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Bioactivity of β-Lactoglobulin and α-Lactalbumin – Technological Implications for Processing

Dereck E.W. Chatterton[1], Geoffrey Smithers[2], Peter Roupas[2] & André Brodkorb[3]*

[1] Arla Foods Innovation, Gustav Wieds Vej 10C, 8000 Aarhus C, Denmark,

[2] CSIRO-Food Science Australia, Private Bag 16, Werribee, Melbourne, Victoria 3030, Australia

[3] Moorepark Food Research Centre, Teagasc, Moorepark, Fermoy, Co. Cork, Ireland.

* Corresponding author: Telephone: +353-25-42222; Fax: +353-25-42340; E-mail: andre.brodkorb@teagasc.ie

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Abstract

The dairy industry faces new technological challenges in order to exploit and maintain some of the bioactive properties of dairy components throughout processing. This review outlines these issues with respect to the two major whey proteins β-lactoglobulin (β-lg) and α-lactalbumin (α-la). Biological activities of both the intact proteins, and peptides derived from the proteins, are discussed e.g. inhibition of angiotensin-converting enzyme (ACE), anti-microbial activity, anti-carcinogenic activity, hypocholesterolemic effect, metabolic and physiological effects. The levels necessary to provide beneficial effects and, if available, evidence from clinical trials are reported. Developments in the purification and enrichment of the proteins are discussed, and the technological implications of industrial processing on the bio-activity of the proteins are examined. The supplementation of infant formulas with α-lactalbumin enriched whey proteins is also discussed in light of its potentially improved bioactive properties.

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Introduction

Whey represents a rich and heterogeneous mixture of secreted proteins with wide ranging nutritional, biological and food functional attributes. The main constituents are β-lactoglobulin (β-lg) and α-lactalbumin (α-la), two small globular proteins that account for approximately 70% to 80% of total whey protein. Historically, whey has either been considered a waste product and disposed of in the most cost-effective manner, or processed into relatively low-value commodities such as whey powder and various grades of whey protein concentrate/isolate (WPC, WPI). Isolation of whey proteins as spray-dried whey powder and, in more limited quantities, as whey protein concentrate/isolate has realized only a small portion of the commercial potential of these proteins. Indeed, whey protein concentrate, once heralded as a value-added outlet for whey solids, is now considered a commodity item. In addition, whey protein-based products have an unfortunate record of inconsistent and unreliable performance in food systems. Thus, expanded utilization of whey proteins will rely on exploitation of individual whey proteins and their derivatives as products with increased nutritional, functional, and/or biological value and thus, increased commercial value to the dairy industry. The emergence of new technologies and methods give a fresh insight into the bioactivity of these proteins and produce new and sometimes surprising results. This review examines the bioactive properties of β-lg and α-la and derived peptides thereof, as well as laboratory- and industrial-scale methods for their enrichment and/or purification.

A) β-Lactoglobulin

Background

β-Lg is the dominant non-casein protein in bovine milk and is found in the milk of most ruminants, but has generally been reported to be absent from human breast milk, although some reports have suggested that minor amounts do occur in human milk (Hambraeus & Lonnerdal, 2003). β-Lg is a small, soluble and globular protein, with a monomer molecular weight of about

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18 kDa at a pH of < ~3. At a pH of between 3 and 7, which includes the pH of Cheddar cheese whey, β-lg exists in solution as a dimer (Creamer & Sawyer, 2003) with an effective molecular weight of about 36 kDa. β-Lg is the major bovine whey protein and generally accounts for ~50% of the total whey protein in ruminants and ~10% of the total protein in bovine milk (Creamer et al., 2003).

β-Lg has a variety of useful nutritional and food functional characteristics that have made this protein and β-lg containing whey protein products, ingredients of choice in the formulation of modern foods and beverages. However, it is the various bioactivities that are increasingly being associated with β-lg and its peptide fragments that are capturing the imagination of food scientists and technologists, particularly when linked with the other functionalities of the protein. Exploitation of these functionalities will rely upon cost-effective processing and isolation technologies that will deliver β-lg-enriched ingredients with maximum performance, both food functional and bioactive, and substantiation of the putative bioactivities, particularly in real food systems.

Food functional characteristics

β-Lg has excellent heat-set gelation characteristics (Holt, 2000). As such, ingredients enriched in this protein find application in areas where water binding and texturisation are required. Examples include manufactured meats and small goods, reformed fish products and a variety of formulated foods. The nature of gels formed from β-lg can also be simply manipulated through control of chemical conditions (e.g. pH and ionic strength) during gelation (Dufour, Robert, Renard & Llamas, 1998). Thus, heat-set gels of β-lg can be formed that are translucent or opaque, and elastic or inelastic. This 'flexibility' in gel formation by β-lg expands the range of applications in which an ingredient enriched in this whey protein can be used.

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β-Lg shows excellent whippability and thereby provides an alternative to egg albumin (egg white) in some food applications. For example, β-lg shows a foam overrun capacity and heat stability equivalent to egg white, even in the presence of sugar. Thus, an ingredient enriched in β-lg should serve as a cost-effective substitute for egg white in meringues and similar products. The foaming properties of whey and egg white proteins and their performance in food applications has recently been reviewed (Foegeding, Luck & Davis, 2006).

