PROTEIN PURIFICATION AND BIOCHEMICAL ENGINEERING

Organizers: William Drohan, Patrick L. Coleman and Richard R. Burgess

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Plenary Sessions *Page*

Poster Sessions

Source Materials for Protein Isolation

X1-001 BACULOVIRUS-MEDIATED EXPRESSION OF APOLIPOPROTEIN GENES IN INSECT LARVAE, Alan D. Attie[1], Daniel G. Gretch[1], Stephen L. Sturley[1], Paul D.Friesen[1], and Nancy E. Beckage[2], [1]Department of Biochemistry, University of Wisconsin-Madison, Madison, WI, and [2]Department of Entomology, University of California-Riverside, Riverside, CA.

Numerous genes from various organisms have been expressed in insect cells infected with recombinant baculoviruses. The major advantage is the high yield of recombinant protein product. Apolipoproteins present a new and special problem. Some of their functions are only maintained when they are assembled into a lipoprotein particle. In addition, some of the apolipoproteins aggregate in the absence of lipid. For these proteins, the widely-used insect tissue culture system is therefore not ideal because it does not have the ability to package apolipoproteins into lipoprotein particles.

Apolipoproteins E and B are ligands for the low density lipoprotein (LDL) receptor. ApoE is a 35 kDa protein with a well-defined receptor binding domain. ApoB is a 515 kDa protein in which the receptor binding domains have not been mapped. An obstacle to the study of apoB is the absence of an expression system that will yield assembled lipoproteins and high levels of expression.

Larvae of the tobacco hornworm *Manduca sexta* produce lipoproteins with structural similarities to mammalian lipoproteins. The tissue that produces insect larvae lipoproteins, the fat body, is also a semipermissive target for infection by baculoviruses. We therefore sought to determine if *Manduca sexta* larvae infected with recombinant baculoviruses harboring apolipoprotein genes would be capable of assembling recombinant products.

Because of its smaller molecular weight, we began by testing human apoE. A recombinant baculovirus with the human apoE gene was used to infect *Sf-21* cells in culture and was injected into fourth instar *M. sexta* larvae. After 60 hr of infection, *Sf-21* media contained 30 μg/ml apoE. Expression levels were much higher in the insect larvae; >200 μg/ml hemolymph after 6 days of infection. Most of the apoE in the insect culture media was in the $d > 1.21$ g/ml fraction of a density gradient, indicating that it was essentially lipid-free. By contrast, one-third of the hemolymph apoE was associated with the $d < 1.02$ g/ml density fraction, indicating its association with buoyant lipoproteins. Only the hemolymph particles were competent ligands for the LDL receptor, reflecting the active conformation of lipid-bound apoE.

Recombinant baculoviruses containing full-length human apoB and two truncated genes, apoB48 and apoB17 were produced. Infection of *Sf-21* cells with all three recombinant viruses led to production of the expected products. Studies are in progress to express the apoB cDNA's in *M. sexta*.

Insect larvae are an excellent system for large-scale recombinant protein expression. For proteins that have lipid binding properties, insect larvae mediate the proper lipid binding of the recombinant proteins to obtain fully active product.

X1-002 PRODUCTION OF HUMAN PROTEIN C IN THE MILK OF TRANSGENIC SWINE, William N. Drohan, Henry Lubon, American Red Cross, Holland Laboratory, Plasma Derivatives Department, Rockville, MD, Tracy D. Wilkins, Transpharm, Blacksburg, VA, and William H. Velander, Virginia Polytechnic Institute, Blacksburg, VA.

Human protein C (hPC) acts as a regulator of hemostasis and is considered to have therapeutic importance in certain disease states including deep vein thrombosis, hereditary deficiency of hPC and septic shock. Immunity affinity purified human plasma sources of this protein appear to be insufficient to meet patient requirements, and expression levels in recombinant mammalian cells have been limited to less than 20 μg/ml. We are investigating the feasibility of using genetically engineered pigs as a supplemental source of human protein C. Using the mouse whey acidic protein (WAP) gene with an inserted cDNA encoding human protein C, we have produced a number of transgenic pigs. The first two transgenic animals produced protein C in their milk at levels of between 200 and 2000 mg per liter. On the average, the recombinant human protein C (rhPC) purified from the milk of transgenic swine has anticoagulant activity approaching that of plasma derived human protein C. The rhPC analyzed by 2-D gel electrophoresis appears to be more micro-heterogeneous than hPC from plasma. In addition, the rhPC possessed a significantly higher fucose content than hPC and also contained N-acetylgalactosamine, in contrast to hPC, which had none of this carbohydrate. Further analysis of rhPC by polyacrylamide gel electrophoresis and amino acid sequencing additionally suggests that swine mammary epithelium processes human protein C in a fashion similar to human liver, but some differences can be seen.

X1-003 FACTORS LIMITING EXPRESSION OF SECRETED PROTEINS IN MAMMALIAN CELLS, Randal J. Kaufman, Andrew J. Dorner, Alnawaz Rehemtulla, Louise C. Wasley, Genetics Institute, Cambridge, MA 02140

Expression of heterologous genes in mammalian cells may be controlled at the level of transcription, processing of precursor mRNAs, mRNA transport to the cytoplasm, mRNA stability and translational efficiency, and protein stability. For most secretory proteins, the rate limiting steps for maximal expression occur within the secretory pathway. Proteins which transmit the secretory apparatus are subject to a variety of steps which regulate folding and secretion of the mature polypeptide. For most secretory proteins, the rate limiting step in secretion is transported from the endoplasmic regiculum (ER) to the Golgi apparatus. Some proteins transiting the ER interact with GRP78, a lumenal stress response protein of the ER. Dissociation from GRP78 requires ATP. We have recently demonstrated by direction firefly luciferase expression in the ER, that ATP can be detected within that compartment. In addition to dissociation from GRP78, a wide variety of post-translational steps are required for appropriate maturation and biological activity of a secreted protein. These steps may be saturated as the expression of the specific protein is increased. One saturable step is proteolytic processing of the precursor polypeptides. Recently, a class of subtilisin-like serine proteases have been identified, which when overexpressed can improve the processing of precursor polypeptides in mammalian cells. These observations should provide the ability to specifically engineer mammalian cells to efficiently perform the required steps for secretion of a desired protein.

X1-004 TRANSMISSIBLE SPONGIFORM ENCEPHALOPATHIES AS CONCERN IN BIOPHARMACEUTICAL PRODUCTION, Richard F. Marsh, Department of Animal Health and Biomedical Sciences, University of Wisconsin, Madison, WI 53706

Fifty three years ago 1260 of 18,000 sheep died of scrapie after being inoculated with a Louping Ill vaccine produced by formalin treatment of brain tissue. This was the first "therapeutic misadventure" observed with these unusual neuropathic agents but, unfortunately, not the last. Creutzfeldt-Jakob disease (CJD) has been iatrogenically transmitted among humans using contaminated EEG electrodes, tissue grafts, and hormone injections.

The recent occurrence of bovine spongiform encephalopathy (BSE) in Great Britain has again raised concerns of possible health risks from the use of products produced from human and animal tissues. To address these concerns it will be necessary to apply our knowledge of the biological and physicochemical nature of these transmissible agents to reduce this potential biohazard to as low a level as possible.

Our main effort must be in obtaining tissues from disease-free individuals. No human tissues should be used from patients dying of CJD or undiagnosed neurologic illnesses. No bovine tissues should be used from countries from which BSE has been reported (Great Britain, Ireland, France, Switzerland, Oman, and the Falkland Islands). But even with these precautions, there remains the risk of using tissues from animals or humans during their long, asymptomatic incubation periods. These infections can not be detected in their preclinical stages.

If an inadvertent contaminated tissue source is used, processing procedures should be incorporated that remove or inactivate as much infectivity as possible. The most effective of these are ultrafiltration and treatment with 1N sodium hydroxide or 8 molar urea. Heat sterilization by itself can only reduce infectivity titers 99% in samples containing high amounts of host protein. Reducing infectivity from $10^7 LD_{50}$ to $10^5 LD_{50}$ is not acceptable. However, terminal heat treatment, as now required by FDA for new bovine products used in humans, can help assure complete safety when used in combination with other processes.

X1-005 PURIFICATION OF PROTEINS FROM MILK OF TRANSGENIC ANIMALS. Tracy D. Wilkins, Virginia Tech and TransPharm Inc. Blacksburg, VA.

Milk is a very convenient material in which to express high concentrations of recombinant proteins, but milk is not a simple substance. It also is not pure and has a lot of variability inherent in the production and milking process. Isolation of recombinant proteins from milk is not necessarily any easier than isolation from any other complex and variable source. The main advantage is that the protein may be produced in much higher concentrations in milk than in cell culture or plasma.

Milk can never be collected in a pure form; it is always contaminated with mammalian cells. Normal cow's milk contains about 500 million white blood cells per liter! Most of these cells are removed by centrifugation, but many lyse and spill their enzymes into milk. All plasma proteins are present in small concentrations in milk because of leakage. For example, plasmin, the main serine protease in milk, comes from plasma. Immunoglobulins and possibly albumin are actively transported into milk. Bacteria also are always present in milk; the contamination rate is dependent on the health of the teats and the method of milking. These bacteria can not be removed by filtration because the casein micelles clog filters.

The caseins as a group make up over 50% of the protein content of milk. They surround calcium phosphate cores and the aggregate makes up the casein micelle. These particles can be removed by either precipitation with low pH or 15% PEG. This results in formation of the "curd" and "whey". Many recombinant proteins will be in the whey, but some bind to the calcium phosphate cores (hydroxylapatite) of the casein micelles and are precipitated. Some hydrophobic proteins are excreted in the membranes of the fat globules and are removed during skimming.

Any isolation process needs to take into account the fact that the recombinant proteins produced by mammary glands of farm animals are not identical to human proteins and differ according to which animal species is used to produce them. They have different carbohydrates attached and sometimes have slightly different processing. Thus conditions which have been developed for purification of the protein from human plasma or tissue cultured cells may not work for isolation of the "same" protein from milk. This is also the reason that spiking human proteins into milk as a model for development of isolation processes has very little use. In the case of human Protein C with which we work, the spiked protein appeared in the whey whereas the recombinant protein bound to the calcium in the casein micelles.

The advantage that milk offers for isolation is the high concentration of recombinant protein. In some cases this can be 50% of the protein in the milk and, when the caseins are eliminated, the recombinant protein may start out 80% pure! Such high concentrations are not likely to be obtainable for complex proteins because the enzymes needed for post translational modifications can become saturated. The amount of modification which the mammary cells can perform has surprised many scientists, but there obviously is a limit. In the future this may be overcome by engineering the mammary cells so that they produce the needed enzymes in high amounts. The current use of "wild-type" farm animals may seem as naive in a few years as the use of wild-type E. coli would today.

