreas; peroxidase from alfalfa; a fungal-derived allergen; and an extracellular xylanase from *Streptomyces rosea.*

Protein Purification and Biochemical Engineering

Initial Crude Harvest Steps-II

K 007 RECENT ADVANCES IN LARGE SCALE CONTINUOUS FLOW CENTRIFUGATION DeLoggio, T.J., Pharmaceutical and Biotechnology Department. Sharples, Inc., 955 Mearns Road, Warminster, PA 18974 A growing number of therapeutic and regulatory proteins produced commercially or for clinical trials are derived via modern rDNA technologies. Notable examples of these are TPA's, insulin, and interferons. The bio-catalysts most often employed to produce these highly active proteins are recombinant bacteria and mammalian cells. The cost-effectiveness of the commercial process can depend on the success of the initial crude harvest steps where cells are separated from the broth and concentrated or classified. Mammalian cells are extremely sensitive to shear forces imposed by the processing equipment. For example, shear stresses of 250 Pa can lead to lysis of Erythrocytes, where as bacterial systems can withstand shear stresses as high as 5,000 Pa. Excessive shear will lead to release of intracellular impurities and complicate downstream chromatographic steps. Shear also leads to the loss of cell viability preventing reuse of the cells. In order for the new protein products to become widely available to the public, equipment manufacturers must meet these new challenges with equipment especially designed for the new bioprocesses. A new centrifugal system has been developed for mammalian cell processing. Recent performance data detailing the principles and capabilities of the system will be presented. Many of the proteins produced by recombinant bacteria, such as E. coli, are contained in inclusion bodies. A new centrifuge has been developed which maximizes the concentration and purity of the inclusion bodies going to downstream processing. Recent performance data for this system will be presented and analyzed. The paper will concentrate on performance of these two new commercial scale continuous flow centrifuges. In addition, issues of aseptic operation, scaleability, validation, and containment will be addressed.

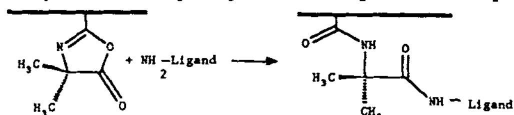
Volume Reduction and Prepurification Technology-I

K 008 **AZLACTONE COPOLYMER BEADS: APPLICATIONS IN BIOSEPARATIONS.** **P.L. Coleman, D.S. Milbrath, M.M. Walker (Bioscience Laboratory), S.M. Heilmann, J.K. Rasmussen, L.R. Krepski (Corp. Res.--Technology Development Lab) 3M Corp., St. Paul, MN 55144.**

I will present results on several chromatographic applications of a novel polymeric support containing the reactive group azlactone. Emphasis will be given to affinity chromatography, among several modes of separation.

Hydrophilic beads are prepared by inverse phase suspension polymerization of vinyldimethyl azlactone and methylene-bis-acrylamide. Bead diameters of 5-200 um and azlactone functionalities of 1-3.5 meq/ml can be prepared by varying ratios and types of monomers and reaction conditions.

The protein coupling reaction proceeds rapidly, reaching half completion in about 5 min. The pH profile is maximum at pH 8-9, but has a significant degree of reactivity below pH 7, so that alkali-unstable proteins are readily immobilized. Sodium sulfate greatly enhances the rate and extent of reaction for most proteins, including BSA, IgG, Protein A, trypsin, and ovalbumin, all of which couple at densities greater than 100 mg of protein per g of polymer.



We have focused our efforts in chromatography using immobilized Protein A since there is a broad interest in this mode of affinity separation. We have prepared immobilized Protein A supports having densities up to 400 mg/g and have tested such supports at several Protein A densities in affinity columns. IgG binding capacities are consistently 1 mole per mole of immobilized Protein A, resulting in as much as 56 mg of IgG recovered per ml of column. The columns have consistently shown low back pressure, high flow rates, low non-specific binding and stable bed volume with changing mobile phase. Model ion exchange, reverse phase, hydrophobic interaction, and size exclusion separations will be included.

K 009 MEMBRANE-BASED AFFINITY SEPARATIONS,
Stephen E. Zale, O. Dile Holton III and Vipin K. Garg, Sepracor Inc., Marlborough, MA 01752
The use of microporous membrane supports for affinity chromatographic separations facilitates rapid, efficient and scalable downstream processing of biomolecules. Fluid residence time requirements can be reduced substantially and more efficient use of ligand can be made when diffusion times are minimized by convecting feed mixtures through the support matrix. The resulting high throughput capability of affinity membrane devices allows affinity separations to be performed on dilute feeds earlier in the downstream purification process.

The effects of variables such as loading, washing and elution conditions on product purity and yield and on affinity membrane device productivity are examined. Examples of Protein A, lectin and immunoaffinity membrane separations are illustrated and the potential impact of affinity membranes on overall downstream processing schemes is discussed.

Volume Reduction and Prepurification Technology-II

K 010 Scale-up considerations for Tangential Flow Filtration processes
Gastón de los Reyes, Millipore Corp., Process Systems Div., Bedford MA. 01730.

Scaling rules and guidelines for Tangential Flow Filtration (TFF) processes are elusive to many users, in particular, those rules that relate to a change of the dimensions of the flow channel (i.e. height, length, as well as screen geometry). As a result, users are forced to do small-scale evaluation of TFF technology with *scaled-down devices* , that is, devices whose flow channels are **identical** to those found in full-scale devices. This approach to scale-up is not the best one to follow: sometimes it is just not possible (since a scaled-down version of the device of interest is not available), at best, it is impractical (since most *scaled-down devices* have too much area and/or too much hold-up volume). An alternative approach to scale-up is presented, which relies on the premise that the only factor that must be kept constant is the membrane (i.e. the membrane must be the same from small- to large-scale), and that all remaining factors can be accounted for according to well established engineering principles.

The key considerations for scale-up of membrane systems are discussed, with emphasis on the chemical engineering aspects of scale-up. Scaling rules, as well as a discussion of product losses are reviewed.

K 011 SALT-INDUCED PRECIPITATION OF PROTEINS, Todd M. Przybycien and James E. Bailey, Department of Chemical Engineering, California Institute of Technology, Pasadena, CA 91125

The effects of salt type and concentration on protein solubility and recoverable activity were studied using α-chymotrypsin (αCT) as a model protein and five salts spanning the lyotropic series. Salt-protein interactions and changes in protein physical properties likely account for discrepancies between experimental data and present solubility theories. Active protein recovery was a function of salt type, but not concentration. αCT precipitates were examined for perturbations in secondary structure via Raman spectroscopy and in active site tertiary structure via electron paramagnetic resonance spectroscopy. NaBr, KBr, and KSCN-induced precipitates had increased β-sheet and decreased α-helix contents; these changes were correlated with active protein yields. Spectra of spin-labelled precipitates indicated that the active site remains intact. Molecular modelling was used to estimate changes in the dipole moment and hydrophobic surface area for the altered precipitates. The generality of these structure changes was explored for twelve different proteins via Raman spectroscopy. KSCN-induced precipitates exhibited increased β-sheet and decreased α-helix contents; structural changes for Na_2SO_4-induced precipitates were less coherent. The β-sheet increase may occur at the expense of α-helix segments. β-sheet increases were correlated with the fraction of charged residues and the surface area of the native protein; α-helix decreases were correlated with the dipole moment and helical content of the native protein. The effects of temperature, protein concentration, salt type, and salt concentration on αCT aggregation kinetics were studied. Stopped-flow turbidimetry indicated that temperature and salt concentration effects are exerted through changes in protein solubility. Protein concentration effects are well-described by Smoluchowski's collision equation. The aggregation of partially inhibited αCT demonstrated poisoning behavior. Solute particle radius distributions determined by dynamic laser light scattering indicated that aggregation depends on the supersaturation. A detailed population balance model, accounting for specific and nonspecific quaternary interactions, was formulated and employed for analysis of the transient aggregation data.

K 012 REVERSED MICELLAR EXTRACTION OF ENZYMES, Matthijs Dekker and Klaas van 't Riet, Wageningen Agricultural University, PO Box 8129, 6700 EV Wageningen

The recovery of an extracellular enzyme usually involves the removal of undesirable compounds from the fermentation broth (e.g. microbial cells, debris, other proteins, salts and water). The conventional processes employed are filtration, ultrafiltration, precipitation and drying while for more purified enzymes chromatographic processes are usually required. It would be very desirable to have access to a recovery step that is selective for the desired enzyme, that gives a considerable increase in the concentration of the enzyme and that can be scaled up easily. A liquid-liquid extraction might serve these purposes. To be able to extract an enzyme from an aqueous phase into another liquid phase, an immiscible phase that can solubilize enzymes is needed.

A water-immiscible phase that can solubilize enzymes is an apolar solvent containing reversed micelles. Reversed micelles are aggregates of surfactant molecules surrounding an inner core of water. Many enzymes have been successfully solubilized by reversed micelles without losing their catalytic activities. These reversed micellar systems containing enzymes have been used to study enzymatic reactions involving apolar substrates. Furthermore it has been demonstrated that proteins can be transferred from an aqueous phase to a reversed micellar phase or vice versa.

To apply the reversed micellar extraction method for the recovery of proteins, a continuous forward and back extraction process can be used. Previously we have investigated the performance of this process in two mixer/settler units. In this way the enzyme α-amylase could be concentrated. During the extraction, a loss of the enzyme activity and a slow loss of surfactant were observed. In this lecture, the mechanism of the enzyme inactivation during the extraction and the modeling of the extraction, is described. As predicted by this model, activity recovery and surfactant loss can be optimized considerably.

The back transfer rate for protein extraction appeared to be relatively slow. This slow mass transfer was ascribed to an interfacial resistance caused by the low coalescence rate of reversed micelles containing a protein molecule with the interface between the organic and aqueous phase. The aqueous phase pH was found to have a large effect on this interfacial resistance. Decreasing the number of groups on the protein with a charge opposite to that of the surfactant headgroups, resulted in an enhanced back transfer rate. In this lecture we describe an alternative process for the desolubilization of protein from the reversed micellar phase. This procedure circumvents the disadvantageous effects of the decreased back extraction mass transfer rate.