β-Lg shows high solubility and clarity over a broad pH range, particularly at low pH (> 97%, pH 3), and is stable to high temperature treatment under these conditions. The protein has a high nutritional value as reflected in an essential amino acid profile comparable to that of egg white. These properties of β-lg have facilitated its use as the active agent in various protein-fortified beverages, such as fruit juices and sports drinks, and in varieties of these beverages with long shelf-life.

Purification and enrichment procedures

A variety of laboratory and industrial-scale procedures for isolation of β-lg (and the other major whey proteins) have been available for some time (Conti, Napolitano, Cantisani, Davoli & Dall'Olio, 1988; Korhonen, Pihlanto-Leppala, Rantamaki & Tupasela, 1998). These procedures all rely upon one or other, or a combination, of the physical and chemical properties of the β-lg protein molecule. Preferential precipitation of β-lg at its isoelectric point, after concentration of the whey source material using ultrafiltration and subsequent demineralisation by diafiltration or electrodialysis, forms the basis of the earliest commercially feasible methodology (Pearce, 1987; Bramaud, Aimar & Daufin, 1997). Selective precipitation of β-lg (and α-la) can also be achieved through the addition of $FeCl_3$ to whey at an appropriate pH, and this phenomenon forms the basis of an alternative fractionation technology (Kuwata, Pham, Ma & Nakai, 1985). Unfortunately, such procedures are not readily amenable to commercial scale-up or to the isolation of tonne quantities of the β-lg isolate, and they can also severely compromise the food functional and/or

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bioactive properties of the isolated protein. However, Bounous and co-workers (1990, 1991, 1994, 1996) have described processes (in patents) for the production of undenatured whey protein concentrates (containing β-lg and α-la) that have a variety of biological actions. These patented processes are primarily based on microfiltration and ultrafiltration methods used in isolation, or in combination.

The growing demand by food manufacturers for cost-competitive and multifunctional ingredients means that the choice of processing/isolation technology for their manufacture is becoming increasingly critical. For these reasons several alternative procedures have been proposed and developed for industrial-scale isolation of β-lg. The most promising of these include liquid chromatography (Ayers & Petersen, 1985; Skudder, 1985; Ayers, Elgar, Palmano, Pritchard & Bhaskar, 2002), and the afore-mentioned methods of selective aggregation and precipitation of α-la from a whey source concentrated by ultrafiltration, under specified conditions of pH and temperature, leaving β-lg in solution and unaffected by the pH/temperature treatment.

Biological activity

Inhibition of Angiotensin-Converting Enzyme (ACE) activity

Angiotensin-converting enzyme (ACE) plays a major role in the regulation of blood pressure and thereby hypertension. Various peptides derived from proteolytic digestion of β-lg have been shown to have inhibitory activity against ACE. It has been shown that unhydrolysed β-lg had very poor ACE inhibitory activity (Mullally, Meisel & Fitzgerald, 1997a, 1997b), but that digests of the protein, generated using pepsin, trypsin, chymotrypsin, or other commercially available proteases, resulted in high ACE inhibition indices (i.e. 73-90%). Furthermore, these workers showed that the active peptides were usually short (< 8 amino acids) and could be enriched from a mixture of protein and other peptides using ultrafiltration with low molecular weight cut-off membranes (Mullally et al., 1997a). A tryptic peptide of β-lg (amino acids 142-148) was further characterized following reversed-phase chromatographic isolation and shown to have an ACE IC_{50} value of 42.6 nM (Mullally et al., 1997a). Similarly, several researchers have demonstrated

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that a number of β-lg-derived peptides have impressive ACE inhibitory activity using a variety of in vitro assay techniques (Abubakar, Saito, Kitazawa, Kawai & Itoh, 1998; Vermeirssen, Deplancke, Tappenden, Van Camp, Gaskins & Verstraete, 2002; Vermeirssen, Van Camp & Verstraete, 2002). In a study where whey proteins were treated with different lactic acid starters and digestive enzymes, it was reported that two peptides from β-lg (amino acids 9-14 and 15-20), following hydrolysis with trypsin or pepsin, and characterization by amino acid and MS-analysis, had ACE inhibitory activity (Pihlanto-Leppala, Rokka & Korhonen, 1998). Four novel ACE-inhibitory peptides have been reported from caprine β-lg, following hydrolytic treatment with thermolysin and purification (Hernandez-Ledesma, Recio, Ramos & Amigo, 2002). It has been demonstrated that a tetrapeptide isolated from β-lg (amino acids 142-145; Ala-Leu-Pro-Met), termed 'beta-lactosin B', had significant anti-hypertensive activity when administered orally to spontaneously hypertensive rats (SHR) and therefore had potential as a natural anti-hypertensive agent for inclusion in foods (Murakami et al., 2004).

