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Recovery of Specific Proteins and Enzymes from Blood and Offal Part 1 – Aprotonin, Transglutaminase, Fibronectin and Related Proteins

Animal offal and blood contain a wide range of proteins that can potentially be recovered. Some of these have commercial value and the techniques for their commercial recovery have been identified.

Aprotinin

Aprotinin is a low-molecular-weight protein that is obtained from the pancreas, lung and blood. This protein is known commonly as trypsin inhibitor, Kalikrien inhibitor or pancreatic trypsin inhibitor. Its function is to inhibit the activity of some members of a group of enzymes known as proteases or proteolytic enzymes. Proteases are enzymes that digest proteins by catalysing the breakdown of the peptide bonds that hold the amino acids together. Aprotinin is comparatively stable to temperature and is commonly used to inhibit the activity of the subgroup of serine-type proteases.

This inhibition effect is important during isolation and purification of intact peptides and proteins, because the presence of undesired proteases usually results in greatly reduced yields of pure and active proteins. This is particularly true for proteins isolated from cellular extracts, since many cells and microorganisms secrete proteolytic enzymes when grown in vitro. The need for effective protease inhibition is therefore essential for proper isolation of purified proteins.

Market Opportunities

A wide range of protease inhibitors is available commercially. They are mostly effective at low concentrations, so a little goes a long way and, in general, they are all very expensive.

AMPC

Aprotinin is generally recovered from lung, although it can also be recovered from the pancreas and from blood. Lung is a cheaper source than pancreas and the level of aprotinin in blood is quite low. Therefore the source tissue of choice is generally bovine lung.

Aprotinin is used extensively as an additive to diagnostic kits to protect against protease inactivation and to prolong the shelf life. It is also used in therapeutics and it is sometimes used clinically to inactivate proteases released into the blood system of shock victims including patients after coronary attacks. It has been used as an additive in cell culture to protect against inactivation of media components. Aprotinin is a high-value generic-use product.

Yield of Product

About 5 grams of aprotinin can be recovered from 50 kg of fresh lung. This is equivalent to 25,000TIU of inhibitor activity. The commercial retail value of 5 grams of highly purified material is around $32,000. To manufacture a kilogram would require 10,000 kg of bovine lung. Recoveries from other species such as pig and sheep would be comparable to the yield from cattle.

Market access

Access to markets requires compliance with the US FDA regulations, the 'Harmonious European Union Code', the Japanese sponsorship approval system, or the GMP guidelines in Australia. Diagnostic application and veterinary use require the aprotinin to be manufactured according to GMP regulations.

Competitors

There is a wide choice of protease inhibitors on the market. General protection against protease effects requires the use of a mixture of inhibitors. Aprotinin has been a popular inhibitor by end users because it is stable and it is very effective. Artificial inhibitors have appeared on the market but have apparently not diminished the market for products such as aprotinin. Another factor that strengthens the value of natural products is the general compatibility of

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these products with cells grown in vitro. Aprotinin can be included in cell-culture medium whereas other synthetic material has a damaging effect.

Aprotinin has been cloned and its production by recombinant techniques could possibly take a share of the market.

Manufacture

The manufacture of aprotinin from lung involves extraction of the protein from minced tissue, separation of solids, clarification, ultrafiltration and concentration and fractionation by reverse-phase chromatography.

Extraction, removal of solids, clarification by alluvial filtration, and ultrafiltration are standard tasks that can be readily carried out by trained workers on a works site. Spent solids can then be easily added back to the rendering plant as no material (that is not of a physiological nature) has been added during the processing. Following concentration, the final yield volume is <10 litres from 100 kg of tissue.

The final fractionation has to be performed in a professional laboratory with graduate staff. This could be done on a works site, or the concentrated liquid could be transferred or sold to a further processor.

The procedure required is simple and effective, easy to perform and is suitable for the recovery of aprotinin from bovine, porcine and ovine lung. It requires the use of a standard processing space as typified in a boning room of a meat works. All the steps could be carried out by suitably trained meat-works staff.

