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Food Research International 73 (2015) 149–161

Contents lists available at ScienceDirect

Food Research International

journal homepage: www.elsevier.com/locate/foodres

Whey as a source of peptides with remarkable biological activities

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ARTICLE INFO

Article history:
Received 15 January 2015
Accepted 25 January 2015
Available online 31 January 2015

Keywords:
Bioactive peptides
By-product
Protein hydrolysis
Whey

ABSTRACT

The dairy industry generates increased amounts of whey from both cheese and casein production facilities. Whey presents an elevated content of lactose and proteins, which are associated with its high biological oxygen demand and decomposing potential. Despite its potential as pollutant, whey has been considered as a dairy by-product due to its nutritional, functional and bioactive properties. The use of enzyme technology may be an interesting strategy to convert whey into added-value products. The hydrolysis of whey proteins can generate bioactive peptides, which are described to perform physiological effects *in vivo*, such as antioxidant, antimicrobial, antihypertensive and antidiabetic activities. Bioactive peptides derived from whey proteins have been also associated with immunomodulatory, anticancer, opioid and hypocholesterolemic activities. This review presents a discussion on the main biological activities of peptides derived from whey proteins.

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1. Introduction

Whey corresponds to the liquid fraction remaining after milk clotting and casein removal during cheese manufacturing. Whey is an abundant by-product of the dairy industry, resulting from either cheese or casein production. This by-product represents about 85–90% of milk volume and retains approximately 55% of milk nutrients (Siso, 1996; Smithers, 2008).

Whey contains the lactose and non-casein proteins of milk, and its elevated content of organic matter is associated with a high biochemical oxygen demand and potential for decomposition. Whey was considered the most important pollutant of the dairy industry, not only due to its high organic loading, but also due to its elevated volume (Walzen, Dillard, & German, 2001). However, the perception of whey as a pollutant has changed with the discovery of its functional and bioactive properties, being considered as an additional product of cheese manufacture (De Boer, 2014; Smithers, 2008).

Despite its elevated nutritional value, the use of whey *in natura* is limited due to its perishable characteristics and elevated dilution of its components. In this way, several technologies have been used to benefit this material. Thus, concentration of whey may be realized by heating and drying (evaporation, spray-drying, freeze-drying) or by reversed osmosis, whereas demineralization can be performed by ion exchange resins or electrodialysis. Membrane separation technologies have been equally used for obtaining protein ingredients from whey (Brans, Schröen, van der Sman, & Boom, 2004).

Alternatively, production of hydrolysates can be an interesting approach to add value to whey. Diverse protein hydrolysates obtained by enzymatic catalysis display biological activities, which are often associated with bioactive peptides. The bioactive peptides are inactive while encrypted in the sequence of original protein but can be released by (a) hydrolysis by digestive enzymes, (b) proteolytic microorganisms, and/or (c) the action of plant or microbial proteases (Korhonen & Pihlanto, 2006). Bioactive peptides are the focus of several investigations mostly related to antioxidant, antihypertensive and antimicrobial activities. Indeed, commercial proteases have been successfully tested for the production of bioactive hydrolysates from milk, including whey proteins. Intensive investigation of antioxidant and antihypertensive peptides derived from hydrolysis of bovine caseins has been performed in the last decades (Corrêa et al., 2011; Daroit et al., 2012; Hernández-Ledesma, García-Nebot, Fernández-Tomé, Amigo, & Recio, 2014; Phelan, Aherne, FitzGerald, & O'Brien, 2009). More recently, important amount of research including other species, such as ovine, caprine and camel, has confirmed the importance of milk proteins, mainly caseins, as source of bioactive peptides (Korhonen, 2009). Although less information is available about bioactive peptides derived from whey proteins, some important biological activities have been associated with protein hydrolysates derived from whey. Angiotensin I-converting enzyme (ACE)-inhibitory activity was observed in whey protein hydrolysates obtained from *Cynara cardunculus* protease (Tavares, Contreras, Amorim, Pintado, Recio and Malcata, 2011; Tavares, Monteiro, Possenti, Pintado, Carvalho and Malcata, 2011). Treatment of caprine whey proteins with gastrointestinal juice resulted in hydrolysates

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http://dx.doi.org/10.1016/j.foodres.2015.01.016
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showing antimicrobial activity (Almaas et al., 2011). The inhibition of dipeptidyl peptidase IV (DPP-IV) by a whey protein hydrolysate generated with food-grade pancreatic enzyme was also described (Nongonierma & FitzGerald, 2013a).

Considering the great potential of whey as a source of bioactive peptides, an effective knowledge on the production and characteristics of these peptides would be very relevant. In addition, taking into account that whey is an ingredient in several food formulations, a whey-derived product rich in bioactive peptides would be an interesting added-value product. The investigation on bioactive peptides generated by enzymatic hydrolysis of whey has a great potential to generate novel products and biotechnological processes. Therefore, this article presents a current evaluation on the major biological activities associated with hydrolysates of whey proteins and some specific bioactive peptides derived from whey proteins.

2. Whey derived products

Hydrolysis of whey proteins has been employed to modify solubility, viscosity, emulsifying and foaming properties, as well as to improve nutritional properties. Special attention has been devoted to the utilization of whey-derived products in sports medicine. It has been suggested that protein hydrolysates providing mainly di- and tripeptides are superior to intact (whole) proteins and free amino acids in terms of skeletal muscle protein anabolism (Manninen, 2009). Thus, consumption of whey-derived products may allow amino acids to be more rapidly absorbed than whole proteins, maximizing nutrient delivery to muscle tissues.

Proteolytic enzymes derived from several sources have been employed in the hydrolysis of whey proteins (Siso, 1996; Zhang, Wu, Ling, & Lu, 2013). As the microorganisms can be cultivated through controlled and well established methods, microbial proteases have been pointed as interesting biocatalysts to the production of protein hydrolysates in commercial scale. Indeed, commercial proteases of microbial origin have been successfully tested for production of hydrolysates from whey proteins (Butré, Wierenga, & Gruppen, 2012).

Hydrolysates of whey protein are considered as ideal ingredients in the formulation of human milk substitutes due to their high nutritional value, low bitterness and low antigenicity. Allergic reactions are often associated with specific sequences of β-LG, the major whey protein. Thus, bacterial proteases have been used for production of hydrolysates with reduced allergenicity. The commercial alkaline protease form *Bacillus licheniformis* Protex 6L was used to hydrolyze whey proteins in a continuous membrane reaction system, resulting in hydrolysates with an estimated 99% reduction of antigenicity (Guadix, Camacho, & Guadix, 2006). Different combinations of proteolytic enzymes, namely trypsin, neutrase, papain and protease S, were tested on the production of low-allergenic whey-derived products. The combination of trypsin with either neutrase or papain was the most effective in the removal of β-LG, producing low molecular mass peptides with reduced antigenic properties (Shin et al., 2007). Hydrolysis of goat acid whey with pepsin was performed in an ultrafiltration membrane reactor. A diversity of peptides were identified in hydrolysates, mostly derived from α-LA, due to the resistance of β-LG towards pepsin. A broad range of peptides, from dipeptides to large peptides containing disulfide bridges, were detected among hydrolysis products (Bordenave, Sannier, Ricart, & Piot, 2000). Thus, improvement of functional and bioactive properties of acid whey can be achieved using this methodology.