Source Materials for Protein Isolation

X1-006 T7 PROMOTER BASED EXPRESSION SYSTEMS FOR OVERPRODUCTION IN E. COLI, Robert Mierendorf, Barbara Morris, Robert Novy, Beth Hammer and Richard Garber, Novagen, Inc., Madison, WI.

The T7 promoter based system developed by Studier and colleagues (1) has proven to be extremely useful for cloning and expression of foreign genes in *E. coli*. Target genes are cloned in pET plasmids under control of strong bacteriophage T7 transcription and translation signals; expression is induced by providing a source of T7 RNA polymerase in the host cell. T7 RNA polymerase is so selective and active that almost all of the cell's resources are converted to target gene expression. The desired product can comprise more than 50% of the total cell protein after a few hours of induction. Perhaps just as important as the strength of the T7 promoter is the ability of the system to maintain target genes transcriptionally silent in the uninduced state. Target genes are initially cloned using hosts that do not contain the T7 RNA polymerase gene, so they are virtually "off" and cannot cause plasmid instability due to the production of proteins potentially toxic to the host cell. Once established, plasmids are transferred into expression hosts containing a chromosomal copy of the T7 RNA polymerase gene under *lacUV5* control, and expression is induced by the addition of IPTG.

Several vector/host combinations that differ in their stringency of suppressing basal expression levels are available, which makes it possible to optimize the yield of individual target proteins. For example, a very toxic single chain antibody requires maximum suppression of basal T7 RNA polymerase activity to be maintained and expressed. In contrast, the highest expression levels of a relatively innocuous protein (β-galactosidase) are observed under less stringent conditions.

Many features have been incorporated into the pET series of vectors, including upstream and downstream sequences designed for periplasmic export, immunodetection, and affinity purification. A vector-encoded stretch of 6 or 10 consecutive histidine residues at the N- or C-terminus allows rapid, economical purification of target proteins by metal chelation chromatography. A significant advantage of this technique is the ability to purify proteins under either native or denaturing conditions. In the latter case it has been possible to renature a target protein by gradual removal of the denaturant prior to elution from the affinity column.

Specialized T7 promoter based expression vectors have also been developed for the construction of cDNA and epitope libraries. In each case the system has been adapted to allow rapid functional screening of expressed protein domains on plaque or colony lifts. These systems have a variety of applications, including the investigation of protein-protein interactions, antibody engineering, epitope mapping, and receptor characterization.

1. Studier, F.W., Rosenberg, A.H., Dunn, J.J. and Dubendorff, J. W. (1990) *Meth. Enzymol.* 185, 60-89.

Initial Processing

X1-007 HIGH THROUGHPUT BIOSEPARATION TECHNOLOGIES, Jay M. S. Henis, Henis Technologies, Inc., St. Louis, MO. 63141.

Membrane and chromatographic separations have been evolving for many decades. In both cases, the technologies were initially thought to be applicable primarily to specialty and small scale applications. Certainly, it is true that it was with such applications that these technologies were originally demonstrated. However, with the passage of time, many improvements and modifications have been made, and even though neither chromatography nor membranes scale well, we can now find many large scale applications in a variety of industries.

The use of cross flow and chemical modification to reduce fouling has enabled membranes to be used in very large scale food and dairy applications. In the case of chromatography the limitations of scale in such applications are particularly severe. High flows can only be achieved in conventional columns at the expense of high pressure, and even at high pressure the bed volume and vessel size can be quite large and cumbersome. Recently, the development of new radial packing procedures to make columns more uniform and the emergence of radial flow chromatography have made very high throughput, low pressure chromatography a real possibility.

Another emerging technology with significant potential is Electrodeionization (EDI). A derivative of electrodialysis methodology, EDI can be used to process high volume fluid streams containing proteins, salts and other materials.

High throughput is particularly important in the dairy industry where processing must take place rapidly and the amount of liquid to be processed is exceptionally large. Advances in these techniques and the results of recent work will be presented with an emphasis on new developments which enhance productivity and throughput.

X1-008 AFFINITY SEPARATION MEMBRANES PREPARED FROM MICROPOROUS POLY(CAPROLACTAM) HOLLOW FIBERS, Elias Klein, Don Yeager, G.B. Harding, and Raghunandan Seshadri University of Louisville, Kidney Disease Program, Louisville, KY 40292

Microporous nylon-6 hollow fibers were modified for use as affinity fibers. The initial amine end-group concentration of 21 μmoles/g fiber was amplified by reacting the polymer with lysine, using standard peptide synthetic methods. The carbohydrate side chains of rabbit polyclonal anti-BSA IgG and a sheep anti-h-IgG were oxidized to link the Abs to the hollow fibers. The covalent links were either to terminal amine groups (from lysine linked to the poly(caprolactam)) or to hydrazide groups. The latter were produced by coupling adipic acid dihydrazide to the amine groups via a glutaraldehyde bridge. Coupling of the oxidized Ab to amine groups required a pH>8.0, whereas the same Ab could react with the hydrazide groups at pH 5.5. The higher pH coupling conditions led to crosslinking of the IgG, presumably between side chain amine groups on the protein and the carbohydrate aldehyde groups. Although significant amounts of IgG could be coupled to the amine groups, the recognition of antigen by such Ab was markedly reduced. In contrast, the coupling of oxidized α-BSA to hydrazide groups, carried out at pH 5.5, gave bound Ab which exhibited the theoretical number of binding sites for its antigen. Capacity of the immobilized polyclonal α-h-IgG was less than theoretical. Equilibrium binding coefficients for BSA showed values ranging from $5.2x10^6$ M^{-1} to $9.7x10^5$ M^{-1}. The molar ratio of BSA to calculated anti-BSA binding site was generally 2:1. Although stripping experiments with SDS at >85 C indicated none of the BSA was covalently linked to either the IgG or the fiber substrate, less than 60% of the adsorbed BSA could be eluted with pH change, salt, chaotropic agent, etc. The same responses were observed with hydrazide modified Sepharose carried through the experiments for comparison.

α-h-IgG bound to PA hollow fibers produced pure h-IgG product when challenged with a mixture of Cohn fraction 2 (0.2 mg/ml) and BSA (1.0 mg/ml) . The dynamic capacity at 12ml/min flow (7.5 seconds residence time) ranged from 4.5-5.5 mg IgG/ml of membrane volume. Similar capacities were found when rProtein A was immobilized in place of the α-h-IgG.

X1-009 SCALING-UP PROTEIN EXTRACTION IN AQEUOUS TWO-PHASE SYSTEMS, Maria-Regina Kula, Institut für Enzymtechnologie, Heinrich-Heine-Universität Düsseldorf, P.O. Box 20 50, W-5170 Jülich, Germany

Liquid-liquid extraction of proteins is possible exploiting aqueous two-phase systems based on the incompatibility of hydrophobic polymers, e.g. polyethylene glycol and dextran in the common solvent water, or by salting out of polyethylene glycol using phosphates, sulfates or citrates (1 - 4). The method is gentle due to the high water content of the two immiscible phases and the low interfacial tension. High activity yields are obtained during extraction.

The technology is especially useful for the isolation of intracellular proteins and the concomitant removal of insoluble cell fragments in large scale or the concentration and enrichment of proteins from whole broth. Partition is governed by the thermodynamic properties of the product, under these conditions separation of solids does not depend on the size of the particle as in mechanically controlled processes such as centrifugation and filtration. Removal of solids is much faster by liquid-liquid extraction. Besides, unwanted byproducts may be removed at the same time.

The selectivity of extraction my be further enhanced using affinity interactions. If required, additional purification steps e.g. by chromatography can be carried out either directly from a separated phase or after suitable conditioning e.g. by diafiltration/concentration using appropriate UF membranes.

The following parameters will be discussed with regard to the requirements for scale-up:
- the kinetics of phase equilibration and partitioning
- methods of mixing
- phase separation by gravity or centrifugation
- batch and continuous processing
- economics of operation
- recycling of phase forming chemicals

Examples will be taken from various case studies performed in the authors laboratories.

1. Kula, MR; Kroner, KH and Hustedt, H (1982) Adv. Biochem. Eng. 24, 73-118.
2. Hustedt, H; Kroner, KH and Kula, MR (1985) in: Partitioning in Aqueous Two-Phase Systems: Theory, Methods, Uses and Applications to Biotechnology (eds. H. Walter, D.E. Brooks and D. Fisher) Academic Press, pp 529-587.
3. Vernau, J and Kula, MR (1990), Biotechnol. Appl. Biochem 12, 397-404.
4. Kula, MR (1990), Bioseparation 1, 181-189.

X1-010 EFFECT OF CULTURE CONDITIONS ON POST-TRANSLATIONAL MODIFICATIONS OF RECOMBINANT PROTEINS PRODUCED IN MAMMALIAN CELLS, Mary B. Sliwkowski, Edward T. Cox and Jane V. Gunson, Cell Culture & Fermentation R & D Department, Genentech, Inc., South San Francisco, CA 94080.

Mammalian cell culture systems are widely used for production of pharmaceutically relevant proteins. The main reason for employing these complex production systems is to exploit their ability to correctly carry out a variety of post-translational modifications, such as proteolytic processing, disulfide bond formation and glycosylation. These modifications are often desirable for their ability to effect biological activity, circulatory clearance rates, solubility and stability. A number of other detrimental modifications, such as proteolytic clipping, aggregation and deamidation can also occur in mammalian systems. Because these post-translational modifications occur with varying degrees of efficiency, they can lead to considerable structural heterogeneity in both natural proteins and their recombinant counterparts. Structural heterogeneity can arise at many steps in the production process but we are specifically interested in characterizing and controlling those modifications which occur during cell culture. By studying more than 20 proteins produced in mammalian cell culture over the past 7 years, we have gained insight into several post-translational modifications which can be influenced by culture conditions. These include: alteration of intracellular glycosylation; remodeling by extracellular proteases, glycosidases and phosphatases; and formation of mixed disulfides between protein-bound cysteine residues and small thiols from the culture medium. Several such examples will be presented. Methods for monitoring and controlling these modifications will be discussed.

