final report

Project Code: A.BIO.0041

Prepared by: Philips Franks
Date published: July 2014

PUBLISHED BY
Meat and Livestock Australia Limited
Locked Bag 991
NORTH SYDNEY NSW 2059

Scientific and Technical Advisor to HVB program 2013-2014

Meat & Livestock Australia acknowledges the matching funds provided by the Australian Government and contributions from the Australian Meat Processor Corporation to support the research and development detailed in this publication.

This project retained Professor Milton Hearn of Monash University Centre for Green Chemistry to assist in the development of new directions for the high value bioactives initiatives. Work included preparation of a "Request for Innovation" (RFI) document calling for submissions for novel blood based bioactive products and applications, advice on the potential next steps in exploiting the chelating properties of haemoglobin and assisting in the delivery of the 2014 Bioactives workshop in Brisbane. The RFI included detailed and suitably referenced exemplars of a range of applications of blood fractions, designed to stimulate respondents to think outside the square. The key elements of the RFI follow:

TAKING INNOVATION TO THE NEXT LEVEL

Meat & Livestock Australia Limited (MLA) delivers marketing and research programs for Australia's cattle, sheep and goat producers. MLA has over 47,500 livestock producer members who have stakeholder entitlements in the company.

MLA conducts research and development (R&D) throughout the red meat supply chain to achieve the core activity of enhancing competitiveness and sustainability and to develop a competitive advantage for the industry.

MLA's R&D programs cover a range of on-farm and off-farm topics. The company also delivers a wide variety of extension and training opportunities. These programs are undertaken by MLA alone, or in partnership with government and industry.

The Vision of MLA is to be a respected provider of marketing and research and development services to the Australian cattle, sheep and goat industries.

The Mission of MLA is to create opportunities across the cattle, sheep and goat supply chains by optimising the return on collective investment in marketing and research and development

MLA has established a Red Meat Innovations programme and is seeking Expression-of-Interest applications from academic researchers, other research teams, consultants or inventors working in the field of bioactive ingredients to work with MLA and associated companies based within and external to the red meat industries and their supply chains. The objective is to develop innovative products and processes that add value, enhance competitiveness, improve productivity and achieve greater resource, energy, water and supply chain utilisation and integration.

As part of its Red Meat Bioactives Program, MLA seeks to facilitate the development of brilliant concepts and practices associated with the generation of value-added products and processes, irrespective of whether they involve new technologies for the extraction of valuable proteins and other biomolecules from blood or other recovered biofluids, production of industrial enzymes and other substances from animal tissues or alternatively used in the manufacture of a diverse range of industrial products, or materials derived from other biomass sources, e.g. bone, cartilage or other offal, hitherto considered to represent low value co-products or waste, in novel large scale ways as supply chain source materials incorporated into consumer products and applications.

Bioactives derived from red meat sources from cattle, sheep and goats represent important sources of value-added products of significant commercial potential within a well regulated

and dynamic market. Their genesis involves integration of scientific, technical, legal and business acumen and expertise. In many cases, the commercial imperatives still heavily favour the manufacture of red meat bioactives from animal tissues or fluids, rather than from chemical or recombinant DNA synthetic methods.

Red meat bioactives can be obtained from four basic types of animal tissues, namely connective, muscle, epithelial and nervous tissues. Assembly of multiple tissues into organs constitutes the body structures. Red meat bioactives can, as a consequence, be sourced from various biological fluids, bone, cartilage and connective tissue, offal and other muscle scraps, and from a range of specialised organs, such as heart, kidney, liver, lung, adrenals, stomach, intestines, pancreas, pituitary, thyroid, spleen, pineal, brain, spinal cord, eye and other glands associated with reproduction, digestion or other biological functions.

Typically, the recovery of a specific bioactive commences with the selection of a particular source material, such as a specific biological fluid, bone, cartilage and connective tissue, muscle or organ, based on some knowledge of the relative abundance of the bioactive within the component tissues that overall form the structural unit of an organ with a defined set of functional properties. All bioactives can, as a consequence, be classified in compositional terms, i.e. in terms of their molecular structures, or alternatively in terms of their functional status. Molecular structure includes elemental composition, physical form and chemical identity of the bioactive and is usually one of the earliest pieces of physico-chemical information about the bioactive that is discovered.