Diverse proteases and procedures have been employed to generate whey-derived products with different degrees of hydrolysis (DH) and bioactivities. Whey proteins can be hydrolyzed by either digestive enzymes, plant or microbial proteases, and then generate peptides that may display a number of physiological roles (Fig. 1). Enzymatic hydrolysis of whey protein concentrate (WPC) was performed with pancreatin, Protamex or Alcalase 0.6L, to produce hydrolysates with 20% DH. Alcalase showed the lowest specificity for β-LG. Considering the protein content from WPC the pancreatin hydrolytic system was the most efficient, since only 4.7% of non-hydrolyzed protein remained in the final hydrolysate, against 8.0 and 9.8% for Alcalase and Protamex, respectively (Pacheco, Amaya-Farfan, & Sgarbieri, 2002). The pancreatin and Protamex hydrolysates showed higher ability to stimulate hepatic glutathione synthesis when administered in mice diets (Pacheco & Sgarbieri, 2005). Naik, Mann, Bajaj, Sangwan, and Sharma (2013) investigated the effect of enzyme/substrate (E/S) ratio, pH and T on DH, antioxidant and ACE-inhibitory activities of WPC hydrolyzed by commercial trypsin. The E/S ratio and pH had a major influence on DH. The resultant hydrolysates were subjected to ultrafiltration, and the permeate and retentate obtained were collected separately and evaluated for bioactivities. Majority of low molecular mass peptides contributed for higher ACE-inhibitory and antioxidant activity from the permeate fraction (Naik et al., 2013). Commercial pancreatin and papain were used to hydrolyze WPC under different E/S ratios. The hydrolysates were subjected to ultrafiltration or not, resulting in 16 different peptide formulations. ACE-inhibitory activity was evaluated and the greatest values were obtained with pancreatin at an E/S ratio 0.5/100, either in the presence or in the absence of ultrafiltration (Silvestre et al., 2012).

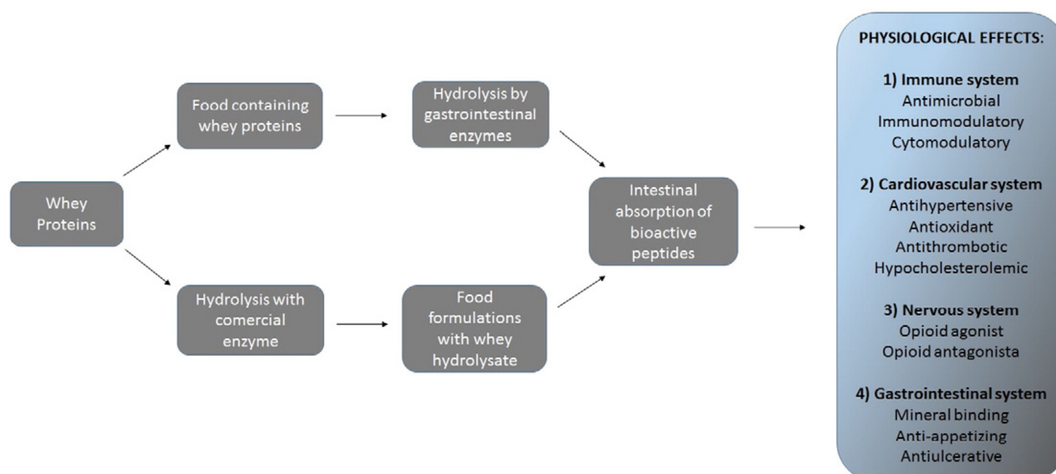

Fig. 1. Physiological effects of bioactive peptides derived from whey proteins. Whey proteins can be hydrolyzed by gastric and/or pancreatic proteases, or by commercial enzymes of plant or microbial origin to release encrypted bioactive peptides causing several physiological effects.

3. Antioxidant activity

In the last years, special attention has been dedicated for searching whey-derived peptides with radical scavenging and lipid peroxidation inhibitory activities. Oxidation of food constituents is a key event in food spoilage. It is well known that lipid peroxidation of food products can cause deterioration in food quality, shorten the shelf life and decrease the acceptability of processed foods. Lipid peroxidation can generate free radicals that can lead to fatty acid decomposition, which may reduce the nutritional value and safety of food by producing undesirable flavors and toxic substances (Niki, Yoshida, Saito, & Noguchi, 2005). Furthermore, free radical-mediated reactions have a significant role in many biological phenomena such as cellular damage and aging by stimulating oxidation of lipids and formation of secondary lipid peroxidation products. Free radicals can also modify DNA, proteins, and small cellular molecules and are believed to play a significant role in the occurrence of diseases, such as cardiovascular diseases, diabetes mellitus, neurological disorders, and even Alzheimer's disease (Stadtman, 2006). Therefore, it is important to retard the lipid oxidation and the formation of free radical in food containing lipids and/or fatty acids (Peng, Kong, Xia, & Liu, 2010).

The use of synthetic antioxidants such as butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), propyl gallate (PG) and tert-butylhydroquinone (TBHQ) has been restricted because of their potential toxic effects on humans. On the other hand, bioactive peptides are considered natural antioxidants and have attracted a great deal of interest because of their safety and wide distribution properties (Zhang et al., 2009).

Peptides from protein hydrolysates are reported to act as antioxidants through mechanisms of inactivation of reactive oxygen species (ROS), free radical-scavenging, inhibition of lipid peroxidation, chelation of metal ions, or a combination of these mechanisms. The major mode of action derives from the inherent amino acid composition and sequence of a peptide (Phelan et al., 2009). Therefore, considering a protein hydrolysate as a complex mixture of peptides and amino acids, distinct antioxidant mechanisms are possibly acting concomitantly, and this highlights the importance of evaluating the antioxidant potential of proteins and their hydrolysates by different methods. Usually, the assays measure the ability of a compound (the potential antioxidant) to transfer hydrogen atoms or electrons to an oxidant. Among the antioxidant capacity assays are the Trolox equivalent antioxidant capacity (TEAC) assay utilizing the 2,2′-azino-bis-(3-ethylbenzothiazoline)-6-sulfonic acid (ABTS) radical, and the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay, which detects both electron transfer and hydrogen atom transfer, and the ferric ion reducing antioxidant power (FRAP) assay, which evaluates electron transfer. These simple and inexpensive methods are widely utilized, although commonly performed under conditions that do not resemble physiological situations. Other assays, such as the oxygen radical absorbance capacity (ORAC), are considered more suitable for detecting the antioxidant potential of protein hydrolysates, since they employ biologically relevant radicals (Conway, Gauthier, & Pouliot, 2013; Huang, Ou, & Prior, 2005). Also, the utilization of food and/or cell model systems of antioxidant capacity is a required approach to better characterize antioxidant potentials (Jin et al., 2013; Kong, Peng, Xiong, & Zhao, 2012).

Antioxidant peptides usually consist of 5–11 amino acids, including hydrophobic amino acids, proline, histidine, tyrosine and/or tryptophan (Zhou et al., 2012). The antioxidant activity of peptides generated through *in vitro* hydrolysis of proteins is related with a greater number of ionizable groups and also the exposition of hydrophobic groups (Sarmadi & Ismail, 2010). Most of the identified antioxidant peptides are derived from αs-casein and have been shown to possess free radical scavenging activities and to inhibit enzymatic and non-enzymatic lipid peroxidation, most likely by being a preferred target over fatty acid free radicals (Rival, Boeriu, & Wichers, 2001). Although antioxidant peptides derived from milk proteins have been mostly associated with bovine casein, hydrolysis of whey proteins may also result in the production of antioxidant peptides (Phelan et al., 2009; Pihlanto, 2006). Some authors relate that the antioxidant activity of hydrolysates of whey proteins corresponds to fractions of low molecular mass (Peña-Ramos & Xiong, 2001), while others associated this activity with high molecular mass fractions (Peña-Ramos, Xiong, & Arteaga, 2004). Conway et al. (2013) suggest that peptides originating from major whey proteins (α-LA and β-LG) were likely responsible for the antioxidant activity of enzyme-hydrolyzed whey concentrate and skim milk, because the casein content of skim milk did not improve its scavenging activity to any significant degree.