Another possible way of extracting enzymes from an aqueous phase into a reversed micellar phase is by membrane extraction. By performing a membrane extraction, it might be possible to utilize in one operation the selectivity by the membrane permeability as well as the selective solubilization by the reversed micellar phase.

Protein Purification and Biochemical Engineering

High Resolution Purification

K 013 METAL AFFINITY PROTEIN EXTRACTION USING METAL-CHELATING POLYMERS,

Frances H. Arnold, Division of Chemistry and Chemical Engineering, California Institute of Technology, Pasadena, CA 91125

Polyethylene glycol derivatives incorporating metal chelates as affinity ligands have been synthesized and used to extract proteins in aqueous two-phase systems. Partitioning in two-phase systems containing Cu(II)IDA-PEG is directly related to the number of histidines accessible on the protein surface. The selectivity of the metal-affinity extraction process can be altered through the design of the polymer-metal chelate. For affinity ligands made from small, neutral chelates such as Cu(II)IDA-PEG, proteins continue to be extracted in proportion to the number of exposed histidines. Dramatically altered selectivities are observed for chiral, charged, or sterically hindered chelates. For example, Cu(II)-(L)Met-PEG exhibits a significantly higher affinity for horse heart myoglobin than the (D)-enantiomer. In fact, horse myoglobin is preferred over whale myoglobin in the presence of Cu(II)-(L)Met-PEG, even though the whale protein contains more surface histidines. The degree to which individual histidines are accessible to the metal is determined by the nature of the chelating ligand. These differences in selectivity can be exploited in the design of affinity extraction separations for specific proteins.

We have also investigated how protein engineering can be combined with metal affinity separations for the purification of recombinant proteins. A synthetic metal-binding site consisting of two histidines placed His-X_3-His in an α-helix and engineered into the surface of S. cerevisiae cytochrome c dramatically increases the partition coefficient of the protein in metal affinity two-phase systems. The metal-binding function does not interfere with the protein's biological activity. Similar sites built into bovine somatotropin at Monsanto Co. have also been characterized by metal affinity partitioning. Stability constants for the protein-metal chelate complex range from 2×10^4 to 2×10^6. The His-X_3-His site in an α-helix confers a unique affinity for metals, a property which can be effectively exploited in the purification of the recombinant protein. The utility of metal affinity extraction has been demonstrated with separations of model mixtures of human hemoglobin and human serum albumin and with the isolation of the synthetic metal-binding cytochrome c from yeast.

K 014 SCALE-UP OF ELECTROPHORESIS: ISOELECTRIC FOCUSING AND ISOTACHOPHORESIS.

Milan Bier, Center for Separation Science, University of Arizona, Tucson, AZ 85721, USA

Scale-up of electrophoretic technology is best carried out in free solutions, in absence of gels or other supporting matrices. Isoelectric focusing is particularly appealing for such development, as proteins become virtually immobilized at their isoelectric point, a highly discriminating parameter. In free solution, of course, control of fluid flow is essential.

Our laboratory has contributed several instruments for large scale focusing. In the most recent apparatus, the Recycling Free Flow Focusing (RF3), the protein solution to be focused is rapidly recycled through a narrow channel interposed between the electrodes, with a residence time of only a few seconds. Remarkable fluid stability is thereby achieved, overcoming some of the difficulties inherent in previous instruments. As a result, the apparatus can be also used for preparative isotachophoresis.

The advantages of preparative focusing are the predictability of results from analytical gels, sharpness of resolution and cleanliness of operation. Isotachophoresis has also the potential of high resolution, even though only a few reliable buffer systems have yet been developed. Supported in part by NASA grant NAGW-693.

K 015 DISPLACEMENT CHROMATOGRAPHY OF BIOPOLYMERS, Steven M. Cramer, Department of Chemical Engineering, Rensselaer Polytechnic Institute, Troy, NY 12180.

Displacement chromatography has been demonstrated to be a powerful technique for the simultaneous concentration and purification of biomolecules. In order for the process to be successfully employed for large scale bioseparations, it is critical that the arsenal of displacers for a variety of adsorbent systems be increased in the near future. Furthermore, the scale-up of the process requires a detailed knowledge of the interplay of various operating parameters and their effect on bioproduct throughput and purity. In this seminar, I will present results from our theoretical and experimental studies on displacement chromatography. Displacement chromatography was successfully employed for the preparative-scale separation of peptides, antibiotics, and proteins. Protein displacements were carried out at elevated flow-rates and the displacement process was scaled-up to relatively large particle and column diameter systems. Model biopolymers were purified by metal-chelate displacement chromatography and non-toxic displacers were identified and synthesized for use in various adsorbent systems. The relative importance of various displacer physico-chemical properties was also investigated.

A mathematical model of displacement chromatography was developed which incorporates the effects of mass transport, axial dispersion, and adsorption/desorption kinetics. The interplay of various operating parameters on bioproduct throughput was investigated and the experimental results compared to the predictions of the mass transport-kinetic displacement model.

The talk will conclude with some comments about critical research areas for the future development of displacment chromatography.

K 016 COUNTERCURRENT CHROMATOGRAPHY - ITS POTENTIAL FOR PROTEIN PURIFICATION, Ian A Sutherland, Brunel Institute for Bioengineering, Brunel University, Uxbridge, Middlesex, UB8 3PH, Uk.

Countercurrent Chromatography (CCC) is a liquid-liquid extraction process which has proved to be invaluable in the fractionation of natural products like antibiotics, particularly those that partition well in aqueous/organic phase systems [1]. Various attempts have been made to extend this technology to the fractionation and purification of macromolecules and cells often utilising aqueous bi-phasic systems [2]. While low interfacial tension and small density differences are major factors in the choice of two phase aqueous polymer phase systems as a suitable gentle host medium for the purification of proteins or cells, they can also be the limiting factors when trying to optimise methods of achieving large scale purification.

Recently Eveleigh [3] has developed immiscible liquid affinity supports utilising perfluorocarbon emulsions, which preserve the aqueous/aqueous nature of polymer phase systems and yet offer physical properties and fluid handling characteristics more akin to aqueous/organic phase systems [4]. The development of high capacity liquid extraction systems using perfluorocarbon emulsions will be described.

1. Countercurrent Chromatography, Theory and Practice (1988) Chromatographic Science Series (N.B. Mandava & Y. Ito eds), Vol. 44, Marcel Dekker, New York & Basel

2. Sutherland, I.A. (1985) Other Types of Countercurrent Distribution Apparatus and Continuous Flow Chromatography Techniques. In: Partitioning in Aqueous Two-Phase Systems (Walter, Brooks & Fisher eds.) pps 149-159, Academic Press, New York.

3. Eveleigh, J.W. (1989) Immiscible Liquid Affinity Supports. In: Separations Using Aqueous Phase Systems (Fisher & Sutherland eds.) pps 447-454, Plenum Press, New York.

4. Sutherland, I.A. & Eveleigh, J.W. (1989) Some Rheological Properties of Perfluorocarbon Emulsions and Their Potential Use in Countercurrent Distribution and Chromatography Separation Systems. In: Separations Using Aqueous Phase Systems (Fisher & Sutherland eds.) pps 477-478, Plenum Press, New York.

Immunoaffinity Purification

K 017 PROCESS IMPLICATIONS FOR METAL-DEPENDENT IMMUNOAFFINITY INTERACTIONS,
W.H. Velander and R.D. Madurawe, Department of Chemical Engineering, Virginia Polytechnic Institute and State University, Blacksburg, VA 24061; C.L. Orthner, J.P. Tharakan, A.H. Ralston, D.K. Strickland and W.N. Drohan, Plasma Derivatives and Biochemistry Laboratories, American Red Cross, Jerome H. Holland Laboratory for the Biomedical Sciences, Rockville, MD 20855.

Immunosorbents have been developed which utilize metal-dependent interactions between monoclonal antibody(s) (Mab) and human plasma Protein C and Factor IX, members of the vitamin K-dependent plasma protein family. The potential process advantages are described for two different types of divalent metal-dependent systems: 1) a Mab-Protein C (antigen) complex which is stabilized in a divalent metal-free environment and 2) a Mab-Factor IX (antigen) complex which is stabilized in the presence of divalent metal ions. In the first example, Protein C binds to the immunosorbent in the presence of EDTA or sodium citrate and can be eluted with buffer containing 25 mM calcium chloride or magnesium chloride. In the second example, Factor IX binds to the immunosorbent in the presence of magnesium chloride and can be eluted with sodium citrate or EDTA. These gentle elution conditions are desirable in order to retain the native structure as well as functional activity of the protein to be immunopurified. Furthermore, unwanted plasma proteins, which may interact nonspecifically with either the agarose matrix or immunoglobulin G (IgG) ligand are less likely to be eluted under these conditions. The calcium-free citrate buffers used for immunosorption by either of these processes preclude the formation of fibrin solids and eliminate eluents which are difficult to process and which interfere with the biological activity of the product (i.e. chaotropes). The immunoaffinity process for Protein C and Factor IX provide high functionality, purity and yield and demonstrates the feasibility of large-scale processes utilizing metal-dependent immunosorbents.

Delivery Systems and Formulation

K 018 RELEASE OF INSULIN FROM pH-SENSITIVE POLY (ORTHO ESTERS),

J. Heller, G. M. Grodsky, A. C. Chang and N. M. Franson, Controlled Release and Biomedical Polymers Program, SRI International, Menlo Park, CA 94025 and Metabolic Research Unit, University of California, San Francisco, CA 94143.