Processing requires the same items of equipment that are required to process albumin from plasma and blood. This includes 'off the shelf' filtration equipment—rotary vacuum drum filter, polishing filter, ultrafiltration equipment. A facility to process one particular biochemical has the processing capability for most other biochemical products.

The 'primary' processing to the stage of a clarified extract would require a processing area that meets established food-processing standards. The purification would require a GMP facility that would be used as a multi-product processing area. Estimated costs are (1996 estimates +2% CPI pa):

- building 50m^2 at \$2,000/m^2 – \$108,000
- tanks, pumps plumbing – \$54,000
- ultrafiltration equipment – \$65,000
- HPLC chromatography unit – \$54,000.

A person with a good knowledge of biochemistry and processing should supervise the process. This person should have a science degree or equivalent qualification.

The estimated cost of manufacture of aprotinin would be \$25 per TIU of activity, based on a batch processing unit of 1000 kg of lung per 5-shift week. Since aprotinin commands a premium price on the market, the profit-to-cost ratio is potentially high.

Alternatively aprotinin can be removed from biological materials by affinity chromatography. In this technique glass matrices are used to stabilise affinity ligands that grab specific targeted compounds from biological solutions. With this method aprotinin could be recovered economically, directly from plasma, if it was carried out as part of a scheme to recover a range of value-added products using a linked series of affinity ligands in a flow-through configuration. About 10,000 TIU units of aprotinin activity could be recovered from 10 litres of plasma.

Transglutaminase (TGase)

Transglutaminase describes a class of enzymes that can bind protein molecules together. TGase does this by catalysing the formation of a bond or cross-link between glutamic acid (in one protein) and a primary amine (in another protein), hence the name (trans-glut-amin-ase from glutamic acid and primary amine). TGases are present in a variety of cells and body fluids.

TGase is present in bovine plasma where it catalyses the reaction in the blood coagulation cascade of reactions resulting in stabilisation of the fibrin clot by cross-linking. Plasma TGase has been isolated by a procedure which utilises ethanol precipitation, defibrination and heating, followed by ion-exchange chromatography. This procedure is time consuming, produces a low yield of product and would be difficult to transform to an economical large-scale process.

Existing applications

Applications for TGase have been reported in medical, nutritional and food-processing areas. TGase is an important component of fibrin glue, which has been used widely in Europe in surgical techniques. Its use in the United States has been limited to autologous plasma sources due to the risk of viral transmission associated with the use of human plasma. The development of synthetic surgical glue using purified components can minimise this risk. Replacement therapy for Factor XIII congenital disorders or acquired deficiencies consists of injections of placental Factor XIIIa. A source of purified TGase, free of blood components, would be of value for the treatment of these conditions. TGase has been reported to have beneficial effects in the treatment of optic nerve damage and has also been used as an immunosuppressant and an accelerant to the transport of pharmaceuticals through the skin.

Market Opportunities

With the increasing demand for high-quality functional proteins as food ingredients, chemical modification has been investigated extensively as a means of improving the nutritional and functional properties of traditional food proteins. Chemical modification has not been widely accepted by the food industry due to concerns over adverse food safety and nutritional effects. The use of enzymatic modification may eliminate many of these concerns, as enzymatic modification requires milder conditions and is unlikely to lead to the formation of toxic by-products. TGase has been utilised to cross-link proteins and incorporate amino acids into proteins to improve their nutritional quality and functional properties.

In the meat industry, utilisation of low-value cuts and trimmings in restructured and extended products may extend the yield of marketable products. Inter- and intra-chain covalent bonds contribute significantly to the tensile strength of these types of meat products. These bonds can be introduced by chemical

modification but this may not be readily acceptable under existing regulations. An alternative procedure involves the use of TGase to cross-link the proteins. An advantage of TGase is that polymerisation and the formation of gels is favoured at lower temperatures (albeit >20°C). Consequently, processing temperatures may be reduced during manufacture, thus maintaining the nutritional quality of the proteins. The use of plasma (as a source of TGase) in the manufacture of a restructured meat product has been patented.