Whey protein isolate was hydrolyzed by different proteases, namely trypsin, pepsin, Alcalase, Promatex, Flavourzyme, or protease N. The hydrolysate generated by Alcalase 2.4L showed the highest antioxidant activities and seven different peptides showing strong antioxidant activities were isolated. The antioxidant peptide WYSL displayed the highest DPPH radical scavenging activity and superoxide radical scavenging activity, with IC_{50} values of 273.63 μM and 558.42 μM, respectively (Zhang et al., 2013). Corrêa et al. (2014) used a *Bacillus* P7 protease to hydrolyze sheep cheese whey, and obtained an antioxidant activity of 51.3% by the 2,2′-azino-bis-(3-ethylbenzothiazoline)-6-sulfonic acid (ABTS) method.

Many bioactive peptides are known to possess multifunctional properties. For example, some antioxidant peptides also show ACE-inhibitory activity (Hernández-Ledesma, Davalos, Bartolome, & Amigo, 2005). The sequence YQEPVLGP was described as antimicrobial, ACE-inhibitory and ABTS radical scavenging (Rizzello et al., 2005; Silva, Pihlanto, & Malcata, 2006). The peptides TTMPLW and VMFPPQSVL are reported as ACE-inhibitory (Hernández-Ledesma et al., 2005; Otte, Lenhard, Flambard, & Sørensen, 2011), and also as immunomodulatory and antimicrobial, respectively (Gobbetti, Minervini, & Rizzello, 2004; Rizzello et al., 2005).

As shown in Table 1, β-LG fragments LQKW f(58–61), LDTDYKK f(95–101), and FNPTQ f(151–155) contain the amino acids Tyr (Y) and Trp (W), which have been described by different authors as mainly responsible for antioxidant activity of peptides, indicating their important contribution on antioxidant properties of permeates from WPC hydrolyzed with thermolysin. Peptide LQKW has been also reported to exert ACE-inhibitory activity and antihypertensive effects on spontaneously hypertensive rats (Hernández-Ledesma, Miguel, Amigo, Aleixandre, & Recio, 2007).

Although the antioxidant properties of whey derived peptides have been extensively investigated, further research about the structure–activity relationship of peptides and synergistic and antagonistic affects among amino acids and other antioxidant compounds should be carried out. Further work is also required to understand the antioxidant potential of hydrolysates generated from whey protein

Table 1
Antioxidant peptides derived from whey proteins.

Origin	Enzyme treatment	Amino acid sequence	Protein fragment	Reference
Bovine α-LA	Thermolysin	INYW	f(101–104)	Sadat et al. (2011)
		LDQW	f(115–118)	
Bovine β-LG	Corolase PP	MHIRL	f(145–149)	Hernández-Ledesma et al. (2005)
		YVEEL	f(42–46)	
		WYSLAMAASDI	f(19–29)	
	Thermolysin	FNPTQ	f(151–155)	Contreras et al. (2011)
		LQKW	f(58–61)	
		LDTDYKK	f(95–101)	
	Trypsin	VAGTWY	f(15–20)	Power, Fernández et al. (2014)
	Alcalase	WYSL	f(19–22)	Zhang et al. (2013)
Ovine β-LG	Protease P7	LAFNPTQLEGQCHV	f(149–162)	Corrêa et al. (2014)

isolate (WPI) in real food systems and their effect on food organoleptic properties.

It will also be important to identify the form in which the antioxidant peptides can be incorporated into food matrices. Compared to pure isolated peptides, crude or semi-purified peptide extracts will be more economically feasible to be used in food products. Furthermore, crude extracts may contain several different peptides that can act synergistically to exert antioxidative action. On the other hand, other components like pigments and trace lipids in crude extracts may cause color and flavor problems. Possible strategies for increasing the cellular permeability of food-derived antioxidant peptides should also be investigated (Anusha, Samaranayaka, & Li-Chan, 2011). Whey hydrolysis could also be promising from a food technology perspective, since transition metal ions promote lipid oxidation and their chelation helps to retard the peroxidation and subsequently prevent food rancidity. This is particularly relevant when considering the potential health risks associated with synthetic antioxidants (Rossini, Noreña, Cladera-Olivera, & Brandelli, 2009). In this perspective, the antioxidant activities of peptides present in whey-derived products could meet the increasing demand for more natural antioxidants aiming human health and food quality.

4. Antihypertensive activity

Hypertension is one of the main risk factors for cardiovascular diseases (Mancia et al., 2009). Indeed, one of the most relevant and studied bioactivity of peptides is the capacity of reducing blood pressure (Hartmann & Meisel, 2007).

Many antihypertensive peptides are characterized by their ability to inhibit ACE (peptidyl-dipeptide hydrolase; EC 3.4.15.1). ACE is a multifunctional ectoenzyme that is located in different tissues and plays a key physiological role in the renin–angiotensin, kallikrein–kinin, and immune systems. The enzyme is responsible for the increase in blood pressure by converting angiotensin-I to the potent vasoconstrictor, angiotensin-II, and by degrading bradykinin, a vasodilatory peptide, and enkephalins (Petrillo & Ondetti, 1982). In particular, ACE is an enzyme that has a key role in the renin–angiotensin system, which in turn regulates the arterial blood pressure and the equilibrium of water and salt in the body. An increase in blood pressure is observed when the enzyme catalyzes the hydrolysis of angiotensin I to angiotensin II, a strong vasoconstrictor agent, and the degradation of bradykinin, which has vasodilative action, to a greater extent than needed (Coates, 2003).

Food scientists and technologists have focused their studies on bioactivities associated with casein and whey protein-derived peptides. ACE-inhibitory peptides have received special attention due to their potential beneficial effects in the treatment of hypertension. Several reports on ACE-inhibitory and/or antihypertensive peptides are associated with peptides derived from bovine milk (Korhonen & Pihlanto, 2006; Phelan et al., 2009). However, in recent years, sheep and goat whey proteins have become an important source of ACE-inhibitory peptides (Recio, de la Fuente, Juarez, & Ramos, 2009). ACE inhibitory peptides usually contain 2–20 amino acid residues, although active peptides with up to 27 amino acids have been described (Saito, Nakamura, Kitazawa, Kawai, & Itoh, 2000).

In their primary structure, milk proteins contain amino acid sequences that, when released by hydrolysis or fermentation processes, exert a significant antihypertensive activity (Erdman, Cheung, & Schroder, 2008). Most of published research on ACE inhibitory peptides is focused on their production and identification (Otte, Shalaby, Zakora, Pripp, & El-Shabrawy, 2007; Ruíz-Giménez et al., 2012). Peptides generated from both α-LA and β-LG are reported to possess ACE-inhibitory properties (Contreras, Hernández-Ledesma, Amigo, Martín-Álvarez, & Recio, 2011; Tavares, Contreras et al., 2011; Tavares, Monteiro et al., 2011). Particularly, it has been shown that non-hydrolyzed β-LG has very low ACE-inhibitory activity (Mullally, Meisel, & FitzGerald, 1997a), but hydrolysis (using pepsin, trypsin, chymotrypsin and/or other proteases) resulted in high levels of ACE inhibition (73–90%). Meisel, Goepfert, and Günther (1997) reported the presence of ACE-inhibitory peptides of low molecular mass in several ripened cheeses.