The principal objective of this work is the development of a bioerodible insulin delivery device that will release insulin in response to the concentration of external glucose where release of insulin is modulated by pH changes resulting from a glucose-glucose oxidase reaction. In this device a pH sensitive polymer containing dispersed insulin is surrounded with a microporous hydrogel containing immobilized glucose oxidase. When glucose diffuses into the device, gluconic acid produced within the hydrogel will decrease the ambient pH thus triggering release of insulin. A major component of such a delivery system is a bioerodible polymer that can reversibly change erosion rates in response to very small changes in the surrounding pH. We have prepared such a polymer by incorporating tertiary amine groups into a linear poly(ortho ester). When insulin is incorporated into this polymer and thin disks subjected to well defined pH pulses, the polymer responds rapidly and reversibly to these pH pulses. Because insulin is a large molecule, the hydrogel must be porous enough to allow free passage of insulin. We are preparing such hydrogels by the free radical copolymerization of hydroxyethyl methacrylate with about 0.5 mole% ethylene glycol dimethacrylate as 25% solutions in water. To avoid diffusion of glucose oxidase from the highly porous hydrogels, the enzyme is derivatized with acrylic anhydride which reacts with its lysine amino groups and the enzyme becomes chemically incorporated into the hydrogel through the double bonds during the free radical polymerization process.

K 019 CONTROLLED-RELEASE DELIVERY SYSTEMS, S.M. Herbig, K.E. Thompson, and K.L. Smith, Bend Research, Inc., 64550 Research Road, Bend, Oregon, 97701. Porous beads made by a phase-inversion process have been developed for the sustained-release delivery of bioactive materials. Typically, these beads have been used to release insect pheromones; however, they have many characteristics that make them versatile controlled-release delivery systems: they release active ingredients at a constant rate, their porous structure can be controlled by controlling process parameters, they easily accomodate high loadings of active ingredient, and they can deliver hydrophobic and hydrophilic active ingredients. These beads constitute a reservoir-type delivery system, with the release kinetics controlled by their porous structure. Beads loaded with insect pheromones have exhibited durations of release of from one to four months, and they have exhibited similar durations of efficacy in field tests.

Another type of controlled-release delivery system, which consists of a bioerodible implant, has been developed for the release of disulfiram to treat alcoholism. The design consists of a drug reservoir coated with a drug-permeable membrane that bioerodes following drug release. This design allows high drug loadings (typically greater than 70 wt%), which minimize the size of the implant. The membrane consists of poly ℓ-lactic acid to facilitate bioerosion and biocompatibility. In a 40-day bioavailability study, ALDH inhibition--the key parameter in determining implant effectiveness--was constant.

K 020 DELIVERY SYSTEMS

by Robert Langer, Massachusetts Institute of Technology, Dept. of Chemical Engineering, Cambridge, MA 02139

Over the past 2 decades, increasing attention has been paid to development of systems to deliver drugs for long time periods at controlled rates. Such systems have been developed for the treatment of eye diseases and birth control. Some of these systems can deliver drugs continuously for over 1 year. However, little attention has been given to developing systems for the controlled release of large molecules (M.W. > 1000) such as polypeptide hormones. In early studies, we discovered that small pellets made of hydrophobic polymers such as ethylene-vinyl acetate copolymer could release many different macromolecules in bioactive form for over 100 days in vitro and in vivo. By combining parameters, such as drug particle size, loading, and coating, release rates for any drug could be changed several thousand fold. Microstructural studies show that the incorporation of powdered macromolecules during polymer matrix casting creates a series of interconnecting channels through which dissolved drug can diffuse. To achieve constant rates, a hemispheric device laminated with an impermeable coating, except for a small cavity in the center face, was developed. Constant release was achieved for over 60 days. Monte-Carlo computer methods are currently being explored to model these systems. By using these techniques, a variety of systems for releasing polypeptides such as insulin, epidermal growth factor, tumor angiogenesis factor, interferon, and protein vaccines have been designed.

Bioerodible polymers, in particular polyanhydrides have recently been explored as vehicles to release both large and small molecules. These polymers are unique in that they show surface erosion and lead to near constant release rates of incorporated drugs. By altering the hydrophobicity of the polymer backbone, release times from 1 week to 6 years can be achieved. They have recently been approved by the FDA for human clinical trials. Twenty-eight medical centers are now testing these polymers in a novel drug delivery application for brain cancer.

Finally, several new biodegradable polymer systems as well as polymer systems that display feedback control will be discussed.

K 021 LYOPHILIZATION. Alan P. MacKenzie, Center for Bioengineering, WD-12, School of Medicine, University of Washington, Seattle WA 98195.
Freeze-drying, or lyophilization involves the conversion of much, and sometimes all the water present in a given system to ice and the sublimation of that ice in vacuo. It involves an additional dehydration where the water remains distributed between ice and one or more additional phases after freezing. Lyophilization requires equipment designed to remove heat from, and to return it to the product. The same equipment needs to facilitate the dehydration of the product by water vapor transfer. The process requires close control of heat and mass transfer. The process also requires the careful choice of freezing procedure and the selection of a suitable freeze-drying cycle. Proteins are often formulated for human or for veterinary injection or for diagnostic application and are therefore frozen and freeze-dried in the presence of one or more other materials required for different reasons. Formulation for best freezing and freeze-drying behavior may present a special challenge in such instances. We will examine the freezing process with special reference to the behavior of dissolved proteins in the presence of simple inorganic salts, inorganic and organic buffer systems, other soluble materials, and surface active agents. We will see how the formulation frequently predetermines the choice of freezing and freeze-drying procedures and how it is that proteins are preserved in active states in practice.

Product Production and Characterization

K 022 REGULATORY ISSUES IN THE GMP PRODUCTION AND PURIFICATION OF BIOLOGIC PRODUCTS, Vipin K. Garg*, Bioresponse Inc., 1978 W. Winton Ave., Hayward, CA 94545; *Current Address: Sepracor Inc., 33 Locke Drive, Marlborough, MA 01752.
In the past decade advances in genetic engineering have greatly increased interest in large scale culture of animal cells and the recovery of secreted proteins from these cells. Monoclonal antibodies, factor VIII, interferons, plasminogen activators, protein C and erythropoietin are a few examples of therapeutic proteins currently being produced in cell culture. As commercialization of these products increases, the regulatory issues associated with the production and purification of biologic products is becoming a critical issue.

The data presented will review these regulatory issues. Several specific production and purification examples for therapeutic proteins (e.g., MoAbs, growth factors, t-PA) will be illustrated. A particular emphasis will be given to evaluating current techniques for large scale purification of proteins. Purification of milligram to multigram quantities of therapeutic proteins using modern separation techniques will be discussed. In addition to protein purity, emphasis will be given to DNA and endotoxin removal from the final product. Regulatory specifications and validation aspects for these variables will also be reviewed.

K 023 HPLC OF BIOPOLYMERS: THE PAST, PRESENT AND FUTURE. , F. E. Regnier and Roman Chicz, Department of Biochemistry, Purdue University, Lafayette, Indiana 47907.

This paper will examine the contribution of microenvironmental effects on chromatographic behavior. Site directed mutagenesis was used to make amino acid substitutions at specific sites in the serine protease subtilisin (MW = 27,500). These genetically engineered variants were shown by X-ray crystallographic analysis at 1.8 A resolution to have the same three-dimensional structure as the wild type enzyme. Chromatographic behavior of variants was examined by IEC, HIC, IMAC, and RPC. It will be shown that substitution of valine for glycine at position 166 changed the pK_a of histine 64 15 A away on the surface and produced a 10 minute change in chromatographic retention. Data from the examination of 20 variants clearly show that amino acids on the surface of a protein may not be viewed as isolated species. Their properties and chromatographic behavior are strongly influenced by a microenvironment encompassing up to 600 A_2 of the surface.

K 024 ISSUES IN THE PURIFICATION AND CHARACTERIZATION OF rDNA PROTEINS FOR THERAPEUTIC USE, Satish K. Sharma, Anne F. Vosters, and David B. Evans, Biopolymer Chemistry, The Upjohn Company, Kalamazoo MI 49001.

Advances in recombinant DNA technology have resulted in various choices for expressing and producing therapeutic proteins. Basically, there are two ways of heterologous gene expression: intracellular and extracellular. The nature of the final protein product is determined to some extent by the decision taken between these two options. Therefore, issues related to purification and characterization of recombinant proteins will be governed by the selected host expression system and the mode of expression. Some of these issues arise as a consequence of heterologous gene expression while others are matters of consideration associated with downstream handling of expressed recombinant proteins (1). These concerns are: improper folding or aggregation, incorrect or heterogeneous N-terminal, blocked N-terminal, presequences, proteolytic clipping, chemical modification, conformational changes, glycoforms, contaminants such as host proteins, cellular DNA, viruses, or endotoxins, and challenges in the final characterization of recombinant therapeutic proteins. This talk will elaborate on these scientific issues with data on some specific examples of rDNA proteins.

(1) S. K. Sharma, Advanced Drug Delivery Reviews, **4 (2)**, 1990.

K 025 PURIFICATION AND CHARACTERIZATION OF RECOMBINANT HUMAN PROTEIN C FROM THREE MAMMALIAN CELL LINES. S. Betty Yan, P. Razzano, Y. Chao, D. McClure, and B. Grinnell, Lilly Research Laboratories, Eli Lilly and Company, Indianapolis, IN 46285.

Human Protein C (HPC), an anticoagulant factor, is a vitamin K-dependent protein that has four post-translational modifications. In an effort to define the functional roles of these modifications, recombinant HPC (rHPC) was expressed in and characterized from three adenovirus-transformed cell lines; 293 (human kidney), AV12-664 (Syrian hamster) and BHK-Ad (Syrian hamster kidney). The rHPC in crude culture medium from the three cell lines displayed anticoagulant activities that were either higher (293), slightly lower (BHK-Ad) or much lower (AV12-664) than that measured for plasma HPC. The rHPC from each cell line was purified and characterized using a novel chromatographic step, termed "pseudo-affinity", which is based on the selective elution of HPC from anion-exchange resin by 10mM Ca^{2+}. All of the 293 and BHK-Ad cell-derived rHPC bound to the anion-exchange column and eluted with 10mM $CaCl_2$. The material exhibited the same specific anticoagulant activity as unpurified rHPC, and contained the same number (9) of γ-carboxyglutamate (Gla) residues as plasma HPC. In contrast, only 60% of the AV12-664-derived rHPC bound to the anion-exchange column was eluted with 10mM $CaCl_2$ and the remainder eluted with 0.4M NaCl. The rHPC in the 10mM $CaCl_2$ eluate contained 9 Gla and has anticoagulant activity similar to that of plasma HPC, while the rHPC in the 0.4M NaCl eluate contained only 6-7 Gla and had only 20% of the anticoagulant activity of plasma HPC. The rHPC from each cell line was properly proteolytically processed into the mature 2-chain heterodimeric form, and had the equivalent number (0.6-1.2) of ε-β-hydroxyaspartate residue as plasma HPC. The glycosyl content of the rHPCs was qualitatively and quantitatively distinct from plasma HPC and each was quantitatively different from each other. Compared to plasma HPC, the fucose content was 3-5 fold higher in each of the rHPCs and GalNAc was present in the rHPCs but not in plasma HPC. The sialic acid content of rHPC from BHK-Ad was twice that of the 293- and AV12-664-derived rHPC, and was equal to that of plasma HPC. The complete removal of sialic acid from 293-derived rHPC resulted in a 2-3 fold increase in anticoagulant activity, suggesting that the variation in the anticoagulant activities between the fully γ-carboxylated rHPCs could be due to differences in sialic acid content.