The functional status and attributes define how the bioactive interacts with or acts upon other substances, be they biological or non-biological in origin. When assessing the industrial potential of specific bioactives, information related to their abundance and the breadth and nature of their functions thus has much greater impact in determining their commercial relevance and potential uses. Applicants pursuing an Expression-of-Interest application may thus wish to emphasise their rationale how the functional utility and attributes of their chosen bioactive(s) can be most appropriately captured in its broadest sense, and not just limited to the physical or chemical properties of the bioactive. Such information about the functional properties provides the basis to better understand the application scope and potential of the bioactive(s) if it is (they are) to be placed on a proper commercial footing.

This potential was recognised by MLA through its Red Meat Bioactives Program over a decade ago, and has been enhanced through a number of subsequently sponsored initiatives and programs, leading to well-staged strategic developments associated with the direct use of the recovered bioactive products or alternatively their incorporation into the manufacture of other consumer or industrial products. In many cases, these developments take advantage of the ability to process so called ‘waste streams’, such as blood (plasma/serum), low value red meat(muscle) off cuts and offal, or bone and cartilaginous connective tissues as the source materials, by a variety of extraction, recovery, purification and processing technologies.

Initially, applications of red meat bioactives, e.g. the manufacture and use of dermatan sulphate, conjugated linoleic acids, globin fractions, etc., have focused on the nutraceutical and functional food markets. However, the unique physical, chemical and functional properties of bioactives from red meat sources provides numerous other opportunities for use in food manufacturing, the construction, building, cosmetic and consumer product

industries, whilst the emergence of new process technologies over the past decade, has also enabled the alternative uses of red meat bioactives at the higher value-added end of the market to be elaborated, such as in electrochemical sensors and optical monitoring scanners, the production of medical implant coatings, etc.

In these regards, one of the key drivers of the Red Meat Bioactives Program strategy established by MLA has been to create opportunities where the functional attributes of the bioactives can be best exploited through development of innovative products and processes. The following exemplars further illustrate the basis of this strategy, where advantage has been taken to capture these distinctive differences between the structural features and the functional attributes of bioactives.

One of the most specialised tissues that can be readily sourced within the red meat industry is blood. Typically, blood represents about 7-8% of body weight of a healthy cow, sheep, pig or goat. The mass density of bovine blood is ~1.05 g/cm3. Blood contains a variety of cellular, e.g. erythrocyte (red) cells, leucocyte (white) cells, and platelet (thrombocytes), components and plasma. Each of the cell types in blood can be independently sub-fractionated and used as a source of specific bioactives, e.g. haemoglobin, superoxide dismutase or catalase from erythrocytes. Blood plasma, and plasma lacking the factors associated with coagulation (i.e. serum), provides rich sources of other bioactives, probably in excess of 40,000 different molecules. Normally, plasma represents about 55% of the blood volume. Besides the presence of numerous low molecular weight saccharides, lipids, steroids, vitamins, hormones, other chemical substances and inorganic compounds, plasma (and serum) contains a diverse range of much higher molecular weight enzymes, antibodies, other proteins and other bioactive molecules..

Typically, bovine plasma contains ~90% water by weight, with 4 proteins, albumin, globulins, lipoprotein and fibrinogen representing ~ 7-8 % of the remaining weight percentage. The emulsifying capacity, foaming properties, water retention capability and rheology of plasma are direct consequences of its protein, lipid and polysaccharide composition. These fluid mechanical properties show pronounced pH and ionic strength dependencies, with a local activity minimum occurring when the pH is close to the pI of the most abundant protein, albumin. Similarly, the apparent dynamic viscosity of plasma also changes with pH, reaching a maximum between pH 5.5-6.0 in the range □ = 0.60-0.95 Pa s at low shear rates. Numerous applications already exist in the food manufacturing industry related to the emulsifying and foam capacity of plasma (and serum). Can these properties of plasma (or serum) be exploited in other new ways? A high impact outcome from a successful Expression-of-Interest Application could lead to the development of new approaches to exploit the rheological or mechanico-chemical functions of plasma (serum) or other biological fluids sourced from red meat animals in existing or new fields of application.

Similarly, the cellular components present in blood or their derived proteins have found application as binders or bio-adhesives, in the development of new formulations of natural colour enhancers or as iron supplements, to name just three examples. Undoubtedly numerous other innovations related to adaptive food manufacturing with the cellular components in blood and their derived proteins can be contemplated. Moreover, other uses, ranging from the generation of cellular concretes with atomised haemoglobin pellets, the manufacture of pharmaceutical products with specific blood cell-derived factors, the use of spray dried plasma as a protein supplement for early weaved calves and piglets, to the

production of amino acid based liquid fertilisers, are all indicative of the multiplicity of functional uses and potential product options available from this raw material. Again, innovations relevant to these or other new fields of use of blood, blood cells or plasma(serum), preferably involving a minimal number of handling steps, could fall within the scope of successful Expression-of-Interest applications.