A potent antihypertensive effect has been reported for a whey protein concentrate hydrolyzed with alcalase (Costa, Almeida, Netto, & Gontijo, 2005). Those authors suggested a pathway involving ACE inhibition as mainly responsible for this effect. Several ACE-inhibitory and antihypertensive peptides have been isolated and characterized from α-LA and β-LG hydrolyzed with digestive enzymes (Chobert et al., 2005; Pihlanto-Leppälä, Koskinen, Piilola, Tupasela, & Korhonen, 2000; Sipola et al., 2002).

Many casein-derived ACE-inhibitory peptides are described, and it has been also demonstrated that the major whey proteins contain encrypted peptides that inhibit ACE. The primary sequences of some ACE-inhibitory peptides derived from α-LA and β-LG are summarized in Table 2. Pihlanto-Leppälä et al. (1999) demonstrated that the synthetic peptides corresponding to the sequences β-LG f(102–105) and α-LA f(50–53) are bioactive peptides presenting ACE-inhibitory activity (Table 2).

ACE inhibitory peptides are inactive within the sequence of the parent protein, but they can be released by enzymatic hydrolysis. Selecting the proper enzyme to hydrolyze the protein is a key factor in obtaining peptides that exhibit greater levels of ACE inhibitory behavior. Trypsin appears as a promising enzyme to release bioactive peptides, since it also improves protein digestibility and decreases protein allergenicity (Mullally et al., 1997a; Pihlanto-Leppälä et al., 2000).

Chobert et al. (2005) investigated ovine β-LG hydrolyzed with trypsin, and yoghurts made from ovine milk using different starters. Those authors identified several peptides responsible for ACE-inhibitory activity in this hydrolysate (Table 2). Didelot et al. (2006) used cheese microbiota to produce several hydrolysates of acid caprine whey with ACE-inhibitory activity and identified the α-LA fragment f(104–108) in the most active fraction. A more recent study also used microorganisms isolated from raw milk cheeses to produce several hydrolysates of acid caprine whey with ACE-inhibitory activity, although no specific peptides were identified (Hamme, Sannier, Piot, Didelot, & Bordenave-Juchereau, 2009).

A β-LG hydrolysate was prepared using thermolysin, and two potent ACE-inhibitory peptides were identified, LLF and LQKW (Hernández-Ledesma, Recio, Ramos, & Amigo, 2002). Subsequently, the antihypertensive effect of these two peptides in spontaneously hypertensive rats (SHR) has been reported (Hernández-Ledesma et al., 2007).

Synthetic ACE inhibitors such as captopril, enalapril, lisinopril and alacepril are remarkably effective at regulating blood pressure and are used as clinical antihypertensive drugs (Ondetti, Rubin, & Cushman, 1997). However, these synthetic drugs have demonstrated diverse side effects, such as allergic reactions, skin rashes, cough, and taste disturbances (Bougatef et al., 2008). Therefore, the search for nontoxic, safer, economical, and innovative ACE inhibitors is required for the control and treatment of high blood pressure. Therefore, various food protein-derived bioactive peptides have been isolated and evaluated for their antihypertensive activity aiming to avoid undesirable side effects of synthetic antihypertensive drugs and to avoid increasing cost of drug therapy.

5. Antimicrobial activity

Bioactive peptides presenting antimicrobial effects possess potential applications in both food quality and safety and human health. In the last years, considering the consumer demands for less processed and more natural and/or functional foods, increasing efforts are focused towards the characterization of whey protein hydrolysates and peptides, postulating their use as food-grade preservatives and functional food ingredients. Given the inexpensiveness and the voluminous

Table 2
ACE-inhibitory peptides derived from whey proteins.

Origin	Enzyme treatment	Amino acid sequence	Protein fragment	Reference
Bovine β-LG	Pepsin and trypsin	GLDIQK	f(9–14)	Pihlanto-Leppälä, Rokka and Korhonen (1998)
		VAGTWY	f(15–20)	
		IIAEK	f(71–75)	Power, Fernández et al. (2014)
		IPAVFK	f(78–83)	Power, Fernández et al. (2014)
	Proteinase K	IPA	f(78–80)	Abubakar, Saito, Kitazawa, Kawai, and Itoh (1998)
	Synthetic	YLLF	f(102–105)	Mullally, Meisel, and FitzGerald (1996)
		HIRL	f(146–149)	
	Trypsin	ALPMHIR	f(142–148)	Mullally, Meisel, and FitzGerald (1997b)
	Pepsin, then trypsin and chymotrypsin	CMENSA	f(106–111)	Pihlanto-Leppälä et al. (2000)
		ALPMH	f(142–146)	
		VLDTDYK	f(94–100)	
		VAGTW	f(15–19)	
	Trypsin	VFK	f(81–83)	
		LAMA	f(22–25)	
		LDAQSAPLR	f(32–40)	
	Protease preparation from *Cynara carduncului*s	DAQSAPLRVY	f(33–42)	Tavares, Contreras et al. (2011), Tavares, Monteiro et al. (2011)
	Crude proteinases from *Lactobacillus helveticus* LB10	RLSFNP	f(148–153)	Pan and Guo (2010)
Bovine α-LA	Synthetic	YGLF	f(50–53)	Mullally et al. (1996)
	Pepsin and trypsin	LAHKAL	f(105–110)	Pihlanto-Leppälä et al. (1998)
	Cheese microbiota	WLAHK	f(104–108)	Didelot et al. (2006)
	Trypsin	WLAHK	f(104–108)	Pihlanto-Leppälä et al. (2000)
		VGINYWLAHK	f(99–108)	
	Pepsin, then trypsin and chymotrypsin	YGL	f(50–52)	
	Protease preparation from *Cynara carduncului*s	RELKDL	f(10–15)	Tavares, Contreras et al. (2011), Tavares, Monteiro et al. (2011)
		DKVGINY	f(97–103)	
		KGYGGVSL	f(16–23)	
		DKVGINYW	f(97–104)	
		KGYGGVSLPEW	f(16–26)	
Ovine β-LG	Thermolysin	ALPMHIR	f(142–148)	Hernández-Ledesma et al. (2002, 2007)
		LQKW	f(58–61)	
	Tryptic hydrolysis	LLF	f(103–105)	Chobert et al. (2005)
		ALPMHIR	f(142–148)	
		IIVTQTMK	f(1–8)	

production of whey as a by-product of cheese and casein production, whey proteins could be considered as an abundant resource to obtain antimicrobial peptides. Among whey proteins, lactoferrin, lysozyme, and their proteolytic fragments, are the most studied regarding antimicrobial activity; conversely, the antimicrobial potential of peptides encrypted within the β-LG and α-LA sequences seems to be less exploited (Chatterton, Smithers, Roupas, & Brodkorb, 2006; Hernández-Ledesma et al., 2014). This section deals with the antimicrobial properties of hydrolysates and peptides obtained from WPC, WPI, and purified β-LG and α-LA, which are the most prevalent whey proteins. Hydrolysis is usually carried out with commercial proteases such as trypsin, chymotrypsin and pepsin. The antimicrobial potential of whey protein hydrolysates and peptides is mainly assessed through the *in vitro* effects on microbial growth. Following peptide identification, sequences are synthesized and tested commonly against bacterial strains to confirm the antimicrobial activity (Table 3).