Anti-microbial activity

Anti-bacterial effects: Proteolytic digestion of bovine β-lg by trypsin has been reported to yield four peptide fragments (amino acids 15-20, 25-40, 78-83 and 92-100) with bactericidal activity (Pellegrini, Dettling, Thomas & Hunziker, 2001). These peptides have been isolated and characterized, and found to exert their anti-microbial effects against Gram-positive bacteria only. Modulation of the peptides via targeted amino acid substitution expanded the bactericidal activity of the peptides to include the Gram-negative organisms Escherichia coli and Bordetella bronchiseptica. The authors concluded that β-lg may exert an anti-microbial function in vivo after its partial digestion by endopeptidases of the pancreas, and that small targeted modifications in the sequence of these peptides could be useful in expanding their anti-microbial function (Pellegrini et al., 2001). Peptide fragments of β-lg, generated through the action of alcalase, pepsin or trypsin, have been shown to be bacteriostatic against E. coli, and against pathogenic strains of E. coli, Bacillus subtilis and Staphylococcus aureus (Pihlanto-Leppala, Marnila, Hubert,

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Rokka, Korhonen & Karp, 1999; El-Zahar, Sitohy, Choiset, Metro, Haertle & Chobert, 2004). For example, the activity of E. coli JM103 in the presence of 25 mg mL^{-1} β-lg (or α-la) hydrolysed with pepsin and trypsin was only 21% of the control after incubation for 6 h (Pihlanto-Leppala et al., 1999). By contrast, the intact β-lg species did not show any anti-microbial activity even at concentrations as high as 100 mg mL^{-1}. It was also shown that ultrafiltration through 10 kDa and 1 kDa molecular mass cut-off membranes may be used to enrich the bacteriostatic properties of the β-lg-derived peptides (Pihlanto-Leppala et al., 1999).

Anti-viral effects: Heterosexual transmission of human immunodeficiency virus type 1 (HIV-1) is the major cause of the ongoing AIDS epidemic worldwide, and application of chemical barrier methods is expected to contribute to control of this epidemic. Several studies have reported that β-lg, chemically modified with 3-hydroxyphthalic anhydride to form 3-hydroxyphthaloyl-β-lg, is effective in inhibiting HIV-1, HIV-2, simian immunodeficiency virus (SIV), herpes simplex virus types 1 and 2, and Chlamydia trachomatis infection in vitro. The authors of these reports conclude that the modified β-lg may be effective as an inhibitor of HIV-1 infection in humans (Berkhout, Derksen, Back, Klaver, de Kruif & Visser, 1997; Neurath, Debnath, Strick, Li, Lin & Jiang, 1997a, 1997b; Wyand, Manson, Miller & Neurath, 1999; Oevermann, Engels, Thomas & Pellegrini, 2003). β-Lg has also been shown to inhibit the replication of rotavirus in a dose-dependent manner (Superti, Ammendolia, Valenti & Seganti, 1997).

Pathogen adhesion effects: The inhibition of microbial adhesion may prevent colonization of pathogens at an early stage of infection, and thus prevent or reduce the impact of the infection. The effect of β-lg on adhesion of pathogens to human ileostomy glycoproteins has been the subject of another study (Ouwehand, Salminen, Skurnik & Conway, 1997). It was found that adhesion of pathogenic strains of Klebsiella oxytoca and E. coli was inhibited by pre-incubation of immobilized ileostomy glycoproteins with β-lg in a concentration dependent manner. Further, the disulfide bridges in the β-lg molecule appear to be important to this activity, and the

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192 inhibition of pathogen adhesion appears to be mediated by β-lg binding, at two distinct sites, to
193 the immobilized ileostomy glycoproteins. High heat-treatment of β-lg appears to adversely affect
194 this anti-adhesion activity of the protein (Ouwehand & Salminen, 1998).
195
196 ***Anti-carcinogenic activity***
197 Whey proteins, including β-lg, have been implicated in providing protection against development
198 of cancer in animal models when delivered orally. Such activity has been investigated in order to
199 establish the role of these proteins in disease prevention, and to contribute to a basis for their
200 inclusion as ingredients in functional foods. Animal feeding trials have compared the efficacy of
201 dietary whey proteins in retarding chemically induced colon cancer in a rat model of the disease.
202 Dairy proteins, in particular whey protein, were found to be efficacious in retardation of intestinal
203 tumours in young rats compared with other dietary proteins (meat, soy) (McIntosh, Regester, Le
204 Leu, Royle & Smithers, 1995). Results also suggested that diets supplemented with β-lg
205 enhanced protection against development of putative tumour precursors (aberrant crypts) in the
206 hind gut wall. The mechanism behind the apparent anti-cancer activity of dietary whey proteins
207 in these studies may be related to their sulphur amino acid content, for which there is a high
208 requirement in the rat, and hypothesized role in protecting DNA in methylated form. In a parallel
209 study, a number of potential functional foods containing whey protein (flavoured milk, pasta, ice
210 cream, dessert pudding, muesli, and savoury dip) have been developed in preparation for human
211 clinical trials. The foods containing whey protein were generally highly acceptable in sensory
212 trials. These products are expected to be suitable as delivery vehicles for dietary whey protein in
213 studies aimed at substantiating the human health benefits of this protein source, including β-lg
214 (McIntosh et al., 1998). β-Lg, among other whey proteins, appears to bind mutagenic
215 heterocyclic amines and thus provide some protection against their carcinogenic properties
216 (Yoshida, Ye & Nishiumi, 1991). The effects of whey proteins from bovine milk on
217 melanogenesis in cultured human melanocytes have been studied. Among the major protein

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components of whey, only β-lg showed a depigmenting effect at a concentration of 1 mg mL^{-1}, and also suppressed the activity of tyrosinase in these cells (Nakajima et al., 1997).