Success Stories

K 026 PURIFICATION OF THERAPEUTIC PROTEIN CONCENTRATES FROM HUMAN PLASMA, William N. Drohan, Shirley I. Miekka and David B. Clark, American Red Cross, 15601 Crabbs Branch Way, Rockville, MD 20855. Advances in biochemistry, availability of higher performance resins and increased acceptance of affinity chromatography are providing for exciting and creative changes in the way that human plasma is fractionated. Traditionally, therapeutic proteins from plasma have been isolated by fractional precipitation in processes such as those developed by Cohn in the 1940's. According to Cohn's procedure, fractions enriched in specific human proteins were precipitated by adjusting the ethanol concentration, pH, temperature, salt and protein concentration of a solution of human plasma. However, for trace proteins such as factor VIII precipitation methods produce fairly low purity products.

By use of a variety of different chromatographic methods, proteins of significantly improved purity and safety have recently been obtained from human plasma. As important, trace proteins in plasma have been recovered in quantities sufficient to develop new therapeutic concentrates. For example, the Vitamin K dependent proteins, factor IX, factor X and Protein C have been concentrated and partially purified, first on ion exchange columns, and then each purified to states approaching homogeneity by a series of monoclonal antibody columns. Antithrombin III has been isolated from the ion exchange column flow-thru stream by a single adsorption/elution on a heparin Sepharose column.

A unified scheme of protein purification from human plasma using column chromatography is discussed and the characteristics of selected purified proteins are described.

K 027 MEMBRANE AFFINITY SEPARATION FOR BIOMOLECULE PURIFICATION AND DIAGNOSTICS. Dennis Pietronigro, Darany An, Vince Forte, Fook Hai Lee, Paul Lin, Kent Murphy and Sara Vasan, NYGene Corp., One Odell Plaza, Yonkers, New York 10701.

Membrane Affinity Separation Systems (MASS^R) is a core technology that exploits a series of proprietary chemical modifications of microporous membranes to produce affinity supports. These modified microporous membranes are used in biomolecule separations and diagnostics.

Specific application of this new technology for the purification of antibodies utilizing a recombinant Protein A ligand covalently coupled to the membrane will be detailed. Performance characteristics of the MASS^R technology for antibody purification are:

Purity = 98%+; Recovery = 85 to 95%; Extremely high flow rates; Process cycle times 4 to 5 minutes; Reusability greater than 100 times; Low ligand leaching; Easy sanitization; Easy storage; Direct scalability; Low cost, economical process; and Simple, rapid operation.

The technical performance criteria of purity, recovery, ease of use and time will be evaluated and compared to both gel and other membrane technologies. Products utilizing MASS^R are available in capacities from 1 mg to over 1 gram. Theoretically, the 1 gram device can purify about 0.25 kilograms per day.

Protein G derivatized membranes and Universal Affinity Membranes (UAM^{TM}) which utilize active ester, epoxide, aldehyde and hydrazide functional groups have also been developed. UAM^{TM} allow a user to covalently attach his own ligand for specific biomolecule purifications and diagnostic applications.

K 028 GENETIC APPROACHES TO FACILITATE PROTEIN PURIFICATION, Mathias Uhlén, Department of Biochemistry, Royal Institute of Technology, Stockholm, Sweden. Gene fusion systems based on the staphylococcal protein A and the streptococcal protein G genes have been developed to facilitate purification of recombinant proteins, both in large and small scale (1). Due to the strong interactions with IgG and serum albumin, respectively, it is possible to recover gene fusion products in a one-step procedure, giving high yield and purity. Different specific enzymatic and chemical cleavage sites have been introduced at the fusion point between the protein A/G derivative and the desired protein. The affinity "tail" can thus be removed from the purified fusion protein, releasing biologically active molecules. The gene fusion products in this system are usually efficiently secreted to the culture medium when expressed in *Escherichia coli* and the product can thus be recovered, purified and concentrated by passing the clarified medium directly through an affinity column. These systems have been used to produce potential recombinant malaria vaccines (2) and different human peptide hormones of therapeutical interest (3).

1. M. Uhlen and T. Moks, *Methods in Enz.* **185**, 129-143 (1990).
2. S. Ståhl et al *J. Immunol. Meth.* **124**, 43-52 (1989).
3. B. Hammarberg et al *Proc.Natl.Acad.Sci.USA* **86**, 4367-4371 (1989).

Solids and Prepurification Processing

K 100 CHARACTERIZATION AND PURIFICATION OF A MEMBRANE-ASSOCIATED GLYCOPROTEIN IN THE HUMAN COLON ADENOMACARCINOMA CELL LINE, COLO-205, DEFINED BY A MOUSE MONOCLONAL ANTIBODY. Per Björk, Ulf Jönsson and Göran Sundblad. Department of Cancer Immunology, Pharmacia LEO Therapeutics AB, S-251 09 Helsingborg, Sweden.
The monoclonal antibody (C-215; IgG2a isotype) used was obtained by immunization of Balb/c mice with the human colon adenocarcinoma cell line, COLO-205. Immunohistochemical studies suggest that it defines a membrane-associated antigen, which is abundantly expressed in colorectal and pancreatic carcinomas as well as in normal epithelium. This investigation aimed at characterization and designing a procedure for isolation of the C-215 antigen. Material was obtained by implantation of COLO-205 cells into Balb/c nude mice. Solubilization of tumors was performed by homogenization in a 1% Nonidet P-40 buffer containing protease inhibitors. The antigen was determined by dot-blot analysis using an AP-conjugated second antibody for color development. The purification procedure included: i) removal of endogenous mouse IgG:s by filtration through a Protein G affinity column; ii) gel exclusion chromatography under native conditions; iii) LiDS-chomatography on immobilized Concanavalin A. Western blot analysis of the purified fraction revealed a single 42 kDa band under denaturing conditions and at 36 kDa after reduction of disulfide bridges. Carbohydrate removal did not affect the antigen recognition by the C-215 antibody. None of two irrelevant antibodies tested (among them 17-1A) was able to react with the purified fraction. Thus, the biochemical characterization indicate that the C-215 mouse monoclonal antibody defines a new tumor-associated but tumor-unspecific antigen.

K 101 PURIFICATION OF (THERMO) STABLE ENZYMES by R.M. Daniel[1], H.W. Morgan[1], A.G. Langdon[2], T.N. Collingwood[2]; Thermophile Research Unit[1] and Department of Chemistry[2], University of Waikato, Hamilton, New Zealand
Most industrially used enzymes are extracellular, largely because of the expense of purifying intracellular enzymes. Enzymes from extreme thermophiles, in addition to having a number of general advantages conferred by their stability, offer the opportunity to use cheaper and simpler purification techniques.

If heat stable enzymes are cloned into mesophile organisms, a rapid simple and effective purification can be carried out by heat treating the cloned organism. This heat coagulates the mesophile proteins and at the same time denatures all contaminating enzymes. We have obtained high enzyme yields (~90%) and good purification factors (7-20 fold) for a variety of cloned enzymes in this way. Scale-up, even under contained conditions, should be straightforward.

High enzyme stability will also enable the use of effective techniques to release/solubilise, in an active form, enzymes which have been adsorbed to colloidal surfaces. The generation of appropriate colloids in, for example, fermentation media enables cheap and simple concentration (of the order of 100 fold) and some purification with reasonable recoveries (70%-80%).

K 102 GENETIC ENGINEERING OF FUSION PROTEIN TAILS FOR USE WITH INEXPENSIVE RECOVERY METHODS, Clark Ford[1], Charles Glatz[2], Zivko Nikolov[3], Malcolm Rougvie[4], Steve Gendel[1], Luojing Chen[1], Jiyong Zhao[1], Diane Parker[2], and Illari Suominen[2], Departments of Genetics[1], Chemical Engineering[2], Food Technology[3], and Biochemistry and Biophysics[4], Iowa State University, Ames, IA 50011.

We are working to develop systems for inexpensive downstream recovery of recombinant fusion proteins containing genetically engineered peptide affinity tails. Two kinds of peptide tails have been fused to the C-terminus of the model protein β-galactosidase (β-gal). 1) The starch-binding domain of Aspergillus glucoamulase has been shown to promote the adsorption of β-gal fusion proteins from crude E. coli extracts to native starch granules. After washing the fusion protein can be eluted from starch at a high level of purity. Starch has many favorable qualities as an adsorbent: it is inexpensive, non-toxic, and easily recovered by sedimentation. Genetic deletion experiments have shown that as few as 80 Polyaspartic acid tails of 5-16 residues of have been shown to promote recovery of β-gal fusion proteins from crude E. coli extracts by polyelectrolyte precipitation using polyethyleneimine. Experiments are in progress testing recovery of charged fusion proteins using other inexpensive charge-based separation methods.