Hydrolysates of whey proteins from camel colostrum, obtained through the action of pepsin and pancreatin as a simulation of gastrointestinal digestion, were also evaluated for antibacterial activities (Jrad et al., 2014). Both non-hydrolyzed and hydrolyzed whey proteins were inhibitory to the growth of *Escherichia coli* XL1 blue and *Listeria innocua* LRGIA01. Particularly, non-hydrolyzed proteins (at 40 g/L) inhibited *E. coli* and *L. innocua* growth by 22% and 16%, and the hydrolysates (at 10 g/L) displayed a growth inhibition of 9% and 11%, respectively, indicating that the natural antimicrobial activity of camel colostral whey proteins (such as lactoferrin) were not affected and/or that antibacterial peptides were released from whey proteins (Jrad et al., 2014). Camel milk whey protein displayed a greater effect on reducing the specific growth rate of *E. coli* Dh1α (16.4%) than bovine whey protein (4.5%) when tested at 0.5 g/L, reflecting the higher contents of antimicrobial factors in the former. Treatment of these protein concentrates with proteinase K resulted in 2.9- and 4-fold increases in antibacterial activities of whole hydrolysates obtained from camel and bovine whey proteins, respectively. Hydrolysis with trypsin, chymotrypsin and thermolysin also showed a trend of increased antimicrobial potential when compared to non-hydrolyzed whey proteins (Salami et al., 2010). Ultrafiltration fractionation of these hydrolysates indicated that permeates of 3-kDa membrane were the most active against *E. coli*. Thus, considering the distinct antibacterial profiles of different whey protein hydrolysates and their ultrafiltration fractions, it is suggested that the protein substrate and the protease employed for hydrolysis affect the generation of antimicrobial peptides (Salami et al., 2010).

The antimicrobial activity against *L. monocytogenes* of the <10-kDa fraction of bovine WPI hydrolysates was obtained after 45–90 min of pepsin hydrolysis, although *E. coli* was not affected and no bacterial inhibition was demonstrated for both strains by trypsin and chymotrypsin hydrolysates. Fractionation of the <10-kDa peptic digest through reversed-phase chromatography yielded five fractions that were differentially inhibitory towards *L. innocua* and *E. coli* (Théolier, Hammami, Labelle, Fliss, & Jean, 2013). Hydrolysis of bovine α-LA with trypsin and chymotrypsin yielded antibacterial peptides (Table 3); whereas peptides derived from pepsin hydrolysis have not demonstrated such potential (Pellegrini, Thomas, Bramaz, Hunziker, & von Fellenberg, 1999). From trypsin hydrolysis, EQLTK (LDT1) and GYGGVSLPEWVCTTF ALCSEK (LDT2) were identified, and the latter fragment is composed of two polypeptide chains linked by a disulfide bridge. Chymotrypsin hydrolysis of α-LA resulted in a different antimicrobial fragment, CKDDQNPH ISCDKF (LDC), also linked by a disulfide bridge. These three α-LA fragments were synthesized and reported to be mostly active against the Gram-positive bacteria tested, especially *Bacillus subtilis*. Interestingly, although EQLTK inhibited *Staphylococcus epidermidis* and *Staphylococcus lentus*, no inhibition was observed against

Table 3
Antimicrobial peptides derived from β-lactoglobulin and α-lactalbumin.

Origin	Enzyme treatment	Peptide sequence[a]	Fragment from original protein	MW (kDa)[b]	Net charge (pH 7)[b]	Antimicrobial activity	Reference
Bovine β-LG	Commercial preparation from bovine pancreas (trypsin + chymotrypsin)	IDALNENK	f(84–91)	0.91	−1	Mainly Gram-positive bacteria (*S. aureus* and *L. monocytogenes*)	Demers-Mathieu et al. (2013a)
		TPEVDDEALEK	f(125–135)	1.24	−4		
	Porcine pepsin	KVAGT	f(14–18)	0.47	1	Mainly Gram-positive bacteria (*Listeria ivanovii*)	Théolier et al. (2013)
		VRT	f(123–125)	0.37	1		
		IRL	f(147–149)	0.40	1		
		PEGDL [or KVGIN from α-lactalbumin]	f(50–54) [or f(117–121)]	0.53	−2 [or 1]	Mainly Gram-negative bacteria (*E. coli*)	
		LPMH	f(143–146)	0.50	0.1	Weak activity against Gram-positive (*Listeria ivanovii*) and Gram-negative (*E. coli*) bacteria	
		EKF	f(134–136)	0.42	0		
	Bovine trypsin	VAGTWY	f(15–20)	0.70	0	Only Gram-positive bacteria	Pellegrini et al. (2001)
		AASDISLLDAQSAPLR	f(25–40)	1.63	−1		
		IPAVFK	f(78–83)	0.67	1		
		VLVLDTDYK	f(92–100)	1.06	−1		
Caprine β-LG	Human gastric juice + duodenal juice	IIVTQTMK	f(1–8)	0.93	1	Weak activity against Gram-negative bacteria (*E. coli*)	Almaas et al. (2011)
		GLDIQKVAGT	f(9–18)	1.00	0		
		SLAMAASDISLL	f(21–32)	1.19	−1		
		DAQSAPL	f(33–39)	0.70	−1		
		VEELKPTPEGNLE	f(43–55)	1.45	−3		
		IIAEKTKIPAVF	f(71–82)	1.33	1		
		VLVLDTDYK	f(92–100)	1.06	−1		
		TPEVDKEALE	f(125–134)	1.13	−3		
		ALKALPMHI	f(139–147)	0.99	1.1		
		LAFNPTQLEGQ	f(149–159)	1.21	−1		
Bovine α-LA	Porcine trypsin	EQLTK	f(1–5)	0.62	0	Mainly Gram-positive bacteria	Pellegrini et al. (1999)
		GYGGVSLPEWVCTTF/ALCSEK	f(17–31)S-S(109–114)	2.25	−1.1		
	Bovine chymotrypsin	CKDDQNPH/ISCDKF	f(61–68)S-S(75–80)	1.65	−1		

[a] Amino acid sequence presented as a one-letter code.
[b] As calculated using the 'Peptide Property Calculator', available at: http://www.innovagen.se/custom-peptide-synthesis/peptide-property-calculator/peptide-property-calculator.asp.

Staphylococcus aureus. In a subsequent work, Pellegrini, Dettling, Thomas, and Hunziker (2001) indicated that bovine β-LG hydrolysis by trypsin released four antibacterial peptides (Table 3). Synthesis and evaluation of their activity range indicated that they were only active against Gram-positive bacteria, mainly *B. subtilis*. None of these peptides were shown to inhibit the fungus *Candida albicans* (Pellegrini, Dettling et al., 2001, Pellegrini, Thomas et al., 1999). In fact, determination of the antifungal activity of peptides released from the major whey proteins seems to be less exploited.

Bovine β-LG and α-LA were treated with trypsin, chymotrypsin, pepsin or pancreatin, and the resulting hydrolysates were shown to possess antimicrobial activity, as evaluated by the stimulation of autolytic systems of 19 bacterial and 5 fungal strains (Biziulevicius, Kislukhina, Kazlauskaite, & Zukaite, 2006). Pihlanto-Leppälä et al. (1999) reported the bacteriostatic activity of α-LA hydrolysates obtained with pepsin or trypsin, and that of β-LG hydrolysates obtained with alcalase, pepsin or trypsin, against a genetically modified luminous *E. coli* JM103 grown at optimal conditions for 6 h. Ultrafiltration of pepsin plus trypsin hydrolysates of α-LA and β-LG suggested that peptides within the <1 kDa fraction were the most effective in inhibiting this bacterium. Additionally, this investigation demonstrated that hydrolysates obtained with different enzymes affected bacterial growth in opposing ways, with some hydrolysates increasing bacterial activity and growth when compared to controls.