New Supports-I (Session Sponsored by 3M Bioapplications)

X1-011 CLEANABLE ZIRCONIA-BASED CHROMATOGRAPHIC SUPPORTS, Michael C. Flickinger[1,2], John E. Morris[1], Lifang Sun[3], John A. Blackwell[3], Wes A. Schafer[3], Michael H. Glavanovich[3], Peter W. Carr[1,3], Francisco Lorenzano[4] and Alon V. McCormick[4], [1]Inst. for Adv. Studies in Biological Process Technology, [2]Dept. of Biochemistry, [3]Dept. of Chemistry, and [4] Dept. of Chemical Engineering and Material Science, University of Minnesota, St. Paul, MN 55108, USA FAX 612-625-1700

Zirconia-based supports offer many potential advantages over carbohydrate or silica support materials used in bioseparations such as: chemical and thermal stability, good mechanical strength, and high density. The solubility of zirconia in acid and base is orders of magnitude lower than that of silicon or aluminum oxide. Thus, zirconia-based chromatographic supports have the potential for repeated steam or hot alkaline regeneration and sterilization to inactivate virus and protein contaminants. The high density of zirconia may be useful for both process scale batch adsorption and fluidized-bed applications. A variety of protein chromatography supports using porous zirconia particles are being developed: ion-exchange, ligand-exchange, concanavalin A-affinity, thiophilic-affinity, and size exclusion. Methods have been developed to coacervate colloidal zirconia to form uniform porous particles which are base-stable and which can be derivatized with non-fouling surfaces. These particles will be useful for process-scale protein separations where a cleanable or sterilizable chromatographic support is required and/or where batch adsorption or fluidized-bed applications are easier than large volume packed-bed chromatography.

X1-012 PERFUSION CHROMATOGRAPHY: A NEW TOOL FOR BIOMOLECULE PURIFICATION AND ANALYSIS, Neal F. Gordon and Noubar B. Afeyan, PerSeptive Biosystems, Inc., Cambridge, MA

Surface-mediated separations via chromatography are used for the purification and quantitative analysis of complex protein mixtures. Porous particles functionalized with the appropriate surface chemistries are used today. Speed, capacity and resolution together determine the overall separation performance and these are strongly related to the morphology of the porous particles.

Since the advent of Perfusion Chromatography®, (the use of flow-through particles to perform high throughput/high speed separations) the direct coupling between the three performance variables has been broken, allowing very high speed, high performance separations (10 seconds to 5 minutes). The major present and future ramifications for research, analytical and preparative applications will be discussed. In addition, this new separation capability allows a novel systematic approach to be used for the development of chromatographic methods, examples of which will be shown.

High speed separations also form the basis for a new technique, ImmunoDetection™, which combines antibody-based assays with a flow-through column format. The result is real time immunoassays performed in seconds or minutes. Application of ImmunoDetection for on-line monitoring of a product and contaminants in a bioprocess will be presented as a case study.

X1-013 A ^{31}P NMR STUDY of IAM SURFACES X. Qiu and C. Pidgeon, Dept. of Medicinal Chemistry, Purdue University, West Lafayette, IN 47907

Immobilized artificial membranes (IAMs) are chromatography packing material containing phospholipids tethered to the silica surface through the lipid alkyl chains. IAM surfaces are unique in that the phosphorous nucleus in the lipid headgroup is a naturally abundant NMR probe. This allows direct study of the motional properties of IAM surfaces using ^{31}P NMR methods. We have performed preliminary ^{31}P NMR line shape analysis and ^{31}P relaxation measurements on the IAM surface denoted as ^{ether}IAM.lysoPC$^{10/3}$. This is is a chemically stable IAM that does not exhibit phospholipid leaching from the silica when suspended in several mobile phases.

The ^{31}P NMR spectrum of the dry powder exhibits two components: (i) a broad symmetric peak centered at 0 ppm; and (ii) an asymmetric powder pattern with a chemical shift anisotropy (~200 ppm). The superposition of these two line shapes in the ^{31}P NMR spectrum of the dry powder suggests that there are two populations of immobilized phosphate groups on the IAM surface. We attribute the asymmetric powder pattern to be caused by ligands exhibiting very slow motion (i.e., due to high ligand packing density) and the symmetric peak to arise from ligands experiencing fast isotropic motion (i.e., low ligand packing density). Two populations of immobilized phospholipid ligands indicates site heterogenicity on the IAM surface.

Suspending IAM in methanol or water caused only a narrow symmetric ^{31}P NMR line shape centered at 0 ppm. This indicates that these highly polar solvents significantly increase the isotropic motion of the immobilized ligands compared to the dry powder. However, suspending IAM in the less polar solvents chloroform, acetone, or acetonitrile, caused the ^{31}P NMR spectra to exhibit an axially symmetric powder pattern. The axially symmetric line shape indicates that these nonpolar organic solvents increase anisotropic motion of the immobilized ligands. Based on ^{31}P NMR line shape analysis the rank order of the isotropic motion of the lipid molecules on ^{ether}IAM.lysoPC$^{10/3}$ are:

$CH_3OH > D_2O > CH_3CN > CHCl_3 >$ acetone > dry powder

The dynamics of the immobilized phospholipids was studied using the spin lattice relaxation time constant (T_1). T_1 measurements were performed at different temperatures on ^{ether}IAM.lysoPC$^{10/3}$ particles that were either 12 μ or 5 μ in size. When suspended in D_2O, the T_1 values of both the 5 μ and 12 μ IAM particles ranged from 0.8 to 1.5 sec in the temperature range from 6 to 60 °C. In addition, both 12 μ and 5 μ IAM particles showed the same T_1 minimum which occurred at approximately ~10 °C. This indicates that the local motion of the ligands themselves, not the overall tumbling of the large IAM particle, determines the rate of ^{31}P spin lattice relaxation. Based on the T_1 minimum, the correlation time (τ_c) of the immobilized phospholipid ligands was 0.8 nsec. Using 12 μ IAM particles, the T_1 minimum in methanol occurred at approximately -10 °C which is 20 °C lower than the T_1 minimum obtained in water. Thus there is significantly more motion of the immobilized phospholipids on IAMs suspended in methanol compared to suspending IAMs in water. This is consistent with the ^{31}P NMR line shape analysis; the line width of IAMs suspended in methanol is much narrower than that in water.

Spin spin relaxation measurements (T_2) were performed in methanol (T_2 = 1.6 msec) and water (T_2 = 1.3 msec). T_2 values for phospholipids in liposome membranes range from 1 to 20 msec. Immobilized phospholipids in IAMs have T_2 values on the low end of this range. These preliminary studies demonstrate that ^{31}P NMR is an excellent probe for the motional properties of IAM surfaces. Although the lipid ^{31}P NMR line shape of ^{ether}IAM.lysoPC$^{10/3}$ in water is different than the ^{31}P NMR line shape of liposomes, both the correlation time, τ_c , and T_2 values of IAMs and liposomes are similar.

New Supports-II

X1-014 **INTRODUCTION OF PROCESS CHANGES IN BIOLOGICAL PRODUCTS: A REGULATORY PERSPECTIVE,** **Richard M. Lewis and Mary Catherine J. Schneider, Center for Biologics Evaluation and Research, Bethesda.**

Biological products are often complex mixtures that cannot be precisely defined. Because the manufacturing process is closely related to the characteristics of the final product, when considering Product License Applications or Amendments the Center for Biologics Evaluation and Research considers not only the final material but the process and the facility as well. Frequently, process changes may include new chromatographic procedures which result in increased purity. Although this is an important and positive goal, biological products which are to be used in therapy must be judged on the basis of their safety and their efficacy and not on their physicochemical characteristics. Physicochemical methods are, however, an important means of controlling manufacturing consistency.

With plasma-derived products, the ability of chromatographic methods to separate virus from the activity of interest is an important consideration and has been demonstrated to contribute to increased safety. The validation of partitioning between either a specific virus or an acceptable model has provided valuable data in support of approval of novel manufacturing process changes. Similarly, if an increase in purity can be demonstrated to increase safety and/or effectiveness, these data are important positive contributions toward acceptance of new technology.

Amendments to licensed products generally do not require documentation to the same extent as the original submission, but factors such as Good Manufacturing Practices and product stability must still be considered. The extent of review and level of scrutiny, however, depends on the complexity of the issue and the thoroughness of the application.

Descriptions of how recent submissions were reviewed and the types of data submitted will be presented. Included will be examples of the types of additional information required for blood-derived products manufactured with the introduction of new chromatographic methods.

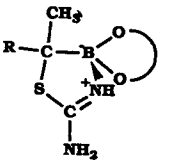
X1-015 BORONATE PROTEIN PURIFICATION, William H. Scouten, Xiao-Chuan Liu, Katchia Adamekova, Zenobia Biedrzycka, Marek Biedzryski, and Vladimir Adamek, Baylor University, Department of Chemistry, Waco, TX.

Boronate chromatography is based upon the ability of boronates to form diesters with 1,2- or 1,3-diols. These are formed at alkaline pH and readily reversible at neutral or acidic pH. Using boronates immobilized on an inert matrix, glycoproteins, RNA, catechol amines and many other biological molecules can be isolated. One particularly valuable use of boronate chromatography is for the analysis glycated hemoglobin, a non-enzymatically glycosylated form of hemoglobin that occurs in elevated amounts in uncontrolled diabetics.

Other uses of boronates is for the immobilization of proteins and other biological molecules. This immobilization is reversible and could form the basis of a readily regenerated immobilized enzyme column. Moreover, proteins immobilized via boronates will be oriented since glycosylation occurs ononly one part of the protein molecule

Major problems in boronate chromatography include the inability to use it at the most desired, i.e. physiological, pH values. logical, pH values. Nonspecific binding is also frequently a problem since boronates are usually hydrophobic, aromatic molecules. Finally, boronate chromatography matrices are not sufficiently stable for many applications.

Our solution to this problem has been to combine three approaches, namely the addition of a coordinating heteroatom near the boron (Mattheson approach), the addition of an acid/base catalytic function, also near the boronic acid (Wulff approach) and finally the addition of electron withdrawing groups to decrease the pK and thus the pH optimum for the stability of boronate diesters (Hageman approach). An example of such compounds is depicted below.



Our initial experiments involved using aliphatic, rather than aromatic ligands in order to minimize hydrophobicity. These worked reasonably well as boronate chromatography materials but were too unstable for repetitive use. It was also found than an electron withdrawing group, such as chlorine alpha to the boronate, is more important than a coordinating group adjacent to the boronic acid. There is some evidence that strong coordination to be boronate may even be negative in that such strongly coordinated materials do not form boronic diesters very readily.

We are now in the process of synthesizing similar materials based upon an aromatic nucleus and testing these for boronate affinity chromatography. An alternate purification of IgG molecules and/or their transient immobilization has been thiophilic chromatography as developed by Porath.

New Separation Methods

X1-016 FREE–FLOW ELECTROPHORESIS IN BENCH-TOP AND LARGE-SCALE BIOPROCESSING
Cornelius F. Ivory, Washington State University, Pullman, WA 99164-2710.

Free-flow recycle zone electrophoresis (RZE) has been demonstrated to recover proteins from binary mixtures, fermentation broths and cell homogenates at processing rates in excess of 1 g/hr. In addition, recent hydrodynamic stability experiments indicate that this technology can be scaled to about 100 g/hr without significant modification. However, the absence of a preparative unit suitable for process development has delayed the application of RZE in downstream processing.