Hypocholesterolemic effect

A tryptic peptide from β-lg (amino acids 71-75; Ile-Ile-Ala-Glu-Lys) has been shown to have hypocholesterolemic activity in animal (rat) trials, and the mechanism of action would appear to relate to inhibition of micellar solubility of the cholesterol, which in turn causes suppression of cholesterol absorption by a direct interaction between cholesterol mixed micelles and the tryptic peptide in the jejunal epithelia. The authors claim that their study provides the first direct evidence of a new hypocholesterolemic peptide derived from β-lg that exhibits a greater hypocholesterolemic effect than β-sitosterol in animal trials (Nagaoka et al., 2001). Further, β-lactotensin, a neurotensin agonist derived from β-lg, shows hypocholesterolemic activity after administration to mice for 2 days at a dose of 30 mg kg^{-1} (i.p.) or 100 mg kg^{-1} (p.o.) (Yamauchi, Ohinata & Yoshikawa, 2003). However, some caution needs to be taken when attempting to extrapolate results from animal studies, particularly using rodent models, to potential effects in humans, as hypocholesterolemic effects can be animal-model specific.

Metabolic and physiological effects

Fatty acid metabolism: Although β-lg can bind in vitro to a variety of hydrophobic substrates, including retinol and long-chain fatty acids, its physiological function is still largely unknown and subject to speculation. The retinol and fatty acid binding of β-lg has been widely implicated in the proposed physiological function of β-lg. Fatty acid binding sites have been characterised on β-lg (Perez, Sanchez, Aranda, Ena, Oria & Calvo, 1992) and it was concluded that β-lg could participate in the digestion of milk lipids during the neonatal period by enhancing the activity of pre-gastric lipase by binding fatty acids that inhibit this enzyme. In addition, it has been shown that β-lg enhanced intestinal uptake of retinol, triglyceride, and long-chain fatty acids in pre-

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ruminant calves (Kushibiki et al., 2001), and it was speculated that the protein may play a role in the absorption and subsequent metabolism of fatty acids.

Mammalian cell growth factor activity: One report suggests that bovine β-lg at high concentration (almost 3 g L^{-1}) exhibits mitogenic activity equal to that of whole whey, as determined by DNA synthesis in hybridoma cultures. This same study indicated that there are variant differences in this mitogenic activity, the B variant of β-lg showing significantly lower activity (Moulti-Mati, Mati, Capiaumont, Belleville, Linden & Nabet, 1991).

Opioid activity: During the past two decades a variety of food protein fragments have been demonstrated to elicit biological effects in various in vitro or in vivo test systems. A considerable number of these bioactive peptides come from milk proteins, and show opioid-like activity, and may be regarded as exogenous supplements to the endogenous opioidergic cellular systems (Teschemacher & Koch, 1991; Teschemacher, 2003). Several whey protein fragments have been shown to behave like opioid receptor ligands (Teschemacher, Koch & Brantl, 1997). Specifically, β-lactorphin, a tetrapeptide (amino acids 102-105; Tyr-Leu-Leu-Phe) derived from β-lg, behaves like an opioid receptor agonist. Recently, β-lactorphin has been shown to improve arterial function in SHR. Notably, β-lactorphin improved vascular relaxation in adult SHR in vitro, and additionally enhanced endothelium-independent relaxation (Sipola et al., 2002). While these reports are interesting, only a minority of the opioid activity has been observed upon oral or intra-gastric administration of these peptides or their precursor proteins, and most studies have been performed in animals (Teschemacher, 2003). A recent study (Roufik, Gauthier & Turgeon, 2006) on bioactive peptides derived from bovine β-lg has supported the view that in vivo studies are essential to validate the physiological effects of bioactive peptides and that long-chain bioactive peptides require protection from gastrointestinal enzymes when orally administered.

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B) α-Lactalbumin

Background

Amounts in bovine and human milk: In mature bovine milk, the concentration of α-la is 1 to 1.5 g L^{-1}, comprising approximately 3.4% of the total protein or 20% of the whey proteins (Swaisgood, 1995). On the other hand, α-la is the predominant whey protein in human milk. Levels of α-la increase from 21% to 34% between day 1 and 14 of lactation (Montagne, Cuilliere, Mole, Bene & Faure, 1999). α-La concentrations in mature human milk (after day 30) are 2.44 ± 0.64 g L^{-1}, determined in a multinational study (Jackson, Janszen, Lonnerdal, Lien, Pramuk & Kuhlman, 2004).

Structure: At the amino acid level, the homology between human and bovine α-la can be described as having 76% fully conserved residues (93 out of 123 amino acids) and 88% similarity when conservation of strong and weak groups are taken into consideration ("ClustalW on-line program", 2006). A similar high degree of homology exists between α-la of most other mammals. However, a new form of human α-la has recently been discovered, which consists of a single nucleotide polymorphism. The biological implications of this new form remain to be determined (Chowanadisai et al., 2005). α-La has a globular structure in aqueous solution. It exhibits a high affinity to metal ions, calcium in particular, at the junction of subdomains at residues 79-88 containing five aspartates (Permyakov & Berliner, 2000). Calcium depletion at low pH causes structural changes to form the so-called molten globule state. This has important implications during purification processes and for the bioactivity of the protein (see later, formation of anti-tumour α-la complexes). Using differential scanning calorimetry, α-la, in the presence of saturating amounts of calcium, is characterised by being quite thermo-stable having a melting temperature (T_m) of 68ºC. However, in the absence of calcium, this protein is very unstable (T_m of 43ºC). Therefore, binding of calcium is of utmost importance for maintaining the structure of this protein. This thermal instability is exploited in one process to purify alpha-

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lactalbumin and will be discussed in later sections.