The sequential hydrolysis of goat milk WPC with human gastric juice (HGJ) and human duodenal juice (HDJ) was studied by Almaas, Berner, Holm, Langsrud, and Vegarud (2008), Almaas, Holm, Langsrud, Flengsrud, and Vegarud (2006) and Almaas et al. (2011) aiming to mimic human digestion. After this treatment, the major part of α-LA and β-LG remained intact. Obtained hydrolysates displayed a 50% inhibition on *E. coli* HMG INF01 growth, and no significant differences were showed in comparison to native WPC and WPC hydrolyzed sequentially with pepsin and trypsin plus chymotrypsin, even though these commercial enzymes resulted in a more extensive hydrolysis of whey proteins (particularly α-LA) and different peptide profiles (Almaas et al., 2006). *E. coli* K12 was significantly inhibited by WPC hydrolyates after 10 h of growth, particularly those obtained by HGJ + HDJ (27% inhibition), and the major peptides appeared to have a molecular mass of >8 kDa (Almaas et al., 2011). WPC hydrolysis with HGJ resulted in significant inhibition of *Bacillus cereus* RT INF01 growth when compared to native WPC and hydrolysates obtained by HGJ + HDJ treatment (Almaas et al., 2006). In a subsequent study, however, WPC hydrolyzed by HGJ + HDJ displayed 44% inhibition of the same strain, when compared to only 2% inhibition by HGJ hydrolysates, after 10 h of active growth (Almaas et al., 2011). HGJ + HDJ hydrolysates of WPC showed to be strongly inhibitory against *Listeria monocytogenes* (Almaas et al., 2008). However, slight inhibition was reported against *S. aureus* ATCC 25923, and no inhibition was observed towards *Lactobacillus rhamnosus* GG and *Streptococcus mutans* LT11 (Almaas et al., 2006).

Regarding antiviral activity, Oevermann, Engels, Thomas, and Pellegrini (2003) reported that α-LA and β-LG (and also other proteins), chemically modified by 3-hydroxyphthalic anhydride (3-HP), resulted in compounds with activity against human herpes simplex virus type 1 (HSV-1). Hydrolysis of native α-LA and β-LG by pepsin, chymotrypsin or trypsin, followed by fractionation through reversed-phase chromatography, revealed diverse peptide pools that demonstrated antiviral activity. Unfortunately, most of the peptide pools were associated to some degree with cytotoxicity towards Vero cells (Oevermann et al., 2003).

The mode of action of antimicrobial peptides derived from α-LA and β-LG are still a matter of research. It seems that the initial step in bacterial killing involves the attachment/adsorption of the peptide to bacterial membranes, which might imply the participation of electrostatic forces. Following such interaction, the amphipathic character might also play an important role aiming the insertion of the hydrophobic peptide region into the nonpolar membrane core. In this context, diverse mechanisms could lead to the formation of transient membrane pores affecting permeability and/or energy generation processes, or result in the breakdown of the plasma membrane. Although intracellular structures and processes could be targeted by the peptides (such as DNA and protein synthesis, enzyme activity, among others), the plasma membrane needs to be affected to allow the incursion of such peptides into the cytoplasm, as reviewed by Benkerroum (2010) and Akalin (2014).

As presented in Table 3, most of the antimicrobial peptides identified so far from the major whey proteins possess a negative net charge at neutrality. These peptides usually display less activity against Gram-negative bacteria, which could be the result of repulsion between the peptide and the negatively charged lipopolysaccharides on the outer membrane of these bacteria (Pellegrini et al., 1999). Demers-Mathieu et al. (2013a) indicated that antimicrobial activity is related to negatively charged peptides with >8 amino acid residues; however, a positively charged peptide (SAPLRVY; β-LG f(36–42)) showed diminished antimicrobial activity towards both Gram-negative and Gram-positive bacteria. Similarly, the peptide IPAVFK (β-LG f(78–83)), which possesses cationic and hydrophobic characters, was only effective against Gram-positive bacteria (Pellegrini et al., 2001). However, Pellegrini et al. (2001) modified the sequence of a β-LG tryptic fragment, VLVLDTDYK (f(92–100); net charge: −1), that was only inhibitory to Gram-positive bacteria, to yield the peptide VLVLDT**R**YK**K** (net charge: +2), that displayed a decreased activity against *B. subtilis* but was able to inhibit *E. coli* and *Bordetella bonchiseptica*, suggesting that a cationic character could be important for peptides to exert activity against Gram-negative bacteria. Almaas et al. (2011) identified 43 β-LG peptides derived from the hydrolysis of goat milk WPC by HGJ + HDJ. Of those, 10 were synthesized, showing distinct features of size and net charge (as presented in Table 3), and none displayed antimicrobial activity against *B. cereus* and *L. monocytogenes*, although all were shown to inhibit *E. coli* K12 growth by 0.7–12% (Almaas et al., 2011).

Théolier et al. (2013) observed the antimicrobial activity of the <10-kDa fraction of bovine WPI hydrolysates obtained after 45–90 min of pepsin hydrolysis against *L. innocua*, although *E. coli* was not affected and no bacterial inhibition was demonstrated for both strains by trypsin and chymotrypsin hydrolysates. Fractionation of the <10-kDa peptic digest through reversed-phase chromatography yielded five fractions that were differentially inhibitory towards *L. innocua* and *E. coli*. Peptide identification from these fractions yielded six sequences with <1 kDa presenting different characters of charge at neutral pH, and cationic peptides appeared to be mostly active against Gram-positive bacteria (as compiled in Table 3). Ultrafiltered (10-kDa cutoff) bovine WPI hydrolysates obtained with trypsin displayed antimicrobial activity against *E. coli*, *S. aureus* and *Listeria* spp. (Demers-Mathieu et al., 2013a). This hydrolysate was further processed by nanofiltration through a polyamide anionic membrane (2.5-kDa cutoff), and the retentate fraction was demonstrated to possess a higher antimicrobial potential than the filtrate fraction. Characterization and identification of peptides in these fractions indicated that antibacterial activity might be related to negatively charged peptides with >8 amino acid residues. Two of the negatively charged peptides identified from the hydrolysates were synthesized, namely IDALNENK and TPEVDDEALEK from β-LG, and their antibacterial effect against Gram-positive and Gram-negative bacteria was much higher than that showed by a β-LG cationic peptide (SAPLRVY). Additionally, it was demonstrated that the negatively charged peptides were mostly active towards Gram-positive bacteria, and that a higher negative charge resulted in a more pronounced inhibition of Gram-positive bacteria (Demers-Mathieu et al., 2013a).