To circumvent this problem, a compact instrument has been designed for benchtop preparative RZE. This apparatus is one-fifth the size and one-tenth the cost of our parent device, yet it can process proteins at a feed rate in excess of 1 g/hr. This is possible because elevated rates of heat and mass transfer allow separation at high power densities while maintaining low electro-osmotic sample distortion. It is also more flexible than the parent device since it can be used for continuous RZE, zone focusing, isoelectric focusing and electro-extraction in two-aqueous phases.

Following a brief review of protein processing and hydrodynamic stability experiments conducted in the parent RZE, the new apparatus will be described and several applications illustrated. It is hoped that access to an inexpensive, flexible and easy-to-use instrument will help open the way for large-scale electrophoresis in downstream processing.

X1-017 COMPETITIVE PROTEIN ADSORPTION IN CHROMATOGRAPHY SYSTEMS, N.-H. L. Wang, Purdue University, West Lafayette, IN 47907

Competitive protein adsorption is generally different from that of small solutes for the following reasons: (1) The intrinsic adsorption rates of proteins can be slow compared to transport rates; (2) The intrinsic adsorption kinetics does not obey multicomponent Langmuir kinetics because of steric hindrance and blockage; (3) Aggregation and denaturation reactions induced by temperature, pH, solvents, chemicals, or solid surfaces can affect significantly the competitive adsorption. As a result, multicomponent protein chromatography can be "incoherent" and "non-Langmuirian". More important, the number of concentration waves, wave broadening, and wave shape can change with solute size, ligand density, sorbent particle size, column length, flow rate, temperature, and gradient conditions. In this study, computer simulations are used to develop improved intrinsic rate laws for competitive protein adsorption. The rate laws are being verified with data from various affinity chromatography systems. A generalized rate theory is also developed to explain a wide range of anomalous protein chromatography phenomena.

Affinity Chromatography-I (Session Sponsored by 3M Bioapplications)

X1-018 PROTEIN PURIFICATION USING AFFINITY LIGANDS DEDUCED FROM PEPTIDE LIBRARIES. George A. Baumbach and David J. Hammond, Plasma Manufacturing Technology Department, Pharmaceutical Division, Miles Inc., Clayton, NC 27520.

A new approach to purification of target proteins will be discussed. Step 1 is to screen a random peptide library for specific peptides which bind to target. Binding peptides are isolated without prior knowledge of peptide binding sequence. The amino acid sequence of binding peptides is determined (step 2) followed by large-scale chemical synthesis of peptide (step 3). Peptides are immobilized on an inert support (step 4) for affinity chromatography (step 5). For illustration, we previously selected a peptide ligand to purify a model protein, streptavidin. The peptide sequence His-Pro-Gln was found to bind streptavidin by screening random peptide libraries with purified streptavidin, itself a biotin-binding protein. We have exploited this information by designing peptide ligands which 1) contained His-Pro-Gln, 2) were end-blocked to resist proteolytic attack, 3) were immobilized through a single primary amine and 4) contained tryptophan to monitor conjugation by u.v. absorption. Streptavidin was added to human plasma in order to simulate a complex source material and then the mixture was applied to the peptide ligand column. Streptavidin was purified in a single step by elution with 1 mM d-biotin to approximately 89% purity and a yield of 62%. We discuss this approach as an alternative to other purification technologies for large-scale production of plasma-derived or recombinant DNA-derived proteins.

X1-019 NEW DEVELOPMENTS IN AFFINITY CHROMATOGRAPHY, Christopher R Lowe, Institute of Biotechnology, University of Cambridge, Tennis Court Road, Cambridge, CB2 1QT

The design, synthesis and chromatographic operation of a new range of stable and selective affinity adsorbents for potential application in the purification of pharmaceutical proteins will be described. Computer-aided molecular design has been exploited to design novel ligands which show a predictable selectivity for the target protein[1,2] and which, when coupled to stable perfluoropolymer supports yield high capacity, low leakage adsorbents for affinity chromatography[3-5]. It is anticipated that these new materials will withstand the rigorous conditions required for sanitisation in situ of industrial processes[6].

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X1-020 IMMUNOADSORPTION: STRATEGIES FOR ANTIGEN ELUTION AND PRODUCTION OF REUSABLE ADSORBENTS, Martin L. Yarmush[1], K. Pickard Antonsen[2], Srikanth Sundaram[1], and David M. Yarmush[1], [1]Department of Chemical and Biochemical Engineering and Center for Advanced Biotechnology and Medicine, Rutgers University, Piscataway, NJ, [2]Miles, Inc., Cutter Biological, Berkeley, CA.

Immunoadsorption is a powerful and generalizable method for protein purification that exploits the fine specificity of antigen-antibody interactions. In spite of its potential utility, the more widespread process scale use of immunoadorption has been limited by the high cost of the antibody and the lack of gentle elution schemes that completely preserve the activity of both the immunoadsorbent and the eluted product. In this report, we review common chemical elution strategies such as pH, ionic strength, chaotropic salts, denaturants, and organic solvents as well as physical techniques such as pressure, electrokinetics, and temperature. In general, selection of elution strategies has largely been an empirical art, balancing stability of the immunoadsorbent and the eluted product and efficiency. The limitations of the available choices demonstrate that more attention must be placed on the antibody. Techniques which assist in the identification or creation of new antibodies with improved binding properties and resistance to degradation, e.g., screening and/or rational protein engineering, are also discussed.

Affinity Chromatography-II

X1-021 HEPARIN AFFINITY CHROMATOGRAPHY OF PLASMA AND RECOMBINANT ANTITHROMBIN III, Michael N. Blackburn, Arun Patel, Paula Boerger, and John Martin, Macromolecular Sciences and Protein Biochemistry, SmithKline Beecham Pharmaceuticals, King of Prussia, PA 19406.

The interaction of antithrombin III with heparin accelerates by 1000-fold the rate with which antithrombin inactivates thrombin and other serine proteases of the complement cascade. The heparin-antithrombin high affinity binding interaction also serves as a convenient and efficient vehicle for the purification of antithrombin from plasma as well as from conditioned media derived from recombinant cell culture. Heparin, however, also binds a number of other proteins with high affinity. We have explored a variety of approaches to optimize the use of heparin for the purification of antithrombin. Heparins from different sources have been coupled to agarose matrices using different chemistries in order to take advantage of the different functional groups available on the heparin chain. These resins were evaluated in terms of ligand density, antithrombin capacity and stability.

We have utilized heparin agarose affinity chromatography to resolve two naturally occurring forms of antithrombin from normal human plasma. The differences in affinity of antithrombins α and β for heparin is evidenced both by elution from a heparin agarose column and by the dependence of antithrombin-thrombin reaction rates on heparin concentration. Heparin agarose affinity chromatography has also been used to isolate recombinant human antithrombins expressed in a CHO cell line.

Finishing Steps-I

X1-022 REGULATORY ISSUES INVOLVED IN TAKING A NEW BIOPHARMACEUTICAL PRODUCT FROM CONCEPT TO MARKET, Nancy J. Chew, NJC Enterprises, Ltd., 700 Washington St., New York, NY 10014 USA

Development of a new therapeutic agent requires nearly a decade and costs hundreds of thousands of dollars. The development and registration process is highly regulated. Technology transfer and the steps required to develop an approvable marketing application will be discussed. FDA's research requirements, quality assurance regulations, and monitoring activities will be described from the point of view of strategic planning. What activities require the most time, which cost the most? What determines which substances ultimately make it to the marketplace?

Before human testing can be done, pharmaceutical development, animal pharmacology and animal toxicology studies must be carried out. Classic small organic molecules have toxicity profiles that are quite different from those of biopolymers. The requirements to show safety and effectiveness must be met differently. Demonstrating the safety of novel biopolymers is unprecedented. The practical regulatory consequences of developing new tests is a crucial strategic element.

The first test in humans is a critical step in the development of a biopharmaceutical. Traditionally, such tests used limited numbers of healthy volunteers to determine side effects. Increasingly, biopolymers are tested for "proof of concept," (effectiveness) during initial human testing. The changing view of regulatory authorities is an important element in the development of new technologies.

The latter phases of development include testing increasingly larger numbers of patients to demonstrate that the product is safe and effective for its intended use. New regulatory pathways have been developed to short-circuit the process for rare, life-threatening, or severely debilitating diseases. Many biopharmaceuticals are being developed for such conditions. What are the practical impacts of these regulatory innovations?

The process of compiling a marketing application for a biopharmaceutical is a lengthy one. Once approved, FDA issues a pair of licenses. The strategy for the development of the biopharmaceutical product must include knowledge of FDA's licensing regulations. The management of manufacturing responsibilities is key to the ultimate profitability of the product.

Once a biopharmaceutical is on the market, what are the regulatory requirements? How does the licensee interact with FDA on an ongoing basis?

X1-023 **PROTEIN PURIFICATION: SCIENTIFIC AND REGULATORY CONSIDERATION ON GLYCOSYLATION, DEAMIDATION AND OXIDATION OF PHARMACEUTICAL PROTEINS, Darrell T. Liu, Division of Biochemistry and Biophysics, CBER, FDA, Bethesda, MD 20892.**

The general properties of glycoproteins, amide bonds and certain labile amino acids associated with protein modification and alteration of activities during purification and storage of pharmaceutical protein will be discussed. Examples will be drawn extensively from pharmaceutical proteins produced by rDNA technology and brief references made on the U.S. Orphan Drug Act and glycoprotein pharmaceuticals. Methods that are scientifically sound and deamed reasonable for the characterization, detection and quantitation of different glycoforms, deamidated species and oxidized form of amino acids will be discussed. The presentation will conclude with regulatory concerns and recommendation for the purification and characherization of pharmaceutical proteins.

Finishing Steps-II

X1-024 METHODS OF CHARACTERIZING PROTEIN PURITY AND INTEGRITY

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The advent of molecular biology has resulted in a tremendous stimulation of protein characterization methods. For example, the routine production of protein pharmaceuticals in large amounts has required determination of protein purity at very high levels. Such an analysis is a complex problem dependent on the structural characteristics and spectrum of potential impurities of the product. Another goal of characterization studies is the detection of protein variants that arise from post-translational modifications, such as glycosylation, phosphorylation and acylations. The ability to carry out high efficiency separations followed by sophisticated structural methods has allowed the detection of very subtle modifications, such as isoaspartate formation and the substitution of methionine with norleucine.