Purification of α-lactalbumin

The starting material for enrichment and purification of bovine α-la is usually whey. Many industrial processes have been reported and methods have been reviewed extensively (Imafidon, Farkye & Spanier, 1997). However, although many of these methods of purification have worked at laboratory scale, scale-up to pilot scale and industrial scale has been difficult, if not disappointing (Gesan-Guiziou, Daufin, Timmer, Allersma & van der Horst, 1999).

Membrane technology: As many processes in the dairy industry are based on membrane technology, this technique has also been exploited to enrich α-la. This can be achieved by performing microfiltration to remove β-lg or alternatively ultrafiltration using a 50 kDa cut-off membrane, thereby passing α-la into the permeate (Uchida, Shimatani, Mitsuhashi & Koutake, 1996). More commonly, enriched fractions of α-la have been obtained by using a two-membrane cascade membrane filtration scheme (Roger, Maubois, Brule & Piot, 1987; Bottomley, 1991; Mehra & Kelly, 2004).

Selective hydrolysis of other milk proteins: A novel approach has been the use of enzymes such as trypsin or alpha-chymotrypsin to selectively degrade β-lg (Kaneko, Kojima, Kuwata & Yamamoto, 1992). A protease of microbial origin has also been used for this purpose (Kaneko, Kojima, Kuwata & Yamamoto, 1994).

Ion exchange chromatography: The advent of more sophisticated means of separating milk proteins at process scale allowed ion exchange chromatography to be chosen for some applications (Outinen, Harju, Tossavainen & Antila, 1995). Chymosin whey has been adjusted to pH 5 or higher where α-la did not bind to the ion exchange matrix and was therefore easily eluted. The fraction was then adjusted to pH 4.0 and ultrafiltered on a narrow molecular weight

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cut-off membrane to separate glycomacropeptide from α-la (Yukio, Masaharu, Ichirou, Suzuka & Masanobu, 1992). In a different approach, α-la was recovered from the whey by heating WPC to a temperature of 75˚C and acidifying using a cation exchange resin in (H^+) form (Rialland & Barbier, 1988).

Purification by isoelectric precipitation: Due to the high costs of ion exchange columns and resins, the majority of isolation procedures utilise isoelectric precipitation, often in combination with heat treatment. This method is cheap and relatively easy to perform and involves whey protein first being desalted and the pH adjusted to pH 3.8-5.5. The resulting solution is heat treated at between 55-70˚C for more than 30 seconds to permit aggregation of part of the whey protein. Thereafter, the solution is cooled to 55˚C to permit flocculation of the aggregates that consisted of α-la. The α-la is then isolated by microfiltration (Pearce, 1995). A similar method has been used in which the protein was destabilized by exposing whey protein to a calcium-binding ion-exchange resin. The pH was then adjusted to between 4.3 and 4.8 and incubated between 10 and 50˚C. The protein was then fractionated to isolate α-la and the pH neutralised (De Wit & Bronts, 1997). By combining isoelectric precipitation and heat treatment, a new method was designed, comprising of heat treatment of a 15% (w/w) whey protein concentrate at 60-80˚C at neutral pH followed by cooling to 45˚C and pH adjustment to 4.2-4.5. α-La was then isolated leading to an α-la/β-lg ratio of more than 0.43 (Hakkaart, Kunst, Leclercq, De Levita & Moonen, 1992).

As mentioned in the earlier section, α-la is sensitive to calcium and adjustment of the pH to around the isoelectric point of α-la results in formation of the molten globule form of the protein. Mild heat treatment causes the protein to precipitate. Unfortunately, the drawback of this approach is that the structure of the protein is irreversibly altered (Chatterton, 2001) compared to that of the more gentle methods of purification (Chatterton, Nielsen, Holst, Bertelsen & Albertsen, 1999). As a result, the bioactivity of the protein could be impaired. The digestibility is

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altered as demonstrated by a study whereby α-la was ingested under conditions similar to that found in early neonatal life (Chatterton, 2001). The peptide 41-52 was released less efficiently when the sample was heat treated according to (Pearce, 1995) compared to that of the non-heat treated sample. This may be of significance as the amino acids 41-53 hold some of the bioactive peptide discussed in the following section.

Bioactivity and Applications

α-La is known for its part of the lactose synthase complex that catalyses the last step of the biosynthesis of lactose and controls the subsequent movement of water into the mammary secretory vesicles. It is therefore critical for lactational control and secretion of milk (Brew, Vanaman & Hill, 1968; Lo, Shaper, Pevsner & Shaper, 1998).