K 103 THE USE OF BIOPROCESSING AIDS IN THE CLARIFICATION PHASE OF PROTEIN PURIFICATION, Ian T. Forrester, Anthony C. Grabski and W. Nick Strickland, Protein Purification Facility, University of Wisconsin Biotechnology Center, Madison, WI 53705.
Protein purification usually requires three fundamental phases, involving (1) the harvesting and clarification of the original extract, (2) concentration of the clarified stream and (3) purification of the target protein. The type and effectiveness of the procedures adopted in Phase 1 will have a direct influence on the efficacy and processing options which can be considered for the Phases 2 and 3. Although every biological system presents the protein purifier with a different set of contaminants, which ideally should be removed in the Phase 1 process, there is some universality to these interfering components. For example cellular debris, nucleic acids, lipid, oligosaccharides and pigmented factors are, to varying degrees, commonly occurring factors. Incomplete removal of these factors may not restrict the completion of protein purification, when conducted at an analytical level. However removal of contaminating factors in an effective and economical manner, assumes greater importance when a process is transformed into a production-level process. We have been investigating the impact of different chemically-based clarification strategies on these broader issues of protein purification. In our program to-date a wide variety of different proteins from quite diverse biological systems, including plant, animal, fungal and bacterial, have been examined. In these studies we have assessed several different chemical options including a range of new materials, the Biocryls -- also referred as bioprocessing aids(BPAs) -- as well as more established chemical agents such as polyethyleneimine and chitosan. These chemically-based, Phase 1, clarification systems have been incorporated successfully into protocols to purify proteins such as: the Mn-dependent lignin peroxidase from *Lentinula edodes* (the shiitake mushroom) when cultured on a commercial wood substrate; trypsin from bovine pancreas; peroxidase from alfalfa; a fungal-derived allergen; and an extracellular xylanase from *Streptomyces rosea.*

K 104 ISOLATION AND PURIFICATION OF BACTERIOPHAGE LAMBDA TERMINASE AND ITS SUBUNITS IN A SOLUBLE FORM FROM OVERPRODUCING STRAINS, Marvin Gold and Wendy Parris, Department of Medical Genetics, University of Toronto, Ontario M5S 1A8 Canada. The lambda terminase subunits (gpNu1 and gpA) are probably the most poorly expressed proteins in E. coli, because their translation is very inefficient. Strains which overproduce the proteins by two to three orders of magnitude have been used as sources for our standard purification protocols (J. Biol. Chem. 258:14619, 1983 and 263:8413, 1988). These methods give high yields of homogenous proteins which however are highly aggregated so that they are insoluble in low ionic strength buffers and behave as very high-molecular weight complexes when solubilized by high salt concentrations. Our studies have suggested that a too rapid and complete removal of nucleic acids from the crude extract promotes aggregation and insolubility for the remainder of the purification procedure. Gradual removal of nucleic acids by a series of steps including hydrophobic-interaction chromatography has enabled us to prepare pure holoenzyme and gpNu1 subunit each in soluble, lower molecular weight forms which will be more amenable to structural studies. Supported by grants from the Medical Research Council of Canada and the National Institutes of Health.

K 105 **THE "T-GEL" AS A GENERAL ADSORBENT FOR THE SEPARATION OF PEPTIDES, GLYCOPEPTIDES AND CARBOHYDRATES,** **Peter Heegaard, The Protein Laboratory, University of Copenhagen, Sigurdsgade 34, DK 2200 Copenhagen N, Denmark**
The mercaptoethanol derivative of divinylsulfone activated solid phases, the socalled "T-gel" was shown previously to exhibit a remarkable, salt-dependent affinity for various proteins, binding to the matrix according to, among other factors their salting-out behaviour.
This prompted investigations into the use of this affinity matrix for the general separation of peptides from glycopeptides and simple carbohydrates. There are only few current methods available for this purpose and they are not always generally applicable (lectin-affinity chromatography, phenyl boronate affinity chromatography).
Pronase digests of pure glycoproteins, digests of glycoprotein mixtures and mixtures of simple carbohydrates and proteins were subjected to T-gel chromatography under specified salt-conditions and the carbohydrate/peptide ratio in run-throughs and eluates were determined. The results indicate, that under specific concentrations of salt, the binding to the T-gel decreases in the following manner: peptides>glycopeptides>carbohydrates.
The T-gel may thus be used as a general adsorbent for nonglycosylated peptides, seperating these from glycopeptides and carbohydrates.

K 106 AFFINITY CHROMATOGRAPHY USING BEADED LIGNO-CELLULOSE, Jeffrey A. Kaster and William H. Velander, Department of Chemical Engineering, Virginia Polytechnic Institute and State University, Blacksburg, VA 24061

One of the major shortcomings of affinity chromatography at large scale is the lack of an inexpensive yet mechanically robust support material. Cross-linked agaroses are the standard supports in industrial applications at this time. Cross-linked agarose is a relatively inert, hydrophilic, and "porous" gel. It is available in beaded form and is easily derivatized for ligand binding. Cross-linked agarose however has very poor mechanical properties (flow rates even for fast flow Sepharose are restricted to less than 15 cm/min in a 1cm diameter column due to bead compression). Commercially available beads that are stronger tend to either have reduced porosity or will not bind as much affinity ligand as compared to agaroses. We are studying the properties of beads made of cellulose from steam exploded wood. This cellulose contains small amounts of covalently attached lignin and the resultant beads are refered to as ligno-cellulose beads. These beads can be produced with a fairly narrow particle size distribution at an average particle size of approximately 200 microns or larger. Ligno-cellulose beads containing 4% solids are able to withstand superficial liquid velocities of up to 120 cm/min with a pressure drop of less than 0.6 psi/cm of column. These beads are easily activated with CNBr and bind wheat germ agglutinin as well as CNBr activated Sepharose-CL 4B. Ligno-cellulose beads appear to have a very open structure permitting the capture of even large proteins (IgG's). They also show very little nonspecific binding. Ligno-cellulose beads appear to be an excellent support matrix for large scale affinity chromatography.

K 107 NEW MATRICES FOR SALT PROMOTED ADSORPTION CHROMATOGRAPHY.Allan Lihme[1], Bente K. Andersen[2], Lotte R. Henriksen[2], Thorkild C. Bøg-Hansen[1]. 1) Protein Laboratory, University of Copenhagen, Denmark. 2) Danish Ingeneer Academy , Lyngby, Denmark."Salt-promoted adsorption chromatography" is a broad term, introduced by Jerker Porath (1), covering a range of different protein purification procedures based on chromatographic matrices which bind proteins at high concentrations of lyotropic salts. Examples of such matrices are the wellknown hydrophobic matrices phenyl-Sepharose and octyl-Sepharose, immobilized metal-chelate matrices and "thiophilic matrices". The latter group of matrices, also introduced by Jerker Porath (2) is based on divinyl sulfone activated agarose coupled with small hydrophilic thiol-containing ligands, e.g. mercaptoethanol giving the so-called "T-gel":

$-O-CH_2-CH_2-SO_2-CH_2-CH_2-S-CH_2-CH_2-OH$

The T-gel has proven to be a very powerful protein separation medium, not only for purification of immunoglobulins but for proteins in general, having high capacity and at the same time giving sharp separations(3).
In this work we present a range of new matrices based on divinyl sulfone activated agarose giving protein separations which resembles the T-gel to different extents. Unlike the T-gel these matrices do not contain a thioether in the ligand, as earlier presumed to be of major importance for effective and selective binding of immunoglobulins (2).
References:1) Porath, J., Biotechnology Progress, 3, 14, (1987).2) Porath, J.,FEBS Letters, 185, 306, (1985). 3)Allan Lihme, Mette Schaumburg Madsen, Peter Heegaard.Submitted.

K 108 LIGANDS AND SOLVENTS FOR PROTECTION AND PRECIPITATION OF PROTEINS, Rex Lovrien and Mark Conroy, Biochemistry Department, University of Minnesota, St.Paul, MN 55108
Isolation of proteins both upstream (crudes) and downstream frequently can be best approached by aiming not at a completely purified protein but at a complex. For example crystallization of number of enzymes and proteins is gotten by crystallizing them as Protein·Ligand$_\nu$ complexes, where ν = number of ligand molecules bound/protein molecule and $\nu \sim 1$ to 5. In fact numbers of 'crystallizable proteins' are not crystallizable if $\nu = 0$, only when $\nu > 1$ or greater. Bound ligands of the right kind (substrate-like, often hydrocarbonaceous, even detergents) act as protein molecule conformation tighteners and also as protective agents. Frequently P·L$_\nu$ complexes are not active but are protected during harsh separations conditions. They regain activity when L is removed, by resin ion exchangers. Protection, conformation tightening, precipitability in amorphous form, crystallizability in ordered liganded form are increasingly seen to be related, increasingly applicable to 'upstream' and scalable processes. There is no concrete reason for believing certain shibboleths, e.g. that crystallization is always a far downstream, hyperpurifying process, as the crystalline lysozyme (directly from egg white) example shows. Numbers of amino acids (A.A.) can also be selectively crystallized out as cocrystals with ligands. Differentiating cosolvents, C_4-C_6 organics, help modulate Pr-L and A.A-L interaction. Preliminary work has been carried out to convert amorphous AA.L$_\nu$ to crystallize A.A.·L$_\nu$. Protecting ligands and cosolvents provide some control over densities, relative buoyancy, also graininess of precipitates, thus requiring simply low speed centrifugation and conventional low tech filtration to separate them.

Protein Purification and Biochemical Engineering

K 109 RECOVERY OF SYNTHETIC METAL BINDING CYTOCHROME C BY METAL AFFINITY EXTRACTION, Robert Todd[‡], Mariana Van Dam[‡], Danilo Casimiro[‡], Barry L. Haymore[†], and Frances H. Arnold[†], [‡]Division of Chemistry and Chemical Engineering 210-41, California Institute of Technology, Pasadena, CA 91125, [†]Chemical Sciences Department, Central Research Laboratories, Monsanto Company, St. Louis, MO 63167.

A metal binding site consisting of two histidines positioned His-X_3-His in an α-helix has been incorporated into the surface of *S. cerevisiae* iso-1-cytochrome c. The resulting metal-binding protein, expressed in yeast in its functional form, has been characterized by aqueous two-phase partitioning with polymer-metal complexes and by immobilized metal-affinity chromatography. Addition of two histidines in this chelating configuration results in a dramatic increase in the protein's affinity for a soluble Cu(II)IDA-polymer complex and a smaller, though significant, increase in affinity for an insoluble Cu(II)IDA chromatography matrix. Synthetic metal-binding cytochrome c can be recovered directly from the lysed yeast cells with significant purification in a single metal-affinity extraction step. This work demonstrates that a chemical property (metal binding) important in protein separations can be significantly altered with a minimum number of amino acid substitutions.