Reversed phase high performance liquid chromatography (RP-HPLC), particularly in combination with mass spectrometry, has played a key role in these developments. This technique can be used to establish identity between recombinant DNA-derived and natural material as well as providing a valuable method for the investigation of proteins in complex matrices. After isolation of a variant, the most common approach is to prepare an enzyme digest and then analyze the peptide mixture by electrospray mass spectrometry on-line with the HPLC separation. This approach is particularly valuable for the characterization of glycoproteins. After separation by RP-HPLC the elution position and structure of the glycopeptide families can be determined by reconstruction of the ion chromatograms using molecular weights that correspond to the masses of each glycopeptide. While this approach can not be used to discriminate isobaric structures, it gives the biochemist a rapid method for mapping the carbohydrate structures in glycoproteins that are available in as little as ug amounts.

X1-025 **REMOVAL OF TRACE BIOCONTAMINANTS: DNA AND ENDOTOXIN**, **Christopher P. Prior, Dave Weber, Rose D'Alisa, Jon Mitschelen, Ted Lee, and Mike Hrinda, Rhône-Poulenc Rorer Pharmaceuticals, Central Research, 680 Allendale Road, King of Prussia, PA, 19406.**

As part of the overall effort to assure product "safety" in humans, it is necessary to reduce DNA and endotoxin to the lowest levels possible as measured at the limits of assay sensitivity. Protein purification and process control strategies will be presented that optimize removal of these trace contaminants to acceptable levels. Three large-scale case studies will be presented describing removal of DNA and endotoxin from biopharmaceuticals purified from plasma, cell culture, and recombinant E. coli.

X1-026 REMOVAL AND INACTIVATION OF MODEL VIRUSES BY METHODS COMMONLY USED IN PURIFICATION OF BIOPHARMACEUTICALS, Alexander F. Sito, Ph.D., Quality Biotech Inc., 1667 Davis Street, Camden, New Jersey

With advances in biotechnology, continuous cell substrates are now being used to manufacture therapeutic proteins (biopharmaceuticals). The use of such cell substrates may result in the potential transmission of retroviruses as well as other adventitious agents to patients receiving biopharmaceuticals. To assure the safety of biopharmaceuticals, manufacturers must validate their purification processes to demonstrate the inactivation and/or removal of harmful contaminants, such as viruses, nucleic acids, mycoplasmas, and scrapie-like agents. Processes used for purifying products of mammalian cells are largely based on a combination of ultrafiltration and column chromatography techniques which are designed to inactivate and remove viral and nucleic acid contaminants without reducing the biological activity of the final product. In order to demonstrate that the purification process can achieve this, it is necessary to spike each stage of the process with host cell DNA and known concentrations of resistant viruses which could be harbored by the production cell line. The choice of viruses and nucleic acids used for spiking experiments depends upon the species of producer cells and the end product's indication. Principles of process validation studies, including risk assessment, experimental design, and safety factors, will be presented.

Late Abstract

A NEW METHOD OF SCALING UP FREE FLOW ELECTROPHORESIS - CAPILLARY FREE FLOW ELECTROPHORESIS (CFFE)

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Free flow electrophoresis (FFE) has been used for the separation of proteins and cells for a long time and has evolved into the most promising method of continuous separation of biomolecules. One of the major drawbacks inherent in free flow electrophoresis in the past, however, is the thermal convection caused by joule heating which occurs whenever a current is applied across a conducting liquid medium. To provide efficient heat dissipation, the cross-section of traditional FFE units is restricted to approximately 1 mm thick, which limits sample throughput. A new continuous FFE apparatus, which internally cools the separation unit by passing water through aligned capillary tubes was developed. This innovation allows scale-up of the separation without thermal convection. This chamber is 20 times thicker than the conventional chamber. Throughput of 1 gm/h of proteins on this unit have been achieved. The bed of aligned capillary tubes does not contribute significantly to band broadening due to electro-osmotic or laminar flow profiles. The primary advantage of CFFE over other free flow electrophoresis units is that it is a continuous separation process as opposed to a batch process. No recycling is required as no thermal convection was observed, even with residence times in excess of 10 minutes. The heat transfer capacity is 1057 W at 3.8 l/min cooling water flow. The purification of several proteins and peptides by capillary free flow electrophoresis is presented.

Initial Stops

X1-100 PREPARATIVE NONDENATURING GEL ELECTROPHORESIS OF 4S-LIMONENE SYNTHASE, A MONOTERPENE SYNTHASE FROM SPEARMINT, William Alonso, Efraim Lewinsohn, Mark Gijzen and Rodney Croteau, Institute of Biological Chemistry, Washington State University, Pullman, WA 99164-6340.

Monoterpene synthases occur in very low abundance in specialized tissues, like most enzymes involved in the biosynthesis of natural products. Consequently, the purification of these enzymes by multiple liquid chromatographic steps generally does not provide sufficient levels of protein to permit N-terminal sequencing, production of antibodies, etc. Thus, a preparative nondenaturing gel electrophoresis system was developed to purify monoterpene synthases directly from crude enzyme preparations, and improve the overall yield of purified protein.

Nondenaturing PAGE is a very selective protein purification technique since separation is based on net charge as well as size and shape of the molecules. The multiphasic zone electrophoresis system [MZE.3328.IV] of Jovin (1973, *Ann. N.Y. Acad. Sci.* 209, 477) has considerable potential for the purification and analysis of many different classes of enzymes because N-terminal modification of proteins is avoided (Moos et al., 1988, *J. Biol. Chem.* 263, 6005), and, in addition, it affords excellent recovery of enzyme activity (Lewinsohn et al., 1992, *Arch. Biochem. Biophys.* 293, 167). Other discontinuous nondenaturing PAGE systems that employ higher pH are not useful for this purpose because the recoveries of enzyme activity are often unacceptably low.

Here we report purification of 4S-limonene synthase approximately 80-fold using the MZE.3328.IV system with the Bio-Rad Model 491 Prep Cell. Initially, slab gel electrophoresis was employed to test the utility of the MZE.3328.IV system for purifying 4S-limonene synthase from a crude enzyme preparation. Subsequently the Bio-Rad Prep Cell was employed to scale up the purification so that higher levels of protein could be obtained, thus facilitating additional analyses.

X1-101 PURIFICATION FOR CRYSTALLOGRAPHIC ANALYSIS OF TWO MONOCLONAL ANTIBODY Fab FRAGMENTS DIRECTED AGAINST *PSEUDOMONAS AERUGINOSA* PAK PILIN Amechand Boodhoo, Randy J. Read and Randall T. Irvin, Department of Medical Microbiology and Infectious Diseases, University of Alberta, Edmonton, Canada T6G 2H7.

Pseudomonas aeruginosa, a significant opportunistic pathogen uses pili to adhere to respiratory surfaces to initiate infections. To investigate the structure/function and antigenicity of pilin, two monoclonal antibodies PK99H and PK3B were generated and papain treated to produce Fab fragments. The Fab isoforms were purified on a FPLC system (Pharmacia) using a Mono P column. The preparation and purification of these mixtures of Fab species to iso-electric homogeneity for X-ray crystallographic analysis will be presented. (Supported by the Medical Research Council of Canada, Alberta Heritage Foundation for Medical Research, the Howard Hughes Medical Institute and the Canadian Cystic Fibrosis Foundation.)

X1-102 THE EFFECT OF CODON CHOICE ON EXPRESSION AND PURIFICATION OF BOVINE PLACENTAL LACTOGEN, Daniel F. Curran, James F. Kane, Bernard N. Violand, Nicholas R. Staten, Kevin L. Duffin, Gregg Bogosian, Monsanto Research Center, St. Louis, MO 63198

Bovine placental lactogen (bPL) is a 200 amino acid protein in the somatotropin/prolactin gene family. The mammalian gene was sequenced and subcloned into an *E. coli* expression vector to produce the recombinant molecule. Fermentation expressed ~1 g/L bPL at 1000L scale. The material was purified and characterized for use in studies to assess the *in vivo* activity of bPL.

Initial characterization of bPL indicated that the amino acid composition and N-terminal sequence was not different from native bPL. Analysis of a bPL tryptic digest yielded an uncharacteristic peptide. Sequencing identified this bPL peptide as missing two internal amino acids, -R-L-. We propose this novel occurrence resulted from an in-frame, two codon translational hop within an open reading frame. The site of this omission is an arginine codon which is not prevalent in E. coli. This led us to modify the codons used for arginine to more abundant ones.

Modifying these codons resulted in use of more abundant tRNA species which improved expression by debottlenecking protein synthesis and also eliminated the mistranslation. The improved expression resulted in five fold increase in bPL produced as indicated by both increase in size and number of inclusion bodies. Efficiency of bPL recovery from cell lysate was also improved, presumably due to an increased sedimentation differential between the inclusion bodies and cell debris. Downstream purification efficiency was unaffected by the codon modification. Product quality improvement was characterized by tryptic mapping and electrospray mass spectrometry which verified the elimination of the two amino acid hop.

X1-103 PURIFICATION OF AN ACTIVE FORM OF THE EXTRACELLULAR DOMAIN OF THE HUMAN ERYTHROPOIETIN RECEPTOR AND ISOLATION OF AN EPO-SOLUBLE RECEPTOR COMPLEX. Dana L. Johnson, Steven A. Middleton, Frank J. McMahon, Daniel Kroon, Eric Tsao, Linda S. Mulcahy and Linda K. Jolliffe. R. W. Johnson Pharmaceutical Research Institute, Department of Molecular and Cellular Biology, Route 202 Box 300, Raritan, NJ 08869

The extracellular domain of the human erythropoietin (EPO) receptor (termed erythropoietin binding protein or hEBP) has been expressed in *E. coli* and is insoluble. The overexpressed protein appears as an insoluble component of the bacterial fermentations regardless of the presence of a bacterial signal sequence. Under specific fermentation conditions, complete processing of the signal sequence is observed to ultimately yield an insoluble protein with a native amino terminus. A method for the recovery, refolding and purification of active hEBP will be described. The most important determinants for refolding of this protein appear to be protein concentration and the time dependent conversion of a folding intermediate into the active form of hEBP. From each gram of starting cells, obtained by high density fermentation, overall yields of 0.5-1.0 mg of active hEBP have been achieved. Utilizing the final product of this process and recombinant human erythropoietin (EPO), a soluble receptor-EPO complex has been isolated and characterized. The details of these experiments will be reported. This reagent should prove useful in biochemical and biophysical studies of EPO/hEBP structure and function.

X1-104 PROTEIN INTERACTION ASSISTED PURIFICATION OF MITOCHONDRIAL ASPARTATE AMINOTRANSFERASE (MAAT) FROM HUMAN PLASMA, AND THE STRUCTURAL COMPARISON WITH ITS TISSUE FORM. Rao V.L. Papineni and Tom R.C. Boyde. Department of Biochemistry, University of Hong Kong, Hong Kong.