The health effects of α-la for human consumption can be subdivided into three groups: those related to (i) the intact, whole protein, (ii) peptides of the partly hydrolysed protein and (iii) the amino acids of the fully digested protein. Great emphasis has been placed on the latter, nutritional aspect, as α-la is a particularly good source of the essential amino acids Trp and Cys as these amino acids are precursors of serotonin and glutathione, respectively. Based on the assumption that the nutritional need of a neonate is fully met by human milk, there is a drive to "humanise" or "adapt" the formulation to adjust for the different amino acid profile of human and bovine milk (Kelleher, Chatterton, Nielsen & Lonnerdal, 2003; Lien, 2003). Bovine α-la, with its high homology to human α-la, is an ideal protein to overcome this discrepancy. α-La enriched whey protein fractions with a reduced β-lg content are therefore of high interest to manufacturers of infant formula.

Inhibition of Angiotensin-Converting Enzyme (ACE) activity and blood pressure-lowering effects:

The peptide with the amino acids sequence Tyr-Gly-Leu-Phe (amino acids 50-53), released from α-la by pepsin treatment was shown to inhibit angiotensin-I-converting enzyme (ACE), having an

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IC_{50} value of 733 μM (Mullally, Meisel & Fitzgerald, 1996). This peptide is termed α-lactorphin (Yoshikawa, Tani, Yoshimura & Chiba, 1986). Interestingly, proteolytic fragments of this peptide i.e. the dipeptides Tyr-Gly (amino acids 18-19 and 50-51) and Leu-Phe (amino acids 52-53) were also observed to have an inhibitory effect, having IC_{50} values of 1523 and 349 μM respectively (Mullally et al., 1996). Other studies detected ACE inhibitory activity in peptides Tyr-Gly-Leu (amino acids 50-52) at similar IC_{50} values (409 μM) (Pihlanto-Leppala, Koskinen, Piilola, Tupasela & Korhonen, 2000). In contrast, peptides with higher inhibitory activity were also detected towards the C-terminus of α-La, i.e. Val-Gly-Ile-Asn-Tyr-Trp-Leu-Ala-His-Lys (amino acids 99-108) exhibited an IC_{50} of 327 μM. The sequence Trp-Leu-Ala-His-Lys (amino acids 104-108) exhibited an IC_{50} value of only 77 μM.

In conscious spontaneously hypertensive rats and in normotensive rats, α-lactorphin lowered blood pressure in a dose-dependent and naloxone inhibitable manner. These effects occurred at 10 μg kg^{-1} dosages. At higher dosages of 100 μg kg^{-1}, maximal reductions in systolic and diastolic blood pressure of 23 $\pm$ 4 and 17 $\pm$ 4 mm Hg, respectively, were achieved (Nurminen et al., 2000)

Anti- carcinogenic activities

Recently, a folding variant of human α-la was discovered, which selectively enters tumour cells and induces an apoptosis like mechanism, probably by binding to histones and thereby disrupting the chromatin organisation in the cell nuclei (Duringer, Hamiche, Gustafsson, Kimura & Svanborg, 2003). This kinetically trapped protein-lipid complex was named HAMLET/BAMLET for Human/Bovine Alpha-Lactalbumin Made Lethal to Tumour Cells (Fast, Mossberg, Svanborg & Linse, 2005). It consists of the calcium depleted apo form of α-la in the afore-mentioned molten globule state, which is stabilised by a fatty acid cofactor. It is noteworthy that the α-la/fatty acid interaction is stereo-specific; only unsaturated cis fatty acids bind to α-la and only the C18:1:9cis fatty acid (oleic acid), bound to α-la in a compact conformation is active against tumour cells (Svensson, Mossberg, Pettersson, Linse & Svanborg,

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2003; Fast, Mossberg, Nilsson, Svanborg, Akke & Linse, 2005). The complex is formed from either co-precipitated α-la in acid-casein or calcium depleted α-la from whey, on an anionic exchange column that was previously conditioned with either the relevant fatty acid or casein from human milk (which also contains traces of the fatty acid) (Svanborg & Svensson, 2003). The active complex is washed off at very high NaCl concentration as it binds tightly to the column matrix. To date, no alternative method of complex formation has been published. In vitro, both the human and bovine forms were shown to induce apoptosis in a wide variety of tumour cells (Svensson, Fast et al., 2003) The specific therapeutic effect of HAMLET in vivo has recently been demonstrated on several examples such as human skin papillomas (Gustafsson, Leijonhufvud, Aronsson, Mossberg & Svanborg, 2004), human glioblastoma (GBM) tumour in mice (Fischer et al., 2004) and mammary cells of mice (Baltzer, Svanborg & Jaggi, 2004).

These newly described α-la compounds can be considered potential candidates for therapeutic or prophylactic treatment. However, to-date, the health benefits for human digestion (in particular in neonates) of milk or dairy products and whether or not such complexes are formed at any stage during digestion remains highly speculative. This requires further scientific and/or clinical investigation.

Anti-microbial activity

The α-la complex described above as HAMLET has also been shown to exhibit anti-microbial activity, in particular against Streptococcus pneumoniae (both antibiotic sensitive and resistant strains) and Haemophilus influenzae. It was pointed out that commercially available α-la samples lacked those biological activities (Svanborg & Sabharwal, 2004), the most likely reason being the purification method for α-la (size exclusion chromatography) whereby compounds of higher molecular weight are discarded and only monomeric α-la retained.