K 110 INDUSTRIAL EXTRACTION OF PROTEINS FROM HARD TO PROCESS SOLUTIONS. Tomas Wikborg[1],Christian Schou[1], Claus S. Nielsen[2], Thorkild C. Bøg-Hansen[3], Allan Lihme[3]. [1)]The Technical University of Denmark, Lyngby, Denmark.[2)]Kem-En-Tec A/S, Symbion, Copenhagen, Denmark.[3)]The Protein Laboratory, University of Copenhagen, Denmark.Traditional methods for initial concentration and purification of proteins from large volumes of solution, often involves procedures such as centrifugation and filtration in order to get rid of debris and other sticky materials before chromatographic steps can be applied. In some cases the nature of the solution makes it a rather difficult task to use these methods. Modern alternatives to centrifugation and filtration procedures include techniques such as liquid phase (affinity) partitioning and mixed bed adsorption, typically by the use of an ionexchange resin. Both of these methods allow an initial fractionation without prior clarification of the protein solution. However, these methods are not generally applicable due to technical difficulties not yet properly solved and a rather scarce commercial availability of appropriate matrices and equipment.

In this work we would like to introduce a new type of high capacity affinity matrix for salt promoted adsorption chromatography of proteins. The matrix[*] is a composite matrix based on divinyl sulfone activated agarose, coupled with a low molecular weight aromatic ligand, and an inorganic carrier. The matrix is specially designed for use in large scale applications, where solutions containing particulate and coloured materials has to be absorped for valuable enzymes, products of genetic engineering or other proteins in a fast and efficient procedure.

[*)]Patent pending.

High Resolution Methods

K 200 THE PURIFICATION OF EUKARYOTIC RNA POLYMERASE II BY IMMUNOAFFINITY CHROMATOGRAPHY USING PROTEIN STABILIZING ELUTION CONDITIONS,

Richard R. Burgess and Nancy E. Thompson, McArdle Laboratory for Cancer Research, University of Wisconsin, Madison, WI 53706. A monoclonal antibody has been isolated that reacts with the C-terminal heptapeptide repeat of the largest subunit of eukaryotic RNA Polymerase II. The enzyme can be immunoaffinity adsorbed from a relatively crude extract, washed and eluted, resulting in high purity and specific activity. Since the epitope is found on RNA polymerase II from almost all eukaryotes, this immunoaffinity column can be used to purify the enzymes from many different organisms. Monoclonal antibodies with similar properties (high affinity but gentle elutability) have been isolated for several other enzymes. Elution conditions are extremely gentle and rely on the use of protein stabilizing agents. Methods for isolating such antibodies and optimizing elution conditions will be described. We believe that the methods are general and will allow the powerful selective technique of immunoaffinity chromatography to be used for the purification of many enzymes, especially labile enzymes that are inactivated by lengthy purification procedures or harsh elution conditions usually employed.

K 201 RECENT ADVANCES IN THE THEORY AND PRACTICE OF DISPLACEMENT CHROMATOGRAPHY, Steven M. Cramer, Department of Chemical Engineering, Rensselaer Polytechnic Institute Troy, NY 12180-3590.

Displacement chromatography has been demonstrated to be a powerful technique for the simultaneous concentration and purification of biomolecules. In this work, we extend our previous research on displacement chromatography of biomolecules using ion-exchange and reversed phase adsorbents to metal chelate affinity systems. The affinity displacement purification of model biopolymer mixtures was carried out and the throughputs in these systems were optimized. This work demonstrates that the use of affinity supports in the displacement mode can significantly increase the throughput and purity obtained with these selective chromatographic systems. Several novel, non-toxic polymers were identified and synthesized for use as displacers for various adsorbent systems. The effects of critical displacer properties such as molecular dimensions, configuration, and "binding density" on the displacement purification of proteins were investigated. The development of novel non-toxic displacers is expected to facilitate the purification of therapeutics by displacement chromatography.

A mathematical model of displacement chromatography was also developed which includes the effects of mass transport, axial dispersion, and adsorption/desorption kinetics. The interplay of various operating parameters on bioproduct throughput was investigated and the experimental results compared to the predictions of the mass transport-kinetic displacement model. Finally, the model is employed for the optimization and scale-up of a significant bioseparation problem.

K 202 PURIFICATION OF MONOCLONAL ANTIBODY SUITABLE FOR PHARMACEUTICAL STUDIES, J. L. Duke, T. M. Reilly, A. T. Nahapetian, N. R. Huckins, E.I. DuPont deNemours Company, Inc., Wilmington, Delaware

Monoclonal antibodies because of their high specificity and affinity are a vital tool in in vivo pharmaceutical research. In vivo research by its nature requires protein preparations with a very high degree of protein purity and very low endotoxin content. Conventional antibody purification techniques such as ammonium sulfate precipitation, ion exchange and affinity column chromatography are often adequate for this purpose. Newer membrane based affinity systems also are sufficient and in some instances are preferred. A comparison of two processes for the purification of a mouse monoclonal antibody, KAA8 anti-angiotensin-II, subclass IgG 1, required for in vivo cardiovascular studies will be presented. The advantages and disadvantages related to the use of different affinity systems will be discussed.

K 203 THE PRODUCTION AND PROPERTIES OF SITE-SPECIFIC RECOMBINANT PIG CITRATE SYNTHASE (PCS) IN E. COLI. Claudia T. Evans, Wang Zhi, Peter Nemeth, and Paul A. Srere. Dept. of Veterans Affairs Medical Center and University of Texas Southwestern Medical Center, Dallas, TX 75216.
Citrate Synthase catalyzes the condensation of oxalacetate and acetyl coenzyme A to form citrate, the first and rate controlling reaction of the TCA cycle. In order to obtain insight into the mechanism, structure and biological function of PCS, six active site recombinant PCS proteins were produced and purified from E. coli. His^{274} and Asp^{375} were replaced by Gly and Arg, and Gly, Asn, Glu and Gln, respectively. The non-mutant and mutant proteins were induced to 0.1-0.4 mg protein/gm cells and purified 400 fold from 300-400 g cells with 40-50% recovery. Greater than 100 mg of each protein was purified in similar fractions by ATP-sepharose chromatography and were judged homogeneous by SDS-PAGE and protein blot analysis. The overall surface structure of the mutant PCS proteins was not different from the native protein when reacted with ten different independent anti-PCS monoclonal IgGs. The specific activities of the mutant proteins were measured and were reduced 10^3-10^4 fold. (Supported by Dept. of Veterans Affairs and NIDDK).

K 204 SIMPLE ELECTROPHORETIC TECHNIQUES FOR BINDING CONSTANT DETERMINATION FOR MONOCLONAL ANTIBODIES AND THEIR ANTIGENS.

Niels H.H.Heegaard[1], Ole J.Bjerrum[2]. [1]The Protein Laboratory, University of Copenhagen, Sigurdsgade 34, DK-2200 Copenhagen N. and [2]Novo-Nordisk A/S, DK-2880 Bagsværd, Denmark.

Monoclonal antibodies have found widespread use as reagents in immunoassays, immunohistochemistry, and in immunoaffinity purification. Some of these applications work best with high-affinity antibodies while others (eg. immunoaffinity purification) require low-affinity antibodies. The binding characteristics of a given antibody is therefore highly important for its usefulness and simple methods for antibody affinity measurements are thus in demand. Classical procedures demand rather large amounts of purified reagents or special properties of antigens and antibodies. These limitations are not shared by affinity electrophoresis procedures. The antigen is here electrophoresed through gels containing different amounts of antibody. The ensuing mobility shifts make it possible to estimate dissociation constants provided the intercating molecules have different mobilities. This principle can be extended to crude antigen-mixtures if a specific detection system (e.g. a second dimension immunoelectrophoresis) is used. Through observations of precipitate-morphology the approach can also be applied for screening of different monoclonal antibody-producing clones. We show affinity electrophoretic studies of monoclonal antibodies against human serum albumin and α_1-fetoprotein with a comparison of experimentally-derived dissociation constants with values obtained by classical binding assays.

K 205 ANALYSIS AND USE OF THE SPECIES DEPENDENT VARIATION IN THE INTERACTION BETWEEN SERUM ALBUMINS AND STREPTOCOCCAL PROTEIN G

Ljungquist, C.*, Nygren, P-Å.*, Trømborg, H.# and Uhlén, M.*

*Dept. of Biochemistry and Biochemical engineering, Royal Institute of Technology, 100 44 Stockholm, Sweden

Apothekarnes Laboratorium, PO Box 158, Sköyen N-0212 Oslo 2, Norway

The serum albumin binding domain from streptococcal protein G, SPG, was found to bind with different strength to serum albumins isolated from different species. The highest affinities to SPG was found with serum albumin from rat, man and mouse. The kinetics of the interaction between HSA and SPG showed to be rapid at the three temperatures (7°C, 22°C, 37°C) investigated. Furthermore the ability of different serum albumins to function as affinity ligands when covalently coupled to Sepharose was tested. SPG derivatives were found to be eluted at various pH when immobilized to different serum albumin Sepharoses. Finally, a concept for the recovery of sensitive proteins by affinity purification, is described, where high yields are obtained under mild elution conditions.

K 206 AFFINITY CHROMATOGRAPHY WITH IMMOBILIZED TRYPTOPHAN: PURIFICATION OF CHORISMATE MUTASE FROM *Sorghum bicolor*.

Wilma Miller, Daniel Siehl, and Philip Haworth, Plant Biochemistry, Sandoz Crop Protection Corporation, Palo Alto, CA 94304.