Bovine serum albumin (BSA) is an abundant multifunctional, non-glycosylated, negatively charged plasma protein, with multiple ligand-binding and transport properties, antioxidant functions, and other biological activities. In healthy animals it accounts for ~75% of all protein molecules in plasma. BSA is a globular (prolate ellipsoidal) molecule of dimensions 140×40×40 Å3, containing 583 amino acid residues, with a molecular weight of 66,463 Da, an isoelectric point of 4.7 in water at 25 °C and an extinction coefficient of 43,824 M−1cm−1 at 279 nm. It contains 17 intra-chain disulfide bonds and 1 free redox active sulfhydryl group, which accounts for ~80% of the thiol activity in plasma. From a physiological perspective, BSA maintains cellular osmotic pressure and microvascular integrity and has a major role in cell adhesion and the generic control of cell signalling substances.

From a functional perspective, the low and high affinity sites in BSA are responsible for binding to many exogenous and endogenous compounds, including fatty acids, metal ions, pharmaceuticals and their metabolites and numerous other compounds. These functional attributes result in BSA having significant roles in drug delivery and efficacy, detoxification, and antioxidant properties including ROS scavenging. Because of its buffering and osmotic regulatory functional properties, BSA has been used clinically in veterinary practice as a plasma expander. As a reagent, BSA has numerous biochemical applications including uses in diagnostics and immunochemical assays, both as a protein standard and additive to minimise non specific binding events. In microbiological, cell and tissue culture or other laboratory applications and protocols, such as improvement in RT-qPCR detection of foodborne viruses on vegetable surfaces, its main functional role, when protein supplementation is necessary but the presence of other components of plasma/serum is unwanted, is to act as an excellent carrier of low molecular weight polar or basic compounds.

Depending on the required level of purity and quality, BSA due to its functional properties has also been used in the manufacture of food products and beverages, cosmetics, and other products derived from or used in industrial biotechnology or other fields of the life sciences, including the production of pharmaceuticals and enzymes. Sulfhydryl group rich peptide fractions derived from BSA have found functional applications as food supplements to support muscle injury recovery and the immune system. Besides these traditional areas of application, BSA, because of its charge group functionality, is increasingly finding other areas of more specialised application including, e.g. uses with gold nanoparticles and nanorods, allowing plasmonic light adsorption properties to be harnessed and tuned for various applications, including personalised, recyclable sanitation and water purification systems.

Numerous methods have been developed for the purification of BSA from plasma or serum, ranging from the classical Cohn Fraction V approach to more sophisticated affinity chromatographic procedures. These extraction methods lead to BSA preparations of different grade, quality and purity, including BSA essential free of deoxyribonuclease and other enzymatic activities. Clearly, further fields of application and modes of recovery and purification of BSA and albumins from other red meat species can be contemplated. A high impact outcome from a successful Expression-of-Interest application could be more cost

effective and efficient procedures for the purification of BSA or other proteins present in bovine plasma (serum) or other sources such as placenta or alternatively new innovative uses of these molecules. The potential for extension of these technologies and product outcomes to plasma (serum) or components present in related tissues obtained from other red meat species would also be relevant.

Red meat tissues contain a large variety of enzymes with diverse functionalities. Many of these proteins are present in relatively high abundances, often preferentially located within or secreted by a specific tissue, which thus facilitates their recovery. These proteins fulfil important functional roles, classified according to whether that act as hydrolases, oxido-reductases, transferases, lyases, isomerases or ligases. Within each classification, numerous exemplars exist with important attributes for use in research or industrial applications. For example, the haem-containing oxidoreductase, catalase, which can be obtained from bovine liver, has the functional capability to decompose hydrogen peroxide to water and oxygen. As a consequence, catalase finds numerous functional applications not only in biochemical assays and biosensors, but more importantly in industrial applications in the cheese manufacturing, textile bleaching and waste sludge pre-treatment industries.