A clinical study using α-la enriched infant formula showed an activity against enteropathogenic E.coli O127 and reduced incidences of diarrhoea comparable to that of breast milk (Bruck, Kelleher, Gibson, Nielsen, Chatterton & Lonnerdal, 2003). This action might be related to

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peptides which are released from α-la during digestion. It is known, that trypsin treatment of α-la has been shown to release two antibacterial peptides Glu-Gln-Leu-Thr-Lys (amino acids 1-5), and Gly-Tyr-Gly-Gly-Val-Ser-Leu-Pro-Glu-Trp-Val-Cys-Thr-Thr-Phe (amino acids 17-31) disulphide-bonded to Ala-Leu-Cys-Ser-Glu-Lys (amino acids 109-114). Treatment using another intestinal enzyme, chymotrypsin, resulted in one antibacterial peptide, namely, Cys-Lys-Asp-Asp-Gln-Asn-Pro-His-Ile-Ser-Cys-Asp-Lys-Phe (amino acids 61-68) disulphide bound to amino acids 75-80. These peptides were mostly active against Gram-positive bacteria, however weaker effects were observed with Gram-negative bacteria (Pellegrini, Thomas, Bramaz, Hunziker & von Fellenberg, 1999). Although pepsin did not release any antibacterial peptides in the study by Pellegrini et al. (1999), a different study indicated that both pepsin or trypsin released peptides from α-la which inhibited the growth of E. coli JM103; the peptide concentration was 25 mg mL^{-1}, whereas unhydrolysed α-la did not inhibit the growth at a concentration of 0.1 g mL^{-1} (Pihlanto-Leppala et al., 2000).

Structural impact on bioactivity: The discovery of bioactive peptides linked via disulphide bonds again highlights the importance of maintaining the structure of α-la using mild processing conditions during purification. Heat-treatment is known to alter the disulphide bond pattern within proteins and/or to cause inter-molecular cross-linking. α-La alone or in the presence of other whey proteins has been shown to induce formation of inter-molecular disulphide bonds between α-la itself, α-la and β-lg, involving Cys 61 (note: Cys 61 is part of the afore-mentioned anti-bacterial peptide) and Cys 111 (Livney, Verespej & Dalgleish, 2003) or α-la and BSA (Havea, Singh & Creamer, 2001; Livney et al., 2003). This would prevent the release of these disulphide linked bioactive peptides.

Growth-promoting and opioid activity

A study has also shown that peptides from hydrolysed α-la have growth-promoting effects on Bifidobacterium longum ATCC 15707 (Kee, Kim, Jung, Yun, Juhn & Hong, 1998). It was

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458 further claimed that α-la could act as a prebiotic agent and be used as such in food and food
459 supplements (Maase & Steijns, 2002).
460 The sequence of the amino acids Tyr-Gly-Leu-Phe (amino acids 50-53), released from α-la by
461 pepsin treatment, has structural similarities to the opioid peptide human leu-enkephalin that has
462 the amino acid sequence Tyr-Gly-Gly-Phe, termed α-lactorphin (Horikawa et al., 1983). An
463 opioid-like effect of this synthetic α-la peptide has been reported, having a weak activity both in
464 receptor assay and pharmaco-dynamic measurements in guinea pig ileum and mouse vas deferens
465 preparations in vitro (Yoshikawa et al., 1986).
466
467 ***α-Lactalbumin in the management of stress***
468 Tryptophan is a precursor for brain serotonin, which may improve the ability to cope with stress.
469 Studies were carried out to investigate whether α-la might alleviate symptoms of stress in adult
470 subjects. However, large neutral amino acids can compete with transport of tryptophan across the
471 blood brain barrier, preventing uptake of tryptophan. The tryptophan to large neutral amino acid
472 ratio in plasma was observed to be 48% higher after an α-la diet than that after a casein diet.
473 Furthermore, in stress-vulnerable subjects, higher prolactin concentrations, decreased cortisol and
474 a reduction in depressive feelings were observed under stress (Markus et al., 2000). In later
475 studies, α-la was observed to improve cognitive performances in stress-vulnerable individuals by
476 increased brain tryptophan and serotonin activity (Markus, Olivier & de Haan, 2002). Other
477 clinical trials suggested that α-la could be used to improve sleep in adults submitted to nutritional
478 disturbances (Minet, Le, Tome & Even, 2004).
479 Another clinical trail on rats demonstrated that α-la can protect against ethanol and stress-induced
480 gastric injury (Matsumoto, Shimokawa, Ushida, Toida & Hayasawa, 2001) such as stomach
481 ulcers with a dose dependent effect (optimum 200 mg kg^{-1}). Interestingly, it exhibits a
482 comparable potency to that of the typical antiulcer agent, Selbex. A subsequent study by the
483 same group found that α-la causes an increase in the gastric luminal pH, an increase in gastric
484 fluid and a delay in gastric emptying (Ushida, Shimokawa, Matsumoto, Toida & Hayasawa,

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2003).