Chorismate mutase (EC 5.4.99.5) catalyzes the conversion of chorismate to prephenate in the first committed step which separates the synthesis of phenylalanine and tyrosine from the synthesis of tryptophan. Two isoforms of choristmate mutase have been observed in most higher plants, an unregulated form (CM-2) and a regulated form (CM-1). CM-2 is found mainly in the cytosol and is insensitive to any regulatory effects of metabolic end products. CM-1 on the other hand is chloroplastic and is feedback inhibited by its end products tyrosine and phenylalanine, but strongly activated by tryptophan. CM-1 is essentially inactive in the absence of tryptophan. This requirement for tryptophan facilitates the use of this amino acid as a highly specific affinity ligand for CM-1 purification. CM-1 from *Sorghum bicolor* has been purified to homogeneity using tryptophan affinity chromatography.

The substrate saturation curve of CM-1 is hyperbolic at a tryptophan concentration of 2.5 μM, but sigmoidal below this concentration. The activation of CM-1 by tryptophan results in a decrease in the K_M for the substrate, chorismate, up to saturating levels of tryptophan. The V_{max} is not altered in response to tryptophan. A possible role of tryptophan in the active site of CM-1 is discussed.

K 207 DUAL AFFINITY FUSIONS TO IMPROVE RECOVERY OF FULL LENGTH PROTEINS

Maria Murby, Per-Åke Nygren, Lena Cedergren and Mathias Uhlén, Royal Institute of Technology, Dept. of Biochemistry and Biotechnology, S-100 44 Stockholm, Sweden

A dual affinity fusion concept has been used where the gene encoding the desired product is fused between two flanking heterologous genes, encoding IgG- and albumin binding domains respectively. Using sequential IgG- and serum albumin affinity chromatography, a full length tripartite fusion protein is obtained. This approach has been used to recover several protelytically sensitive proteins, e.g. human proinsulin, human secretin and rat protein disulfide isomerase (PDI). Compared to bipartite fusions, increased stability is often obtained for tripartite fusions. After site specific cleavage of the tripartite fusion protein, the desired product is obtained by collecting the flow through after passage through IgG- and serum albumin columns. This concept thus provides a powerful tool to recover material of full length gene products susceptible to protelytic degradation.

K 208 Purification and Partial Characterization of Different Forms of Recombinant Human Activin A by RP-HPLC, Charles H. Schmelzer, Louis E. Burton, Ralph H. Schwall*,and Cathleen M. Tamony, Department of Recovery Process R & D and Department of *Pharmacological Sciences, Genentech, Inc., South San Francisco, CA. 94080. We have used C_{18} RP-HPLC to separate three forms of activin A from a preparation partially purified using gel filtration and cation-exchange chromatographies (Schwall, R. H. et al. (1988) Mol. Endocrinol. 2, 1237-1242). Based on SDS-PAGE, the three forms of activin are designated as slow activin 'sActA', M_r ~28,000; main activin pool 'ActA', M_r~27,000; and fast activin 'fAct', M_r~26,000. Typically, ActA is present at 80-90% of total activin A forms expressed in tissue culture. All three forms of activin blot with a goat antibody prepared against ActA. Both ActA and fActA have the same specific activity as determined using a pituitary cell bioassay, and contains identical amino terminal sequences and similar amino acid compositions. ActA has a pI of ~8.5 as determined by chromatofocusing in 6 M urea. ActA elutes from a Superose 12 column in 6 M guanidine hydrochloride corresponding to M_r 12,000. After reduction all three activin species have the same molecular weight on a 12.5% SDS-polyacrylamide gel, suggesting fActA and sActA may-be misfolded forms of activin.

K 209 THE RECOVERY OF THERAPEUTIC PROTEINS FROM MILK, W.H. Velander, T. Morcol, Department of Chemical Engineering; R.M. Akers and P.L. Boyle, Department of Dairy Science; J.L. Johnson, Department of Anaerobic Microbiology; Virginia Polytechnic Institute and State University, Blacksburg, VA 24061. W.N. Drohan, C. Pittius and J. Long, Jerome H. Holland Laboratory, American Red Cross, Rockville, MD 20855.

Recent advances in molecular biology have resulted in the production of animals who will express heterologous proteins in milk. Mammary tissue specific expression may lead to the use of large production animals as bioreactors for the biosynthesis of therapeutic proteins. Associated with these studies is the recovery of the intact protein from milk. In addition to the naturally occurring proteolytic activity, the three phase structure (lipid, casein and aqueous whey fractions) have been shown to complicate protein purification. Milk-doping of I^{125}-labeled, Protein C (hPC) has been used to optimize both detection and isolation. Both porcine and bovine milk have been evaluated. An immunoaffinity process employing a metal-dependent monoclonal antibody has been developed for the recovery of human Protein C which has been doped into porcine milk. Decreases in recovered hPC activity as a function of residence time in milk have been attributed to proteolytic activity in milk.

Protein Purification and Biochemical Engineering

Large-Scale Processes

K 300 LARGE SCALE PURIFICATION OF MONOCLONAL ANTIBODIES, Blank, Gregory & David Vetterlein, Process Recovery R&D, Genentech, Inc., S. San Francisco, CA 94080. Murine IgG_1 monoclonal antibody (MAb) was purified from fermentation culture fluid at the 1200 liter scale using Protein A affinity and ion exchange chromatography. The harvest fluid was concentrated ~10 fold by binding to a S-Sepharose FF column at pH 4.1. The MAb was step eluted with a high salt buffer (1.5M Glycine, 3.0M KCl, pH 9.0) suitable for MAb binding to Protein A. This step provides > 5 logs of viral (X-MuLV) clearance and gives recoveries of 85%. The S-Sepharose eluate was purified by Protein A affinity chromatography. MAb could be eluted with low pH buffer or with low salt buffer at pH 6.5. Protein A column size was minimized to reduce cost by performing multiple cycles using an automated system. The Protein A step gave > 4 logs viral clearance. Protein A eluates were pooled, concentrated and buffer exchanged by ultrafiltration/diafiltration against 30mM Tris, 25mM NaCl, pH 8.5. Recoveries through UF/DF were 75%. The MAb dialysate was applied to DEAE Sepharose FF and step-eluted with 30mM Tris, 150mM NaCl, pH 8.5. Viral clearance by the DEAE column was > 5 logs. MAb was 0.2μm filtered and stored at -60°C. The process overall had recoveries of 71% and > 14 logs viral clearance. MAb purity was > 99% (SDS-PAGE) with endotoxin levels of less than 0.1 EU/mg. Protein A and Bovine IgG were less than 100 ppm.

K 301 PURIFICATION AND CHARACTERIZATION OF RECOMBINANT BABOON AND CYNOMOLOGOUS MONKEY PROLACTINS. Edward S. Cole, Joseph Dunn, Edward H. Nichols, Kevin Lauziere, and John McPherson., GENZYME Corp., 1 Mountain Rd., Framingham, MA, 01701

Prolactin is a hormone produced mainly in the anterior pituitary. It displays a wide range of biological activities with over 85 reported effects in various vertebrate species. Cynomologous monkey and baboon prolactin were each produced in mouse C127 cells by recombinant techniques. A three step purification scheme was devised involving fast flow S-Sepharose and fast flow Q-Sepharose chromatographies followed by gel filtration on S-200 resin, which were used to purify both types of prolactin. This process was scaled-up to purify gram quantities of prolactin in good yield, from serum free media, to greater than 95% purity based on SDS-PAGE. The purified recombinant proteins were each heterogeneous in nature, similar to the heterogeneity previously reported for human and animal tissue derived prolactins. SDS-PAGE revealed complex banding patterns with major bands at 25 kDa(a doublet), 29 kDa(a doublet), 16 kDa, and 8 kDa which all blotted with anti-Prolactin antibodies on Western blots. Carbohydrate compositional analysis indicated that approximately 50% of the molecules were glycosylated minimally with a biantennary, complex oligosaccharide. Amino terminal sequence analysis identified the separate proteins as monkey and baboon prolactins thru 20 amino acids. We have also identified two proteolytic clips at AA#10 near the amino terminus and AA#133 in the large disulfide loop which may have biological significance and which, along with variability of glycosylation, account for some of the heterogeneity observed in these preparations.

K 302 LARGE SCALE PURIFICATION OF GLUCOSE OXIDASE FROM RECOMBINANT YEAST Annie de Baetselier*, Michèle De Beukelaer*, Philippe Dohet*, Vin Ha-Thi*, Katherine Frederick#, Amit Vasavada#, Steven Rosenberg#. *Institute of Cellular and Molecular Pathology, 75 avenue Hippocrate, B-1200 Brussels, Belgium, and #Chiron Corporation, 4560 Horton Street, Emeryville, CA 94608. A yeast strain was transformed with the expression plasmid pSGO2 which directs the expression of glucose oxidase under the control of the ADH2-GAPDH promoter (Rosenberg et al., J. Cell. Biochem. 13A, 77, 1989). Production of the enzyme during fermentation was monitored by one-line size-exclusion HPLC analysis. Transformants secrete into the medium more than 1g/L of active glucose oxidase. Purification of the enzyme to homogeneity was accomplished solely using filtration methods without resorting to chromatographic techniques. Comparison of the yeast derived enzyme with that from *Aspergillus* showed it to be of comparable specific activity, more heavily glycosylated and completely free of catalase contamination.

K 303 PURIFICATION AND CHARACTERIZATION OF HUMAN RECOMBINANT INSULIN LIKE GROWTH FACTOR II (IGF-II) AFTER EXPRESSION AS AN IgG-BINDING FUSION PROTEIN IN E. COLI, Maris Hartmanis, Erik Holmgren, Kristina Kalderén, Anders Ekebacke, Henrik Wadensten and Michael Tally°, KabiGen AB, S-112 87 Stockholm, Sweden and °Department of Endocrinology, Karolinska Institutet, S-104 01 Stockholm, Sweden.
Human recombinant IGF-II was expressed at high levels as a secreted 22.5 kDa fusion protein in E. coli. The 15 kDa fusion partner is a synthetic IgG-binding protein derived from Staphylococcus aureus protein A. The expression vector contains the protein A promoter and signal sequence. After fermentation and filtration, the medium was passed over an affinity column containing IgG-Sepharose. The purified fusion protein was cleaved at a methionine residue in the linker region employing cyanogen bromide in formic acid. IGF-II was then purified to homogeneity using a two step procedure involving ion exchange chromatography and reverse phase HPLC. N- and C-terminal sequencing in combination with plasma desorption mass spectrometry indicated a correctly processed growth factor. The purified IGF-II exhibited full biological activity in a cell growth stimulation assay and in a radioreceptor assay using human placenta membranes.