Surimi, a minced fish product, is an ingredient in the manufacture of the traditional Japanese food, kamaboko, and in seafood analogues. It has been added to restructured meat products as a binding agent. Surimi has a strong gel-forming capacity due to the action of TGase present in the fish tissue. Surimi-like products have been produced from low-value cuts of poultry. TGase has also been incorporated in the manufacture of traditional surimi.

Edible films can be used as food coatings to retard bacterial spoilage, retain moisture and maintain product quality and functionality. Current procedures for producing edible films in general require the addition of plasticisers. The use of TGase would eliminate the need for such additives. Quality enhancers such as flavours, antimicrobial agents, antioxidants and food colours could be incorporated into the film to enhance the appearance and quality of the product. In addition, these films could provide an alternative to the current packaging materials used for food products. Edible films do not have the environmental effects of plastic. Increased cost penalties for plastic waste disposal in some countries is making the use of plastic film uneconomical in these markets.

Bacterial TGase has potential applications in the preparation of food films, as these enzymes are not dependent on calcium for activation. Consequently, calcium chloride, which can impart a bitter taste in food products, could be eliminated—resulting in improved organoleptic quality.

Commercial Developments

The Ajinomoto company has pioneered the use of transglutaminase for developing new food products and now sells a bacterial TGase as a stabilised powder mixture in Asia and in North America. The bacterial TGase has an advantage in that it is stable at low temperatures, requires less salt in the environment to perform and does not require activation.

Tgase from bovine plasma and other animal sources has been used in muscle-food preparation (restructured meats), the production of biofilms as protective wraps, and the production of tailor-made proteins such as emulsifiers.

Market access

Unfortunately, bacterial TGase is well established and has set the market expectation. Users, who are aware of the benefits of the bacterial enzyme, will compare it to the plasma TGase. If plasma TGase is to compete it will have to be marketed in the same simple, easy-to-use presentation that the Japanese now use for the bacterial enzyme. The existing patents that cover the use of plasma TGase and the problems associated with labelling blood products that has minimised the application of plasma for edible use in Australia, will need to be addressed before production of TGase could be considered.

Manufacture

Food-processing applications, biofilm production and many of the other uses referred to above do not require purified enzyme. Plasma TGase in a crude but active form can be removed from plasma by acid titration of ethanolic solutions of plasma. A mixture of fibrinogen, fibronectin, albumin and transglutaminase precipitates in the cold and the paste can be recovered by continuous centrifugation. The paste can be processed to recover the separate ingredients or it can be dried and packaged for use in enzyme formulations.

Table 1. TGase recovery from bovine plasma

Product	Yield	Formulation	Manufacture cost	Use
Crude (0.2% TGase)	5 kg/tonne of plasma	Spray dried powder mix	$50/kg	Food Bioresource
Pure TGase	4g/tonne of plasma	Freeze dried	$100–500/g	Scientific reagent Immunology

While the crude transglutaminase can be recovered simply using standard techniques, purification of the enzyme by batch processing could need to be shared by a meat works and a biotechnology company. Pure enzyme can be recovered by using immobilised antibody against TGase with the use of immobilised supports on glass beads. The enzyme can be recovered in high yield by using ligands on glass supports to recover the TGase directly from diluted plasma.

Estimated capital costs (1996 est. +2% CPI pa) for the recovery of crude transglutaminase are:

- building space 100m^2 – $216,000 (A standard area in a meat processing plant would be adequate)
- decanter – $49,000
- holding tanks and plumbing – $54,000
- for the recovery of TGase using continuous affinity ligands coupled to a plasma stream: affinity resin – $65,000; plumbing and tanks – $16,000.

If ovine plasma in food manufacture could be accepted by the consumers, crude ovine TGase could be used to supplement the gelling properties of plasma-derived preparations and avoid the market resistance that is faced with bovine products, although its use is not documented.