MAAT may prove a better discriminant in diagnosis of certain diseases than crude measurements of total enzyme activity. It is present in normal human plasma has been considered paradoxical in view of its rapid clearance rate. Apparently, little is known about its physicochemical properties when in circulation.
Considering the low levels of the enzyme (10μg/l) in the plasma, a sensitive method is needed for isolation. Boyde (Biochem J 1969;59:111) demonstrated that MAAT binds to α2-macroglobulin (α2M) in human plasma by a reversible coulombic interaction, and the binding is strongly potentiated by dilute borate buffers. We took advantage of this phenomenon, isolating the enormous molecular complex by size-exclusion chromatography. The interaction between α2M and MAAT was reversed with the change of buffer conditions, and the MAAT was separated from α2M by a second size-exclusion chromatography step.
The first purification step yielded a 145% yield with a purity of 125 fold. The second size-excusion chromatography resulted in two sets of MAAT fractions depending on the binding to α2M viz. the rigidly bound MAAT of 1840 fold purity and the releasable MAAT with a 324 fold purity. The yield was 70 and 25% respectively.
Our results, suggesting that the molecular properties of MAAT in human plasma are different from that of the tissue form will be discussed.

X1-105 SOLUBILIZATION OF GROWTH HORMONE AND OTHER RECOMBINANT PROTEINS FROM *E. coli* INCLUSION BODIES USING A CATIONIC SURFACTANT, Nirdosh K. Puri†, Enzo Crivelli*, Michael Cardamone*, Rodney Fiddes‡, Joseph Bertolini*, Barry Ninham§, and Malcolm R. Brandon‡.
†SmithKline Beecham Animal Health, Lincoln, Nebraska, USA; *Bunge Scientific and Technical Services, Melbourne, Victoria, Australia; §Department of Applied Mathematics, Canberra, ACT, Australia; and ‡Centre for Animal Biotechnology, Parkville, Victoria, Australia.
Recombinant pig growth hormone (rPGH) was solubilized from inclusion bodies (IBs) using the cationic surfactant cetyltrimethylammonium chloride (CTAC; $(CH_3)_3N^+C_{16}H_{33}Cl$). The solubilizing action of CTAC appeared to be dependent on the presence of a positively charged head group, as a non-charged variant was inactive. Relatively low concentrations of CTAC, 1-5%, were required for solubilization, and protein-bound CTAC was easily removed by ion-exchange chromatography. Compared with solubilization and recovery of rPGH from IBs with 7.5 M-urea and 6 M-GnHCl, the relative efficacy of solubilization was lower with CTAC. However, superior refolding efficiency resulted in final yields of purified rPGH being in the order of CTAC > urea ≥ GnHCl. Detailed comparison of the different rPGH preparations as well as pituitary-derived growth hormone by HPLC, native PAGE, CD spectral analysis and radio-receptor-binding assay showed that the CTAC-derived rPGH was essentially indistinguishable from the urea and GnHCl preparations. The advantages of CTAC over urea and GnHCl for increasing recovery of monomeric rPGH, by minimizing aggregation during refolding *in vitro* were also found with recombinant sheep interleukin-1β and a sheep insulin-like growth factor II fusion protein. In addition, the bioactivity of the CTAC-derived recombinant interleukin-1β was approximately ten-fold greater than that of an equivalent amount obtained from the urea and GnHCl preparations. It is concluded that CTAC represents an excellent additional approach or a superior alternative to urea and in particular GnHCl for solubilization and recovery of bioactive recombinant proteins from IBs.

X1-106 HUMAN N-MYRISTOYLTRANSFERASE: EXPRESSION AND PURIFICATION FROM *E. coli* AND FROM BACULOVIRUS-INFECTED INSECT CELLS, David C. Wood, Rory F. Finn, Margaret D. Huang, Kevin L. Duffin, Nancee M. Kimack, Roger C. Wiegand, and Verne A. Luckow, Monsanto Corporate Research, St. Louis, MO 63198
Purification of useful quantities of rare proteins often requires overexpression of the protein coding sequence in a suitable host organism. The level of expression and cellular localization of the protein are host- and protein-dependent and are not predictable from DNA or amino acid sequence. We have expressed the gene for human MyristoylCoA:Protein N-Myristoyltransferase (NMT) in *E. coli* and in baculovirus-infected insect cells. In *E. coli*, NMT expression was distributed between inclusion bodies and a soluble, cytoplasmic form. In baculovirus-infected insect cells, soluble NMT was found in the culture medium and in the intracellular fraction. From both hosts, purification of the soluble NMT from cell lysates was accomplished by means of S-Sepharose and hydroxylapatite chromatography steps. The resulting protein was a single band by SDS-PAGE colloidal Coomassie staining analysis, and was produced with a yield of ~0.7 mg per gram (wet weight) cells. NMT from both hosts had the same specific activity using GNAASARR-NH_2 as peptide acceptor for the myristoyl moiety. For NMT derived from *E. coli*, the expected N-terminal sequence was obtained by gas phase sequence analysis as well as the expected molecular weight by electrospray mass spectrometry analysis. However, in the case of baculovirus-infected insect cells, electrospray analysis yielded a mass ~50 amu greater than predicted, and gas phase sequence analysis indicated a blocked N-terminus. The chemical identity of this apparent N-terminal blocking group is currently under investigation.

X1-107 PURIFICATION AND CHARACTERIZATION OF BOVINE AND PORCINE BONE MORPHOGENETIC PROTEIN. Yi Sheng Ni, Yong Dai. Department of Biology, Peking University, Beijing 100871 China.

Bone morphogenetic protein (BMP) is an acidic polypeptide and induces differetiation of mesenchymaltype cells into cartilaage and bone in soft parts and bone defects in vivo. We report here on the bovine BMP (bBMP) and porcine BMP (pBMP) are extracted from decalciferous bone matrix with 0.5ml/L $CaCl_2$, 6mol/L Urea or 4mol/L Gdn—HCl. Both of them have apparent MW of 18 KD. A 34KD protein is Separated by extraction with Triton X—100. A 24KD protein is disassociated form BMP by precipitation in 1.5mol/L Gdn—HCl. Aggregates of BMP and a 14KD protein are insolouble in aqueous media. Implantation of composites of crude BMPs and de—BMP decalciferous bone matrix (DDBM) in muscles of rat induces ectopic bone formation DDBM aets as a carrier which alone dose not induce bone formation. The same funetion of ectopic osteo—induction of bBMP and pBMP in rat Suggests that BMP is not species specific.

Processing

X1-200 A RAPID PURIFICATION PROCEDURE FOR HUMAN SYNOVIAL PHOSPHOLIPASE A2 USING PERFUSION CHROMATOGRAPHY™. Byron Ellis, Pamela De Lacy, William Burkhart, Anne M. Hassell, Steven R. Jordan, Daniel Kassel and Michael Luther, Department of Structural and Biophysical Chemistry, Glaxo Research Institute, Research Triangle Park, NC 27709

Phospholipases A2 are a diverse family of enzymes that hydrolyze phosphoglycerides producing free fatty acids and lysophospholipids. PLA2s are involved in mediating the inflammation response and have been associated with many pathological conditions. In order to understand their role in inflammation, it is important to gain knowledge of the structure, catalytic action and mode of action of PLA2. This abstract describes a rapid, efficient, scalable purification process for the production of human synovial PLA2 (hsPLA2) for structural studies yielding enzyme which is > 95% pure. The perfusion chromatography procedure we used offers a distinct advantage over previously published procedures. HsPLA2 was expressed in baculovirus, lysed using an acid extraction of the transfected cells followed by cation exchange chromatography on a Poros® II HS column. This column could be run in excess of 20 ml/min without loss of capacity or resolution. We recovered greater than 50% of the hsPLA2 activity and achieved a 50 fold purification. The fractions containing hsPLA2 activity were pooled and desalted on a Sephadex® G-25 column followed by chromatography on a C-4 reverse phase column. 80% of the applied PLA2 activity was recovered from the C-4 column for an overall yield of 40%. The Poros® II HS column allowed us to complete the entire purification procedure from cell lysis to purified enzyme in 8 hours. The enzyme was shown to be homogeneous by SDS-PAGE, amino acid composition, N-terminal sequence analysis and mass spectroscopy. The baculovirus expressed hsPLA2 has a MW of 13,900 +/- 2 by electrospray mass spectrometry. The highly purified hsPLA2 forms diffraction quality crystals suitable for various structural studies. We find Poros® media to be an effective tool for designing an improved purification method for hsPLA2.

X1-201 OPTIMISING A FIRST CAPTURE STEP FOR MONOCLONAL ANTIBODIES FROM CELL CULTURE SUPERNATANT

Hazel Griffiths, Fiona Crisp, Cora Henwood and Denise Rowland, Biotechnology Development Laboratories, Wellcome Research Laboratories, Langley Court, Beckenham, Kent, England.BR3 3BS.

Several alternative chromatography techniques have been assessed for effectiveness at capturing humanised IgG_1 monoclonal antibodies from cell culture supernatants with a view to use in a therapeutic protein production process. The criteria assessed were: suitability for scale-up, cost effectiveness, low ligand leaching, high recovery and purity of product.

The capacity, robustness, ligand leaching and pressure / flow rate characteristics were assessed for Protein A Sepharose 4FF (Pharmacia), Protein A Agarose (ACL), PROSEP A (Bioprocessing Int.) and POROS A/P (Perseptive Biosystems) all of which were protein A affinity gels. Other methods for capturing the antibody from cell culture supernatants were assessed including ion exchange chromatography, dye-ligand chromatography and affinity chromatography based on a protein A mimic Avid AL (BioProbe Int.). The results from these studies indicated that the CNBr activated support gave low protein A leaching, as determined by in-house assay, compared to other activation chemistries which were reputed to be more stable. The protein A based methods reproducibly produced immunoglobulin of high purity with, in the case of Protein A Sepharose 4FF, POROS A/P and PROSEP A, capacities greater than 15mg IgG per ml gel, without the necessity to alter the start material. The pressure / flow rate analyses showed that the controlled pore glass support was able to be run a higher flow rates than the other matrices at low pressures. The majority of the other methods investigated did not bind the immunoglobulin effectively from cell culture supernatant without manipulation of ionic strength or pH.

X1-202 INITIAL DOWNSTREAM PROCESSING IN EXPANDED BEDS BY USE OF A NOVEL ADSORBENT. Rolf Hjorth, Ann-Kristin Barnfield Frej, Hasse Hansson, Karl-Arne Hansson, Susanne Kämpe, Pharmacia BioProcess Technology AB, S-751 82 Uppsala, Sweden.

The technique of expanded bed adsorption offers promising advantages in the initial recovery of proteins from crude fermentation broths. The process steps clarification, concentration and capturing can be performed in one unit operation and thus permit omissions of the traditional mechanical separation steps. Novel adsorbents are developed to enable the practical use of the expanded bed technology in production processes.