K 304 ISOLATION OF APOLIPOPROTEIN A-I FROM WASTE FRACTIONS OF COLD-ETHANOL PLASMA FRACTIONATION; CHARACTERIZATION OF A THERAPEUTIC CONCENTRATE, J.-J. Morgenthaler[1], P.G. Lerch[1], R. Bolli[1], H. Friedli[1], G. Hodler[1], H.-J. Heiniger[1], M.C. Peitsch[2], H. Isliker[2], [1]Central Laboratory, Swiss Red Cross Blood Transfusion Service, Bern, Switzerland, [2]Institute of Biochemistry, University of Lausanne, Lausanne, Switzerland.
Apolipoprotein(s) are usually isolated from high and low density lipoprotein fractions (HDL and LDL) obtained by ultracentrifugation of blood plasma, to which salts have been added in order to adjust its density. This method does not allow the preparation of large amounts of apolipoprotein(s). A preparation which contained >90% apolipoprotein A-I (apoA-I), together with some apoA-II, could be obtained by ethanol extraction and precipitation from certain waste fractions of a cold-ethanol plasma fractionation method. The product was further worked up by dissolution in guanidin hydrochloride, pasteurization, diafiltration, sterile filtration, and lyophilization. The final product fulfilled the requirements for an injectable drug. ApoA-I prepared by this method exhibited an identical pattern in isoelectric focusing as apoA-I obtained from HDL; CD spectra were also indistinguishable. Both preparations activated lecithin-cholesterol acyltransferase (LCAT) to the same extent. Activation of LCAT seems to depend on the monomer content of the apoA preparation. Upon incubation with plasma, isolated apoA-I incorporated into HDL; this incorporation was limited by the amount of available lipid and could be increased by addition of lipid together with apoA. The preparation could also be incorporated into proteoliposomes, which floated at the same density as HDL.

K 305 LARGE SCALE PURIFICATION OF HIV-1 ENVELOPE PROTEINS gp160 AND gp120 EXPRESSED IN BACULOVIRUS, J.A. Myers, C.A. Beard, S. Almeda, D.J. Peters and O.J. Konarkowski, Repligen Corporation, One Kendall Square, Building 700, Cambridge, MA 02139
We have developed large scale procedures for the purification of recombinant HIV-1 gp120 and gp160 expressed in baculovirus. gp160 is expressed as the full length protein and is neither processed nor secreted. Cells are infected in roller bottles. Our purification protocol entails the following steps: mechanical lysis using a continuous flow method, concentration and extraction using a tangential flow membrane, lentil lectin chromatography and gel filtration chromatography. We can routinely perform large scale purification using this method to $\geq$ 90% purity. During expression of our gp120, the signal sequence is cleaved and the protein is secreted into the media. Starting with the media, we purify the protein using an ion exchange column, an antibody column and lentil lectin column chromatography. The gp120 purified in this way retains its ability to inhibit syncytia formation with CD4+ cells. We have characterized the purified proteins in a number of ways. In Western blots, our purified proteins react with antibodies directed against the major neutralizing envelope epitope. Our scaled up procedure of these two purification protocols allows for the production of large amounts of highly purified protein.

K 306 AN IMPROVED PROCESS FOR THE MANUFACTURING OF ANTITHROMBIN III CONCENTRATE, H.A. Nunez, D. Gee and W. N. Drohan, American Red Cross, Rockville, MD 20855.
The American Red Cross is producing an AT III concentrate that will benefit AT III genetically deficient patients. This product has been shown to be efficacious for the prevention of thrombosis following surgery, during pregnancy and parturition, in severe infections and in the treatment of acute and recurrent thrombosis. None of 10 patients receiving the AT III concentrate as their only blood product have shown hepatitis B seroconversion. The current manufacturing process entails two affinity chromatography purification steps on heparin-Sepharose (HS) and a PEG precipitation step to remove protein impurities. The process is lengthy, labor intensive and relatively low in AT III yield. Consequently we have modified the process without changing the viral inactivation step (heating in solution at 60 C for 10 h). In the improved process the protein impurities bound to the HS are removed prior to the desorption of AT III, thus eliminating the need for readsorption onto HS or further purification. In this way a twofold yield increase and a 30% reduction in the processing time has been achieved. Importantly, the final product is identical to the product obtained with the lengthier process. The product contains 7 2 mg protein per ml with a specific activity of 5.5 1.5 IU/mg. SDS-PAGE at loads of 1, 5 and 10 ug in 9% gels of 1.5 mm thickness show a single protein band when stained with Coomassie blue. Molecular sizing HPLC indicates that more than 98% of the concentrate is in the monomeric form. Both crossed immunoelectrophoresis and molecular sizing HPLC experiments demonstrate that approximately 75% of the AT III retains the ability to bind to heparin following the pasteurization and freeze-drying steps.

K 307 LARGE-SCALE PURIFICATION OF RECOMBINANT HIV-1 GP120 ANTIGENS, Carl Scandella, William Lidster, Christopher Marion, Jaleh Kilpatrick, Peter Brown, Kathelyn Steimer and Nancy Haigwood, Chiron Corporation, Emeryville CA 94608.
A method has been devised for the production of gram quantities of recombinant HIV-1 gp120. The envelope glycoprotein genes from HIV-SF2, HIV-IIIB, HIV-SF33, HIV-162, and HIV-Zr6 have been engineered for secretion of the external gp120 portion of gp160. Permanent Chinese Hamster Ovary (CHO) cell lines were developed for high level expression of the gp120 from each of the HIV-1 isolate types. Additionally, variants of the gp120 gene deleted in one or more of the hypervariable regions were engineered for high level expression, and CHO cell lines expressing these variant antigens were isolated. Cell lines were scaled up in continuous suspension culture. The secreted glycoproteins were concentrated by ultrafiltration, then purified by ion exchange, hydrophobic interaction, and gel filtration chromatography. The purification process, which was developed for the recombinant SF2 gp120, was applied to the purification of the gp120 from other isolates and the deletion variants with minor modifications. The purified proteins retained activities dependent upon conformation, including receptor (CD4) binding and the ability to bind neutralizing antibodies from HIV-positive human sera directed against putative conformational epitopes.

K 308 PURIFICATION OF A MULTICOMPONENT RECOMBINANT MALARIA VACCINE CANDIDATE FROM E.COLI. B.Takacs, P.Caspers, H.Etlinger, M-F. Girard, E.Hochuli, H.Matile, J.R.L. Pink and D.Stüber, Central Research Units, F. Hoffmann-La Roche Ltd. Basel, Switzerland.
In view of the global resurgence of malaria, increasing efforts are being made to define parasite antigens that could be incoporated into a malaria vaccine. Although immunization with attenuated parasites can lead to partial or complete protection against malaria, the use of parasite cultures is not a convenient way to produce large quantities of vaccine candidates. Malaria vaccines must be produced through peptide synthesis or recombinant DNA technology. Synthetic vaccine candidates, based on the repetitive (NANP) sequence of the circumsporozoite protein of P. falciparum have protected a small number of volunteers from malaria challenge. The efficacy of such vaccines could be improved by the inclusion of a blood stage component of the parasite. We have combined the gene coding for a blood stage antigen, Ag 5.1, with DNA coding for $(NANP)_{19}$ sequence. To facilitate purification of the recombinant protein, which is well expressed in E.coli, DNA coding for a hexahistidine sequence was introduced at the 5'end of the gene. Recombinant proteins containing hexahistidine have high affinity for Ni^{++}-chelate columns even in the presence of a 8M Urea or 6M Guanidine HCl. The recombinant protein, His_6-Ag 5.1-$(NANP)_{19}$, with an apparent molecular size of 40'000 Daltons, was purified to homogeneity by a combination of four steps: 1) release and solubilization of the recombinant fusion protein from E.coli in the presence of 6M Guanidine HCl; 2) precipitation of over 60% of the bacterial proteins by the addition of ammonium sulfate to 50% saturation; 3) affinity chromatography on a novel Ni^{++}-chelate column in the presence of 6M Guanidine HCl; 4) adsorption onto a cation exchange resin in the presence of 6M Urea, and elution with an increasing NaCl gradient. The final product is freely soluble in saline and can be quantitatively adsorbed to $Al(OH)_3$ adjuvant. Compared with the previously tested Tetanus toxoid-$(NANP)_3$ malaria vaccine, this protein elicits an antibody response which more closely resembles that evoked by native sporozoites. In addition, the recombinant vaccine induces the production of antibodies against the blood stages of the malaria parasite.

K 309 COMPARISON OF VARIOUS RESINS IN THE PURIFICATION OF FACTOR IX BY IMMUNOAFFINITY CHROMATOGRAPHY, John Tharakan, Frank Highsmith, David Clark, and William Drohan, American Red Cross, Jerome H. Holland Laboratory for the Biomedical Sciences, 15601 Crabbs Branch Way, Rockville, MD 20855

We have developed an immunoaffinity chromatography method to purify Human Coagulation Factor IX (FIX) to high levels of purity and specific activity. The process that is used currently employs Sepharose CL2B as the immunoaffinity ligand support matrix. This is a soft gel that has a low crushing pressure and poor performance characteristics in large scale processes. In this study, we have examined several additional resin supports as well as several coupling chemistries. The supports examined include other Sepharoses, highly cross-linked agarose as well as an azlactone copolymer bead. Coupling chemistries include cyanogen bromide (CNBr), 2-fluoro-1 methylpyridinium toluene-4-sulfonate (FMP), aldehyde groups reduced by $NaCNBH_3$ to form secondary amine linkages as well as the amino linkages formed in the azlactone copolymer bead. Our results compare the binding capacity and efficiency of the affinity ligand as well as the recovery of antigen. In addition, we examine the kinetic and transport phenomena associated with the purification process using the different support matrices.