As reported by Pellegrini et al. (2001), increased hydrophobicity of an α-LA fragment (GYGGVSLPEWVCTTF ALCSEK; f(17–31)S-S(109–114)), obtained by replacing Leu by Ile (resulting in the sequence GYGGVS**I**PEWVCTTF ALCSEK), decreased its antimicrobial activity, indicating that hydrophobicity alone is not a critical determinant for the antibacterial effect of this fragment. In fact, attempts to explain the antimicrobial activity by analyses of single peptide features are, in the majority of cases, unfruitful. Considering the diversity of antimicrobial peptides from α-LA and β-LG, it is difficult to ascertain which factor is the most important for observed activities. Probably, the antimicrobial potential of the identified peptides is the result of multiple features, such as the amino acid sequence, size and composition, secondary structure (particularly helicity), net charge, isoelectric point, charge distribution, and amphipathicity, which could contribute differentially to the inhibitory effects observed towards bacteria (Akalin, 2014; Benkerroum, 2010; Demers-Mathieu et al., 2013a). From a technological perspective, both the protein substrate and enzyme specificity, by acting on different peptide bonds in the protein substrate and resulting in distinct peptides, play a significant role on the antibacterial profile of hydrolysates.

El-Zahar et al. (2004) demonstrated that peptic hydrolysates of ovine α-LA and β-LG inhibited *E. coli* HB101, *E. coli* Cip812, *B. subtilis* Cip5265 and *S. aureus* 9973. However, no antimicrobial activity was observed towards *Salmonella enterica* Cip5858, *L. innocua* R1007 and *S. mutans* Cip103220T. Results from reversed-phase chromatography of β-LG hydrolysates indicated that the presence of hydrophilic and hydrophobic peptides was required for observation of antimicrobial effects.

Whey protein hydrolysates and peptides possessing antimicrobial activities are postulated to act as natural food biopreservatives. Despite the demonstration that antimicrobial peptides could be produced from whey proteins by generally regarded as safe (GRAS) enzymes, much effort is needed to approach this topic in a more practical manner. However, the few available results are promising. For instance, incorporation of an anionic peptide-enriched extract, obtained from nanofiltration of a tryptic WPI hydrolysate (Demers-Mathieu et al., 2013a), was demonstrated to inhibit the growth of *L. monocytogenes* in reconstituted Cheddar cheese, an approach that might contribute to food safety within the hurdle technology concept (Demers-Mathieu et al., 2013b). Nevertheless, little is still known about the suitability and efficacy of their incorporation into food products (or even in model food systems), including a potential allergenic effect of the peptides, particularly those released from β-LG (Benkerroum, 2010). Therefore, these seem to be major research challenges aiming to establish the feasibility of the application of antimicrobial peptides derived from whey proteins in food technology and also in human health.

6. Antidiabetic activity

Type 2 diabetes is a metabolic disorder characterized by impaired insulin secretion by β cells and insulin resistance in tissues, a condition that is associated with the development of several complications, including hypertension and cardiovascular disease. Considering the prevalence of type 2 diabetes (estimated to affect 370 million people) and its increasing trend, different strategies are developed to properly treat hyperglycemia (Kahn, Cooper, & Del Prato, 2014).

It is reported that oral administration of whey proteins and their hydrolysates positively affects blood glucose control and insulinotropic responses in humans (Jakubowicz & Froy, 2013). Similar effects are described in animal models of diabetes and BRIN-BD11 pancreatic β cells (Gaudel et al., 2011). Nongonierma et al. (2013) observed that whey protein hydrolysates enriched in free amino acids and hydrophilic peptides could have been responsible for the increased insulinotropic response of BRIN-BD11 cells. Although not completely understood, the

effects of whey proteins and their hydrolysates on glycemia appear to be mediated, *in vivo*, by the release or the presence of bioactive peptides and amino acids from whey proteins that could stimulate the secretion of gut hormones, and also act as dipeptidyl peptidase IV (DPP IV) inhibitors (Jakubowicz & Froy, 2013).

6.1. Inhibition of dipeptidyl peptidase IV (DPP IV)

Glucagon-like peptide 1 (GLP-1) and the glucose-dependent insulinotropic polypeptide (GIP) are hormones released from the gastrointestinal tract upon food ingestion, increasing insulin secretion by β cells. This characterizes the incretin response, which contributes for up to 70% of insulin release in healthy subjects. In type 2 diabetes, although the incretin effect is greatly depressed, the action of GLP-1 seems to be preserved; however, both hormones are substrates of DPP IV, a ubiquitous enzyme found to be associated with cell surfaces and as a circulating form. Since plasma levels of GLP-1 and GIP decrease rapidly because of DPP IV activity, DPP IV inhibitors could increase the half-life of active GLP-1, potentiating the insulinotropic effect and glycemic control (Power, Nongonierma, Jakeman, & FitzGerald, 2014).

Diverse proteins are considered as precursors of DPP IV-inhibitory peptides (Power, Nongonierma et al., 2014). *In silico* approaches revealed several peptides encrypted within the amino acid sequences of dietary proteins that could act as DPP IV inhibitors (Lacroix & Li-Chan, 2012a; Nongonierma & FitzGerald, 2013b, 2014), including bovine, ovine and caprine whey proteins (Tulipano, Cocchi, & Caroli, 2012). Treatment of whey proteins by proteases is thus investigated to generate hydrolysates able to inhibit DPP IV activity. Such approach represents a valuable strategy from both basic and applied perspectives, expanding our knowledge on DPP IV-inhibitory peptides (Table 4), also suggesting that hydrolysates and peptides might be useful in the management of type 2 diabetes.

Lacroix and Li-Chan (2012b) performed the *in vitro* simulated gastrointestinal digestion of sodium caseinate, WPI, milk protein concentrate, and skim milk powder using a pepsin–pancreatin system, and evaluated the DPP IV inhibitory activity of the hydrolysates. WPI hydrolysates, obtained after 60 min of pepsin treatment, displayed the higher inhibition of DPP IV (IC_{50} of 0.075 mg/mL), and further hydrolysis with pancreatin decreased the DPP IV inhibitory potential. Other 11 commercial proteases were also employed to hydrolyze WPI (60 min treatment) and, although peptic hydrolysates remained as the best DPP IV inhibitor (78% of DPP IV inhibition at 0.375 mg/mL), hydrolysates obtained with thermolysin and Umamizyme K also showed high DPP IV inhibitory activity (63 and 61%, respectively, at 0.475 mg/mL). Therefore, protease specificity affects the DPP IV inhibitory activity of the resulting hydrolysates. Sequential ultrafiltration of WPI hydrolysates was demonstrated to increase the DPP IV-inhibitory activity of hydrolysates obtained with pepsin (1–3 kDa), thermolysin (<1 kDa) and Umamizyme K (1–3 kDa), indicating that smaller peptides were not the main contributors for DPP IV inhibitory activity in all cases (Lacroix & Li-Chan, 2012b). Hydrolysates of bovine WPC and β-LG, obtained with a protease preparation from *Cucurbita ficifolia*, were ultrafiltered and further fractionated by reversed-phase high performance liquid chromatography (RP-HPLC). Fractions of <3 kDa tended to display higher DPP IV inhibitory activities, especially those from WPC hydrolysates (Babij et al., 2014).

Hydrolysis of whey protein by commercial gastro-intestinal preparations resulted in hydrolysates able to competitively inhibit DPP IV activity, with IC_{50} values in the range of 0.99–1.43 mg/mL, also demonstrating superoxide radical scavenging activity (Nongonierma & FitzGerald, 2013a,c). Treatment of the hydrolysates with DPP IV, followed by evaluation of DPP IV inhibition, caused no significant effect on the IC_{50} value (Nongonierma & FitzGerald, 2014). Also, simulated gastrointestinal digestion of the whey protein hydrolysate increased its DPP IV inhibitory potential (IC_{50} = 1.02 mg/mL) (Nongonierma & FitzGerald, 2013a). Fractionation of whey protein hydrolysate (IC_{50} of 1.33 mg/mL) obtained with Corolase PP by ultrafiltration through 5 and 2 kDa cutoff membranes resulted in enrichment of DPP IV inhibitory peptides (IC_{50} of 0.48 mg/mL); also, fractionation through a solid-phase extraction cartridge indicated that the hydrophilic portion displayed higher DPP IV inhibition (IC_{50} of 1.11 mg/mL) (Nongonierma & FitzGerald, 2013a).