Bovine serum albumin was recovered directly from yeast cell fermentation. The yield of BSA in laboratory scale experiments exceeds 90%. The recovery of recombinant intracellular anticoagulant protein annexin V from *E. coli* unclarified homogenates in laboratory and pilot scale yielded more than 95%. The eluted material could then be directly applied to a chromatography column.

This novel adsorbent for expanded bed adsorption is derivatized with SP or DEAE groups on a matrix of cross linked agarose with a mean particle size of 200 µm, and high enough density to make expanded bed adsorption a true possibility in purpose designed columns.

The dynamic binding capacity is typically 70 g lysozyme/l adsorbent and 40 g BSA/l absorbent at 300 cm/h in expanded beds for SP and DEAE derivative respectively.

X1-203 MATRIX COPRECIPITATION-COCRYSTALLIZATION (MCC) OF PROTEINS USING ORGANIC IONIC LIGANDS. Rex Lovrien, Tim Richardson, Wei Xie, Mark Conroy. Biochemistry Dept. Univ. of Minnesota, St. Paul 55108.

Organic ligands are designed to tighten conformation, then force proteins out of solution as coprecipitates to isolate proteins:

Conformationally motile (floppy) proteins in solution $\xrightarrow[y = 1 \text{ to } 4 \text{ ligands/protein}]{}$ Tightened Molecules in solution

Tightened protein molecules still in solution $\xrightarrow[\text{Increase y, 5 to 20}]{}$ Protected, coprecipitated or cocrystallized protein

MCC ligands bind strongly to many proteins. The tightening reaction partly dehydrates them so coprecipitates are dense and protected. The organic groups associate to form a matrix, a ligand-ligand network host. Protein molecules are guests. The agglomerate coprecipitates and sometimes cocrystallizes because the proteins become ordered, of uniform conformation. MCC ligands are easily removed by resin exchange, releasing enzymes with good activity yields. Fifteen enzymes have been MCC isolated. Some enzymes are remarkably protected against pH, thermal, and prooxidation stress. The ligand matrix method is: (i) Used upstream and downstream. (ii) Scaleable. (iii) Protective. (iv) Simple to research. (v) Scavenging from 0.01 - 0.10% solution of proteins. (vi) Dewatering. (vii) Densifies products, enabling filtration/centrifugation under low pressure/gravities. (viii) Easily reversible. (ix) High yielding. MCC is application of basic molecular principles to industrial process problems.

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X1-204 PURIFICATION OF PROTEASES BY IMMOBILISED-BACITRACIN A AFFINITY CHROMATOGRAPHY,
Wojtek P. Michalski, Jennipher K. Crooks, Stephen J. Prowse, Jan M. Tennent and Anthony W. D. Lepper, CSIRO Division of Animal Health, Animal Health Research Laboratory, Private Bag No.1, Parkville, Vic. 3052, Australia
Many pathogens produce proteases which are considered to be virulence factors. Some proteases are known to be important in the pathogenesis of protozoan parasites: *Plasmodium knowlesi* and *Eimeria spp* (Fuller & McDougald, 1990) or some bacteria: *Dichelobacter (Bacteroides) nodosus* (Kortt *et al.*, 1982). Many proteases are immunogenic and the application of a simple and specific procedure for their purification would be of advantage.
Bacitracin A is an antibiotic polypeptide produced by *Bacillus subtilis* that inhibits proteases from all four classes, viz. aspartic-, serine-, cysteine-proteases and metalloexopeptidase (Mäkkinen, 1972). This observation was exploited in designing a protease purification procedure. Bacitracin A was coupled to CNBr- or Tresyl-activated Sepharose 4B (Van Noortand, *et al.*, 1991) and used for the affinity purification of proteases from *Eimeria tenella* (a protozoan parasite causing coccidiosis in poultry) and *Moraxella bovis* (a pathogen responsible for bovine keratoconjunctivitis). Protein fractions containing proteolytic activity were eluted from the affinity column with an increasing pH gradient. Fractions were pooled and further purified by size exclusion chromatography. This relatively simple, two-step procedure resulted in over 90% recovery of proteolytic activity.
A serine-type protease was purified from *E. tenella*. It was electrophoretically homogeneous with an apparent molecular mass of 20kDa. Two high molecular mass proteases were purified from cell free filtrates of *M. bovis*.

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X1-205 A NEW APPROACH FOR THE SYNTHESIS OF AFFINITY RESINS: ENZYMATIC SYNTHESIS OF POLY(ADP-RIBOSE)-AGAROSE BEADS. *P. L. Panzeter, B. Zweifel and F. R. Althaus.* Institute of Pharmacology and Biochemistry. University of Zürich, CH-8057 Zürich, Switzerland.

Polymers of ADP-ribose are formed in nuclei by poly(ADP-ribose) polymerase. This enzyme transfers an ADP-ribose moiety from NAD^+ to an acceptor protein and subsequently elongates it to form polymers of up to 200 ADP-ribose units. These protein-attached polynucleotides provide very strong, non-covalent binding sites for chromatosomal histones [Panzeter et al. (1992) *Biochemistry 31,* 1379-1385]. To further characterize binding of nuclear proteins to ADP-ribose polymers, we have synthesized a poly(ADP-ribose) affinity resin. Previous methods for making affinity resins entailed preparation of the appropriate ligand followed by its covalent linkage to a suitably activated resin. However, with this approach the researcher often has no control over ligand orientation with respect to the resin and consequently with respect to the target species. Since we wanted the poly(ADP-ribose) oriented on the resin in such a way as to simulate that found covalently modifying proteins, we attempted an enzymatic approach to affinity resin synthesis by using poly(ADP-ribose) polymerase and NAD^+- or (ADP-ribose)-agarose beads. Both resins were recognized as acceptors by the enzyme, which elongated the existing ligands to form polymers closely resembling those modifying proteins. Addition of ADP-ribose residues depended upon a) poly(ADP-ribose) polymerase activity, b) time of incubation, c) the concentration of free NAD^+ available as substrate, d) the amount of derivatized agarose, and e) the chemical moiety through which the ligand was linked to the agarose. Fractionation of rat liver nuclear lysate over the poly(ADP-ribose) resin revealed a strong affinity of H1 for ADP-ribose polymers. This resin can also be used to purify the catabolic analogue of poly(ADP-ribose) polymerase, poly(ADP-ribose) glycohydrolase, and to study polymer-binding proteins from other species. Such an enzymatic approach to synthesizing affinity resins, when possible, could improve binding efficiencies and capacities by optimizing ligand orientation.

X1-206 THE RAPID DEVELOPMENT OF HYDROPHOBIC INTERACTION CHROMATOGRAPHY PURIFICATION OF A MURINE MONOCLONAL IgG_{2a} $F(ab')_2$ FRAGMENT,
Douglas W. Rea, Terry L. Mayes, Michiel E. Ultee, CYTOGEN Corporation, Princeton, N.J. 08540
The new technology of perfusive chromatography (BioCAD) was applied to rapidly develop methods for purification of both a monoclonal antibody from ascitic fluid and the $F(ab')_2$ fragment from peptic digestion of the antibody. The purification of the $F(ab')_2$ fragment by hydrophobic interaction chromatography (HIC) required the most development work. As fragment was produced by peptic digestion of antibody in the presence of high salt, use of HIC for purification of fragment was a logical first step for the process.
Initially, the purification performance of three different media was tested, using several different salts and pHs. Of the three, POROS PE (phenyl ether) had the best resolution and flow characteristics. Among several salt solutions tested, phosphate-buffered sodium sulfate at pH 8 gave the best resolution of the $F(ab')_2$ fragment.
An initial linear gradient indicated the salt concentrations necessary to remove the impurities. A series of step gradient runs based on the linear gradient values finalized the conditions to be used.
These conditions were scaled up from 2 to 25 to 2100 mL size columns using bulk resin. Results were similar at all scales, with 70 to 80% yields. The only impurity detected was about 5% residual undigested antibody. While this had been removed by an analytical pre-packed column, this removal was less efficient with bulk resin. A small Protein A column prior to the HIC column completely removed residual antibody without any further buffer manipulation. The final $F(ab')_2$ fragment was pure by both HPLC-GPC and by SDS-PAGE analysis.
The development and scale up of the HIC purification took six weeks. The entire process, from receipt of ascitic fluid, through the development of both the purification of the whole antibody and the $F(ab')_2$ fragment, to the end of a 7.5g pilot scale run required only 3 months.

X1-207 AUTOMATED, CONTINUOUS ELUTION, MICROPREPARATIVE ELECTROPHORESIS: HIGH RECOVERY OF BIOLOGICAL ACTIVITY AND PROTEIN MASS,
Barnett B. Rosenblum, Ben F. Johnson and Iqbal M. Zaidi, Applied Biosystems, Inc., Foster City, CA 94404
Electrophoresis has been regarded as one of the best techniques for analysis of protein purity due to the high resolving power and versatility of the different electrophoretic procedures. However, electrophoresis has generally not found wide use in the preparative purification of proteins except for protein sequencing. Purification using slab gel electrophoresis is a time consuming process, due to the requirement for identifying the protein of interest (staining) and electroeluting the protein. Recovery of protein from SDS slab gels ranges from 5% to nearly 100% depending on the nature of the protein and the experience of the user. Recovery of biologically active proteins from either non-denaturing gels or from detergent containing gels tends to be poor and is used as a method of last resort. The use of either non-denaturing electrophoresis or SDS electrophoresis on the ABI Model 230A, an automated, continuous elution, micropreparative electrophoresis system, allows the purification of proteins with high recovery of biological activity and of protein mass. Proteins are swept from the bottom of the gel by elution buffer, through a sensitive UV detector and then delivered to a fraction collector. Acrylamide or agarose gels are used depending on the application. Using an acrylamide gel with tris-phosphate-SDS buffers, we evaluated the recovery of bovine serum albumin. We achieved greater than 90% recovery of the applied protein. High levels of recovery of enzymatic activity is desired from preparative non-denaturing gel electrophoresis. Recovery of *Aspergillus niger* amyloglucosidase from a tris-phosphate buffered acrylamide gel was evaluated. The two isozymes of amyloglucosidase were isolated and separated from each other with recovery of 100% of the enzyme activity in the eluted fractions. The Model 230A is a versatile automated electrophoretic system for the purification of proteins by size and/or charge, with high recovery of protein mass and biological activity.

X1-208 GENE REPLACEMENT LEADS TO AN *E. COLI* STRAIN WITH EPITOPE-TAGGED ESSENTIAL ENZYME: FACILE PURIFICATION OF MUTANT GLUTAMINYL-tRNA SYNTHETASE, Hans-Ulrich Thomann and Dieter Söll, Department of Molecular Biophysics & Biochemistry, Yale University, New Haven, CT 06511.