Similarly, the serine proteases, besides their importance in different fields of in vitro biochemical and life science research, in diagnostics or disease monitoring, are responsible in vivo for co-ordinating key physiological functions, including digestion, blood coagulation, reproduction and the immune response. Representative examples of the serine proteases are the pancreatic chymotrypsin, trypsin or elastase and the blood derived enzyme, thrombin. A common functional feature of the serine proteases is their ability to cleave peptide bonds in proteins, exploiting the characteristics of a catalytic triad, involving three essential amino acids: histidine, serine and aspartic acid juxta-positioned in the folded structure of the protein to form the catalytic site. Since more than 1500 different commercially relevant enzymes, falling within the 6 enzyme categories, are currently known to exist in different bovine tissues, it is not surprising that these proteins individually or in tandem have the capability to facilitate a multiplicity of different chemical reactions that have industrial manufacturing relevance. For example, the functions of lingual or pancreatic lipase, which also utilises the catalytic triad of aspartic acid, histidine and serine, permit the hydrolysis of long-chain triglycerides into partial glycerides, mainly diglycerides, and free fatty acids. When either free or immobilised, these lipases have scope for applications as biocatalysts for the conversion of, inter alia, used vegetable-based cooking oils into fuels, in various areas of food manufacturing, including baking and flavour enhancement in cheese manufacturing, when controlled release of very long chain polyunsaturated fatty acids from fish oils is required, and in the synthesis of organic compounds where a high level of regio-selectivity is essential, to mention several applications.

Because of its functional ability to cleave peptide bonds in proteins, trypsin, expressed as the inactive zymogen, trypsinogen, has similarly found increased usage in the free state or when immobilised, e.g. as chitosan gels, as important biochemical products and in various biotechnological processes, including tissue engineering and the production of recombinant proteins, in addition to its more traditional applications to add value to food products, such as processing of sweet whey or fish protein hydrolysates, skin removal and roe processing, as an auxiliary in the tanning and bating of different types of leathers, in the recovery of various carotenoid pigments and flavouring compounds, and a large variety of biochemical and cell culture procedures.

The above enzyme exemplars are representative of the product and biological functionality diversity of red meat sourced enzymes. Clearly, numerous other possibilities and options exist for innovation in the extraction of the above illustrative enzyme examples, or other commercially relevant enzymes selected from within the six enzyme categories. A high impact outcome from a successful Expression-of-Interest application could lead to new cost-effective technologies for the recovery of specific enzymes from red meat tissues or, alternatively, new value-adding product applications where the functional attributes of these classes of proteins are innovatively employed.

Besides opportunities for new technologies and product differentiations to be garnered with proteins from red meat sources, other compound classes also represent viable commercial options. Conjugated fatty acids, including the conjugated linoleic acids (CLAs) and sterols, are examples of these other classes of compounds where significant commercial opportunity exists, due in part to their anti-carcinogenic, anti-oxidative and weight management functional properties or potential as chemical precursors in the synthesis of other nutraceuticals or pharmaceuticals. Collectively, CLAs includes all octadeca-dienoic acids with conjugated double bond structures, i.e. the 56 geometric isomers with different cis or trans configurations, with the cis9-, trans11- and the trans10-, cis12-CLA isomers the most abundant in meat. CLAs are biosynthetically derived mainly in the rumen of cows by the isomerisation of dietary linoleic acid by the enzyme, linoleic acid isomerase, generated by Butyrivibrio fibrisolvens, or by the desaturation of trans-fatty acids in adipose tissue or the mammary gland. All meat lipid sources, e.g. muscle or adipose tissues, can be used as a source material for the extraction and recovery of these molecules. Because CLA's have been given the "Generally Recognised as Safe" (GRAS) status by various national regulatory authorities, including the United States Food and Drug Administration, the use of CLA in food products has become widespread with high levels of industrial demand. Purity and yield are thus essential commercial requirements. Similar considerations apply to the sterols that can be obtained from bovine or other red meat tissues and organs. A high impact outcome from a successful Expression-of-Interest application could lead to the development of new cost-effective high yield technologies for the extraction of these molecules, with minimal isomerisation or structural change, and avoidance of the use of harsh conditions for their recovery.

Also included in these classes of non-protein bioactives are the sulphated glycosaminoglycans (GAGs), collectively known as "chondroitin sulphates", which typically involve a chain of more than 100 alternating monosaccharide units involving D-glucuronic acid (GlcA) and D-N-acetylgalactosamine (GalNAc)), repeated as an unbranched polysaccharide chain, sulphated at variable positions. A major source of the chondroitin sulphate molecules is bovine trachea, which contains on a dry weight basis about 10% of these GAGs and other related complex polysaccharides. Depending on the site of sulphation five discrete structural forms are known (chondroitin sulphate A-E, with chondroitin B also known as dermatan sulphate). This structural diversity of the chondroitin sulphates underpins their functional properties and differentiates these molecules and their end-use applications.