Fibronectin

Fibronectins are cell-surface and blood glycoproteins that promote adhesion of cells to the extra-cellular matrix. They are often included in culture medium for mammalian cells. Fibronectins act as cell-attachment factors, which are necessary for the growth of a number of anchorage-dependent cells. Fibronectin was first identified in plasma and is present in the co-precipitate that constitutes crude transglutaminase. The plasma level of fibronectin is relatively high at about 300 mg/mL and, as a result, plasma has proven to be a convenient source for its isolation.

The worldwide market for fibronectin is estimated to be of the order of $5m. The market is significant but not large. Recovery of fibronectin is therefore an incremental advantage for a plasma processor but not likely to be viable in itself.

Market opportunities

Fibronectin is predominantly used in cell culture. It is a mature product with major markets in North America and Europe.

Competitors

The need to add fibronectin to cell-culture medium may, for many applications, be offset by the addition of high levels of Foetal Calf Serum. The market for Foetal Calf Serum is often oversupplied—having some depressive effect on the price of fibronectin and other growth factors.

Manufacture

Classical approaches to the recovery of this protein use multi-step procedures; however, an immobilised ligand on a glass support has been used to avoid these steps and thereby enhance the recovery yield. The advantages of using a glass support derive from a combination of three main features: the matrix, the immobilisation chemistry and the immobilised ligand. Porous glass offers significant advantages for use in large scale, downstream processing compared to traditional materials. It is incompressible and very durable and does not shrink or swell in different solutions. It has a narrow pore-size distribution coupled with a large internal size area. Nonspecific binding is very low which allows many proteins to be recovered almost pure by just one single pass through the absorbent.

Fibronectin could be purified to homogeneity directly from plasma using this affinity system. High yields and consistent flux rates of plasma through the resin have been obtained. About 30g of fibronectin are yielded from 100 litres of bovine plasma using this technique, while maintaining high flux rates.

The fibronectin prepared in this manner is extremely pure (>99% protein purity) and biologically very active in proliferation tests with human fibroblast cells. Commercial recovery is possible using in-line capture-cartridge technology plumbed directly into the plasma supply line. The affinity cartridges can be plumbed into the feed line in parallel, and be changed on a fixed lifetime basis. This would involve removal of the spent cartridge, and replacement with an active cartridge. The cartridges could be stored at chiller temperature and subsequently the bound fibronectin could be recovered by elution from the cartridge, dialysis and freeze drying in a specialised laboratory.

This technology need not be difficult to operate but would have to be under the control of a person with a science qualification and would best be operated as a collaborative venture with a biotechnology company.

The cost of recovering the fibronectin is largely due to the cost of the resin, which is made with the appropriate ligand attached. A commercial system capable of producing kilogram quantities would require several cartridges. A 5-litre cartridge could bind 5 kg of fibronectin, which is enough to treat 17,000 litres of plasma. The affinity matrix, which is the material in the cartridge doing the capture, costs about $27,000 per cartridge. As the ligand slowly leaches off the support so the capacity decreases with time. Hence, costs for the treatment of plasma must include a write-down cost over the cartridge's lifetime. The estimated cost of manufacturing fibronectin is $20 per gram.

Capital costs to carry this out in an established co-recovery processing area would include the cost of the affinity matrix ($65,000), tanks, plumbing, metering equipment ($27,000). (1996 estimates +2% CPI pa)

As the plasma is not harmed when the fibronectin is recovered, other than the fibronectin being removed, it can be reused for the removal of other products and finally for the isolation of albumin.

Management of the production process and for the final product specification requires a range of analytical techniques. Most of these tasks can be performed in-house routinely and require simple equipment—but some expertise—to perform the tests. Sufficient understanding of basic biochemistry is required to interpret the results and to take corrective action. HPLC equipment is now quite cheap, is easy to operate and would cost about $15,000. Other tasks could be outsourced through veterinary pathology laboratories at reasonable charges.

Further reading

This information is a summary of information from the following project funded by the Meat Research Corporation.

- Project UGR.002: Value Added Proteins and Enzymes Recovered from the Meat Industry

Further detail is available from the final project report of this project which is available from Meat and Livestock Australia.

Related information is given in the following MLA Co-products brochure.

- Recovery of specific proteins and enzymes from blood Part 2 – Growth factors

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