Supplementation to infant formulas

Formula-fed infants have been shown to have disparities in plasma amino acids compared to breast-fed infants (Raiha, Minoli & Moro, 1986; Raiha, Minoli, Moro & Bremer, 1986; Heine, Radke, Wutzke, Peters & Kundt, 1996; Sarwar & Botting, 1999), particularly in the levels of tryptophan that have been demonstrated to be lower. To prevent this, the levels of protein in infant formulas have been adjusted to be far higher than in human milk. However, such high protein levels can be unhealthy for infants. Therefore, there is now a trend in lowering the protein content of infant formulas to approach the level found in human milk. Unfortunately, this will only enhance the disparities in the plasma amino acid profile, unless a protein rich in essential amino acids, tryptophan in particular, is added to the formula. As α-la is rich in essential amino acids, this protein is ideally suited to this purpose. This has been shown to be the case in a pre-clinical study in infant rhesus monkeys (Kelleher et al., 2003). More recently, α-la supplemented to reduced-protein infant formulas has been observed to supply adequate nutrition for infants despite a reduction in the protein content of the formula and additionally were better tolerated than control formula (Lien, Davis & Euler, 2004). As mentioned before, clinical trials with α-la enriched infant formula showed anti-microbial activity (Bruck et al., 2003).

Allergenicity of α-lactalbumin and ß-lactoglobulin

The prevalence of allergies to cow's milk in the general population depends on geographical location and ethnicity and varies from 1 to 3%, being highest in infants and lowest in adults (Bahna, 2002). Almost all milk proteins have been implicated in allergic reactions (Chatchatee, Jarvinen, Bardina, Beyer & Sampson, 2001; Chatchatee, Jarvinen, Bardina, Vila, Beyer & Sampson, 2001; Järvinen, Chatchatee, Bardina, Beyer & Sampson, 2001; Busse, Järvinen, Vila, Beyer & Sampson, 2002; Wal, 2002; Cocco, Jarvinen, Sampson & Beyer, 2003). In patients with persistent allergy to cow's milk; four IgE- and three IgG-binding regions have been identified on

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α-la, while seven IgE- and six IgG-binding epitopes were detected on β-lg. In patients likely to outgrow their allergy, three of these IgE-binding epitopes were detected on β-lg and none on α-la (Järvinen et al., 2001). A proteomics approach in combination with immunoblotting, indicated that all major milk proteins including β-lg were allergens, though no evidence was found for α-la (Natale et al., 2004). Consequently, there is no consensus between studies regarding the allergenicity of α-la. The lack of agreement between studies might be related firstly to the thermal history of the protein. As mentioned earlier, there are several techniques available to purify both β-lg and α-la at process scale. It is also known that the allergenicity of a protein can be changed by thermal processing. Although allergenicity can be lost by heat treatment, the converse also applies; namely that heat-denatured proteins can also present new antigenic sites, which are uncovered by the unfolding process or created by new chemical reactions with other molecules present in the food, e.g. β-lg associating with α-la in milk (Davis & Williams, 1998; Livney et al., 2003) and β-lg/α-la/BSA disulphide cross-linking (Havea et al., 2001). Therefore, further studies are necessary to take into account the thermal history of the proteins in milk, in particular α-la when supplemented to the new generation of infant formulas. Secondly, the degree of allergenicity of a milk protein can be related to the type of techniques used. For instance, tryptic peptides from bovine α-la have been reported to have a specific IgE binding capacity and therefore linked to development of allergenicity (Maynard, Pierre & Maubois, 1989). However, as trypsin is not the only proteolytic enzyme in the gastrointestinal tract, it is highly likely that these peptide sequences are cleaved further to smaller peptides in vivo, potential allergenic epitopes are thereby broken up. Indeed, extensive hydrolysis of proteins to small peptides or even amino acids is used to make dairy proteins for commercial hypoallergenic milk products (Sampson, Bernhisel-Broadbent, Yang & Scanlon, 1991; Crittenden & Bennett, 2005).

Conclusion

β-Lg provides the food industry with a unique ingredient material, a cost-effective protein with attractive properties in food functionality. It exhibits a growing number of biological activities

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including anti-hypertensive, anti-cancer, hypocholesterolemic, opioidergic, and anti-microbial effects, among others. This major bovine whey protein thus demonstrates true multi-functionality providing the industry with a plethora of opportunities for development of novel foods and beverages containing this protein. Manufacturing techniques are simple and cost-effective, relying upon the physico-chemical properties of the β-lg protein, and resulting in isolates of varying levels of purity for specific applications. While the bioactivities reported for β-lg and its peptide fragments are exciting and provide an opening for the inclusion of β-lg as the active ingredient in a range of functional foods and beverages, progress needs to be cautious, as many of these bioactivities are only putative.

Bovine α-la is ideally suited as an ingredient for infant nutrition, based on its high degree of amino acid homology to human α-la. The biological function of bovine α-la and its peptides include those mentioned above for β-lg but also stress reducing and sleep improving properties. However, the type of industrial purification or enrichment of this protein from milk and whey can be of critical importance for maintaining some of the biological effects. Heat treatment applied during processing can alter the structure of the protein and thereby contributing to lower digestibility and changing the biological activity of the protein. Other whey proteins that are usually in the presence of α-la enriched products have been shown to cross-link to α-la, thereby irreversibly altering the molecular structure, which is likely to affect the bioactivity of the protein. Therefore, the determination of the intactness of the molecular structure in its native state seems of crucial importance in order to assure full preservation of its bioactivity. There is still a strong need for clinical trials in order to support some of the afore-mentioned health claims. Finally, this review clearly shows that further research is needed to both independently confirm the reported bioactivities and to better understand the mechanism of action at a molecular level.

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