From commercial and abundance perspectives, chondroitin sulphate A (sulphated at the carbon 4 of GalNAc, i.e. chondroitin-4-sulphate) and chondroitin C (sulphated at the carbon 6 of GalNAc, i.e. chondroitin-6-sulphate) possibly represent the more important targets currently as dietary supplements in humans for the treatment of osteoarthritis and as

veterinary products. Two other GAGs, one with sulphate substituents arrayed in complex and variable yet cell-specific patterns, keratan sulphate, found in bone, cartilage, cornea and other neural tissues, and heparan sulphate, a non-sulphated GAG, found in kidney, brain, intestinal mucosa, aorta, and virtually all cells in the body, are finding increasing application in cell biology, veterinary medicine and disease monitoring due to their cell adhesion properties and more specific functions in blood (anti-thrombolytic) coagulation and regulation of growth factor signalling.

Besides the claimed functional benefits of these GAGs as nutraceuticals and their established use in enzyme-linked immunochemical kits and cell culture procedures, indicative potentials also exist in cosmetics manufacture and clinical applications, including use in regenerative medicine, the treatment of thrombophlebitis or micro-angiopathy, as a carrier for delivery of tumour-targeted therapies, and roles in the control of food intake, gastric acid secretion, energy homeostasis, tissue 3D printing research, and as glyco-drug precursors in haematopoietic stem cell transplantation.

The structural polydispersity of these GAGs, and their fundamentally different functional roles thus should provide ample opportunities for the development of new technologies for their isolation by processes, which enable enhanced efficiency at the industrial scale and at the required level of purity and yield to meet the requirements of GMP manufacturing and to satisfy current national regulations for use either a prescription medicines or as FDA approved dietary supplements. A high impact outcome from a successful Expression-of-Interest application could be manifested as new platform technologies for the extraction of these molecules and their use in additional application fields, including those outside traditional fields of use.

The basis of the strategic alignment for the commercial development of various bioactives accessible from red meat sources, preferably based on the establishment of innovative, proprietary platform technologies, with the MLA Vision and Mission has been summarised in a variety of MLA Reports including ‘Realising the potential’1, ‘Evaluating our opportunities’2, ‘Bioactives – Bioprocessing cost estimation’3, ‘Seizing

the Opportunity’4, and ‘We can do this’5, whilst the attributes of various bioactive products have been detailed in the MLA Bioactives Compendium and associated documents6,7,8. These developments have been driven by major shifts over the past decade by the public

1 MLA Workshop Report: Realising the potential, 25 November 2005. http://www.mla.com.au/redmeat-innovation/project-reports/report-categories/co-products/bioactives.

2 MLA Workshop Report: Evaluating our opportunities.1-2 June 2006. http://www.mla.com.au/redmeat-innovation/project-reports/report-categories/co-products/bioactives.

3 MLA Workshop Report: Bioactives – Bioprocessing cost estimation. 19-20 April 2007. http://www.mla.com.au/redmeat-innovation/project-reports/report-categories/co-products/bioactives.

4 MLA Workshop Report: Seizing the Opportunity. 9-10 October 2009http://www.mla.com.au/redmeat-innovation/project-reports/report-categories/co-products/bioactives.

5 MLA Workshop Report: We can do this. 11-13 October 2011. http://www.mla.com.au/redmeat-innovation/project-reports/report-categories/co-products/bioactives.

6 MLA Bioactives Compendium. http://www.redmeatinnovation.com.au/project-reports/report-categories/co-products/bioactive-opportunities-for-the-Australian-red-meat-industry, (2011) ISBN 9781741915457, 1-27.

7 MLA Workshop Reports: Bioactive Choices, Business Models and Breakthrough Technologies for the Sustainable Manufacturing of High-Value Added Bioactive Products from Red Meat Sources: Part I-IV. http://www.mla.com.au/redmeat-innovation/project-reports/report-categories/co-products/bioactives, 1-33

8 MLA Meat Technology Update: Bioactives, Nutraceuticals and Functional Foods. http://www.mla.com.au/TopicHierarchy/InformationCentre/Coproducts/Bioactives/default.htm, 1-8

demand for improved quality of life, better health outcomes, greater productivity and improved industrial responsiveness to environmental issues that are linked to the increasing community concern about the long term sustainability of petrochemical-based products that are dependent on continued availability of suitable raw materials and access to low power, water and compliance/ regulatory costs. Bioactives from red meat sources provide one avenue for different industry sectors, including the food, consumer product and cosmetic industries, to progressively move towards these more sustainable targets.