Purification of mutant enzymes is a prime requirement of biophysical and biochemical studies. For our investigations on the mechanisms of tRNA glutaminylation by the *E. coli* GlnRS we need to purify stable as well as labile mutant enzymes away from any contaminating wild-type protein. Therefore we constructed an *E. coli* strain in which the chromosomal *glnS* gene copy is replaced by an *HAI-glnS* fusion gene, leading to the expression of GlnRS which is tagged at its N-terminus with the antigenic determinant of the Influenza virus hemagglutinin (1,2). During the construction of the new strain, called HAPPY 101, we replaced the wt *glnS* gene of *E. coli* JC7623 via homologous recombination with the *HAI-glnS* gene construct bearing the gene for kanamycin resistance (3) within its close 5' flank. By selection for kan^r and further confirmation by Southern blot analysis we obtained clones with the correct gene replacement. As *E. coli* JC7623 is not recommended for use in plasmid amplification we transduced the *kan::HAI-glnS* gene locus via bacteriophage P1 to *E. coli* AB1157. Using monoclonal mouse anti-HAI antibodies purified from 12CA5 cell supernatants (2), S100 extracts from *E. coli* HAPPY 101 could be shown to contain only the HAI-GlnRS fusion enzyme. Extracts obtained from this strain can be depleted from the epitope-tagged enzyme by affinity chromatography using mouse anti-HAI mab sepharose. In a second affinity chromatographic step involving rabbit anti-GlnRS sepharose overexpressed mutant enzymes are purified to homogeneity.

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Finishing

X1-300 **RESTORATION OF MALONYLCoA SENSITIVITY TO CARNITINE PALMITOYLTRANSFERASE PURIFIED FROM RAT HEART MITOCHONDRIA**

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We previously demonstrated (Biochemistry [1990] 29, 4326-4334) that malonylCoA sensitive carnitine palmitoyltransferase (CPT) is associated with enzymes of the beta-oxidation complex when rat heart mitochondria are solubilized in octylglucoside. The investigations of the malonylCoA sensitive CPT activity have been extended. In addition to three of the four enzymes of beta-oxidation, this complex also contains the carnitine/acylcarnitine translocase protein with a molecular weight of 33,000 daltons in addition to three proteins which bind malonylCoA. When the beta-oxidation enzymes and other proteins associated with this complex are separated from CPT, addition of these proteins plus phosolipid to purified rat heart mitochondrial CPT confers malonylCoA sensitivity to CPT. Subfractionation of the malonylCoA binding proteins associated with this complex shows that the 86 kilodalton protein exhibits significant reduced binding of malonylCoA in the presence of 50 nM etomoxiryl-CoA; such data indicates that this protein is involved in conferral of malonylCoA sensitivity to purified CPT. For most experiments the combination of 5- nM etomoxiryl CoA plus malonylCoA is much more effective at exhibiting CPT than malonylCoA. These data show that CPTi can be inhibited by malonylCoA in the presence of malonylCoA binding proteins. These data indicate the malonylCoA binding proteins are regulatory subunits of a macromolecular CPTo complex.

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X1-301 **THE USE OF HIGH PERFORMANCE CAPILLARY ELECTROPHORESIS AS A VERSATILE TOOL IN THE PROTEIN CHEMISTRY LABORATORY**, D. Brazel R. Doenges, P. Hermentin, Research Laboratories of Behringwerke AG, D-3550 Marburg, FRG.

The advent of High Performance Capillary Electrophoresis (HPCE), especially the more recent available instruments with autosamplers, allows the rapid analysis of proteins and peptides with different modes. Results will be presented with respect to:

- the influence of different buffer compositions (cations, anions, modifier) in capillary zone electrophoresis (CZE) on the resolution and migration time of a protein,
- a comparison of the detection limits of protein in HPCE, ultrathin and vertical slab (1.5 mm) silver-stained SDS-PAGE, respectively,
- HPCE-isoelectric focussing (simplicity/reproducibility),
- the analysis of a peptide mixture in different capillaries varying the length, inner diameter and surface modifications, respectively,
- a CZE system to seperate F_{ab}-F_c split product from the intact Mab,
- the highly sensitive mapping of underivatized complex sialylated oligosaccharide structures (femtomole range) derived from glycoproteins by hydrazinolysis in a neutral buffer system.

X1-302 THE USE OF DISPLACEMENT CHROMATOGRAPHY TO SEPARATE VARIANTS OF RECOMBINANT DNA-DERIVED HUMAN GROWTH HORMONE, Rosanne C. Chloupek, William S. Hancock and Jana M. Jacobson*, Genentech, Inc., 460 Pt.San Bruno Blvd., S. San Francisco, CA, 94080 and *BioWest Research, P.O. Box 135, S. San Francisco, CA 94080.

The potential of displacement chromatography for the resolution of low levels (<5%) of closely related protein variants from a highly purified native protein was explored with a degraded sample of recombinant human growth hormone (rhGH). The rhGH sample was heated at 45°C for 30 days in the dry state. The displacement separation of 250 mg of the sample was achieved on an anion exchange support (DEAE TSK-5PW) and BioWest Research displacer A-5. Fractions were analyzed for degradation products by size exclusion chromatography coupled with low angle laser light scattering photometry (SEC-LALLS), HPLC analytical anion exchange chromatography, HPLC tryptic mapping and mass spectrometry.The displacement method successfully resolved a deamidated variant, an iso-aspartyl variant, the two-chain species and aggregates from native rhGH. The variants were also enriched to concentrations easily detected by the routine HPLC analytical methods available for rhGH characterization.

Displacement chromatography promises to be a powerful tool for isolation and characterization of closely related protein variants existing at very low levels in purified protein products. This was particularly demonstrated by the resolution of the iso-aspartate variant from native sequence rhGH, despite the very small difference in pKa between aspartic acid and beta-aspartic acid .

X1-303 STRUCTURE OF SELF-INCOMPATIBILITY GLYCOPROTEINS FROM THE FEMALE SEXUAL TISSUES OF AN ORNAMENTAL TOBACCO, *NICOTIANA ALATA*, Shaio-Lim Mau, David Oxley, Marilyn M. Anderson, Antony Bacic and Adrienne E. Clarke, Plant Cell Biology Research Centre, School of Botany, University of Melbourne, Parkville, Victoria 3052, Australia.

The female sexual tissues of the self-incompatible ornamental tobacco plant, *Nicotiana alata*, contain relatively high concentrations (up to 10 mg/ml) of an allelic series of self-incompatibility glycoproteins. These glycoproteins are secreted by cells of the central transmitting tract of the female stylar tissues. The glycoproteins have been isolated and structures of their N-glycosyl chains established by chemical analyses and NMR and FAB-MS. Genomic clones and cDNA clones corresponding to the protein backbones of these glycoproteins have also been isolated and sequenced. cDNA clones show conserved regions, particularly around two histidine residues, and a series of cystine residues. The clones have homology with the fungal RNases from *Aspergillus oryzae* and *Rhizopus niveus*. The isolated glycoproteins are RNases. There are also regions which are highly variable between different alleles - these are known as the hypervariable regions. Analysis of the genomic clones shows that in two clones corresponding to the S_2- and S_6-allelic glycoproteins, there is a single intron. This intron lies close to the major hypervariable region. The glycosylation sites are outside the hypervariable regions and some are conserved between different alleles. Different alleles bear different numbers of N-glycan chains.

X1-304 TWO-PHASE REGULATION OF THE CD4-p56^{lck} COMPLEX: ASSEMBLY AND AGGREGATION Monika Raab and Christopher E. Rudd Division of Tumor Immunology, Dana-Farber Cancer Institute, 44 Binney Street and the Department of Pathology, Harvard Medical School, Boston, MA 02115

p56^{lck} has been expressed in the baculoviral expression system in the presence or absence of its receptor CD4, and assessed for changes in enzymatic activity. The binding of *lck* was monitored by comparing *in vitro* kinase activity relative to the quantity of enzyme as detected by anti-lck immunoblotting. Enzyme was purified either by antibody affinity chromatography ,or DEAE chromatography, or directly assessed in whole cell lysates. Although several Mr forms of *lck* were expressed, an intermediate Mr form preferentially associated with CD4. Activity associated with the receptor bound form of the kinase was some 10-fold higher than that found in preparations of unbound kinase. These data indicate that the mere binding of p56^{lck} to CD4 is sufficient to activate the kinase and therefore indicate that the CD4-p56^{lck} complex is regulated differently from conventional tyrosine kinase receptors. The second stage of regulation was shown by the fact that antibody-mediated crosslinking further increased activity by 2 to 5 fold. We are presently investigating the molecular details of the mechanism of CD4-p56^{lck} regulation.

X1-305 PREPARATION OF CLINICAL GRADE RECOMBINANT PROTEINS, Bela J. Takacs and Marie-Françoise Girard, Department of Biology, Pharmaceutical Research-New Technologies, F. Hoffmann-La Roche Ltd, Basel, CH-4002, Switzerland

The preparation of large amounts of recombinant proteins in highly purified and well-characterized form that satisfy current regulations concerning the production of clinically acceptable therapeutic products has become a major task for protein chemists. High purity is essential since these proteins are produced in heterologous host cells that are rich in antigens and toxins. In this communication we present methods for the production of clinical grade malaria vaccine candidates expressed in E. coli by recombinant DNA technologies. The essential features of the purification protocol consist of (1) mechanical breakage of host cells and solubilization of the recombinant protein in 6 M guanidine hydrochloride; (2) ammonium sulfate fractionation; (3) metal chelate affinity chromatography in the presence of 6 M guanidine hydrochloride; and (4) cation exchange chromatography in the presence of 6 M urea. The use of undesirable chemicals (PMSF, DFP, TFA, acetonitrile, mercaptoethanol, etc.) was avoided rather than demonstrating their complete removal after the purification steps. An essential feature of the purification protocol described is the testing of chromatographic fractions not only for the presence of the recombinant protein in question but also for contaminating E. coli proteins. Elimination of fractions with high E. coli protein content was found necessary to obtain a final product that contained less than 0.01 % of host derived proteins. The recombinant proteins were renatured from 8 M urea by increasing the pH to 10.5 in the presence of glycine and EDTA, reduction with DTT, dilution to a protein concentration below 1 mg·ml^{-1}, and dialysis against saline. By using the four-step purification protocol described above we were consistently able to obtain recombinant proteins that contained less than 0.01 % of host derived proteins, less than 10 pg of DNA and less than 300 endotoxin units per dose of vaccine. The integrities of the proteins were greater than 95 % and the vaccine candidates contained less than 5 % of degraded or aggregated forms of the antigens.