Tryptic hydrolysates of bovine β-LG were reported to decrease blood glucose level in mice when compared to controls after an oral glucose tolerance test. This hydrolysate was able to inhibit DPP IV *in vitro* (IC_{50} of 210 μM), and the peptide VAGTWY was suggested to be the major compound responsible for this effect, displaying an IC_{50} of 174 μM (Uchida, Ohshiba, & Mogami, 2011). TPCK-trypsin was employed to cleave bovine β-LG, and the resulting hydrolysate inhibited DPP IV with an IC_{50} value of 1.6 mg/mL, also demonstrating antioxidant and ACE-inhibitory activities (Power, Fernández et al., 2014; Tables 1 and 2). Diafiltration permeates of 5 and 2 kDa membranes displayed

Table 4
Whey protein-derived peptides with dipeptidyl peptidase IV (DPP IV)-inhibitory activity.

Origin	Enzyme treatment	Peptide sequence[a]	Fragment from original protein	MW (kDa)	Net charge (pH 7)[c]	Isoelectric point	IC_{50} (μM)	Mode of inhibition[d]	Reference
Bovine β-lactoglobulin	*In silico*	IPA[b]	f(78–80)	0.30[c]	0	6.01[c]	49	Competitive	Tulipano et al. (2011)
	Trypsin	VAGTWY	f(15–20)	0.70	0	5.93[c]	174	n.d.	Uchida et al. (2011)
							74.9		Power, Fernández et al. (2014)
		IPAVF	f(78–82)	0.54	0	6.01[c]	44.7	n.d.	Silveira et al. (2013)
		IPAVFK	f(78–83)	0.67[c]	1	10.10[c]	149.1	n.d.	Power, Fernández et al. (2014)
							143.0		Silveira et al. (2013)
		TPEVDDEALEK	f(125–135)	1.24[c]	−4	3.50[c]	578.7	n.d.	Power, Fernández et al. (2014)
							319.5		Silveira et al. (2013)
		VLVLDTDYK	f(92–100)	1.06[c]	−1	3.88[c]	424.4	n.d.	Silveira et al. (2013)
	Pepsin	LKPTPEGDL	f(46–54)	0.97	−1	4.07	45	Un-competitive	Lacroix and Li-Chan (2014)
		LKPTPEGDLEIL	f(46–57)	1.32	−2	3.83	57	Un-competitive	
		IPAVFKIDA	f(78–86)	0.97	0	5.84	191	Competitive	
Bovine α-lactalbumin	Pepsin	WLAHKAL	f(104–110)	0.83	1.1	8.76	286	Non-competitive	Lacroix and Li-Chan (2014)
		WLAHKALCSEKLDQ	f(104–117)	1.64	0	6.74	141	Un-competitive	
		LAHKALCSEKL	f(105–115)	1.21	1	8.21	165	Competitive	
		LCSEKLDQ	f(110–117)	0.93	−1	4.37	186	Non-competitive	
		TKCEVFRE	f(4–11)	1.01	0	5.81	166	Un-competitive	
		IVQNNDSTEYGLF	f(41–53)	1.50	−2	3.67	337	Non-competitive	
		ILDKVGINY	f(95–103)	1.03	0	5.83	263	Competitive	

[a] Amino acid sequence presented as a one-letter code.
[b] Peptide synthesized following *in silico* digestion of β-LG.
[c] As calculated using the 'Peptide Property Calculator', available at: http://www.innovagen.se/custom-peptide-synthesis/peptide-property-calculator/peptide-property-calculator.asp.
[d] n.d.: not determined

increased inhibition of DPP IV, with IC_{50} values of 0.58 and 0.53 mg/mL, respectively, although diafiltration retentates had no effect on DPP IV, indicating that low molecular mass peptides appeared to be responsible for the observed bioactivity. From this hydrolysate, Power, Fernández et al. (2014) also identified VAGTWY as the most potent DPP IV inhibitor (IC_{50} of 74.9 μM), and the DPP IV inhibitory activity of IPAVFK and TPEVDDEALEK was demonstrated (Table 4). Interestingly, VAGTWY seems to be a multifunctional bioactive peptide, since it also displayed significant antioxidant and ACE-inhibitory activities. Also, VAGTWY, IPAVFK, and TPEVDDEALEK were previously reported to possess antimicrobial properties against Gram-positive bacteria (Table 3). A hydrolysate of a bovine WPC obtained with trypsin was reported to inhibit DPP IV activity, with an IC_{50} value of 1.51 mg/mL (Silveira, Martínez-Maqueda, Recio, & Hernández-Ledesma, 2013). Sixteen β-LG peptides were identified from this hydrolysate and, from the six peptides synthesized, IPAVF and IPAVFK displayed higher DPP IV inhibition (Silveira et al., 2013; Table 4). Additionally, VLVLDTDYK (from β-LG) was previously demonstrated to possess antimicrobial action (Table 3).

The inhibition of DPP IV by various whey proteins hydrolyzed by pepsin for 60 min was reported by Lacroix and Li-Chan (2013). Hydrolysates derived from α-LA presented higher inhibition (IC_{50} of 0.036 mg/mL), whereas WPI, β-LG, and lactoferrin displayed higher IC_{50} values. After sequential fractionation of the peptic WPI hydrolysates by cation-exchange chromatography (CEC), size-exclusion chromatography (SEC), and RP-HPLC, two fractions displayed the higher DPP IV-inhibitory activities, with IC_{50} values of 0.216 and 0.081 mg/mL, respectively, when compared to the whole (unfractioned) WPI hydrolysate ($IC_{50} = 0.075$ mg/mL) (Lacroix & Li-Chan, 2014). Regarding α-LA hydrolysates, after sequential SEC and RP-HPLC, two fractions presented IC_{50} values of 0.019 and 0.016 mg/mL, respectively, which were more effective than the unfractionated α-LA hydrolysate ($IC_{50} = 0.036$ mg/mL; Lacroix & Li-Chan, 2013). From these experiments, higher DPP IV inhibition was displayed by hydrolysate fractions enriched in non-polar peptides. Subsequently, 24 α-LA peptides and 11 β-LG peptides were identified from the most active fractions of WPI and α-LA hydrolysates, and among the synthesized peptides, LKPTPEGDL and LKPTPEGDLEIL from β-LG, and WLAHKALCSEKLDQ from α-LA displayed the higher inhibitory potential towards DPP IV (Lacroix & Li-Chan, 2014; Table 4).

From the studies summarized in Table 4, DPP IV-inhibitory peptides identified from hydrolysates usually have molecular masses below 2 kDa, and most contain proline and/or hydrophobic amino acid residues within their sequence (Lacroix & Li-Chan, 2012a). Length, net charge, and polarity of whey-derived peptides do not appear to have, *per se*, a predictable impact on inhibitory action or potency; nevertheless, amino acid sequence seems to play more important roles for DPP IV inhibition (Lacroix & Li-Chan, 2014). Peptide inhibitory capabilities seem to result from competitive, un-competitive or non-competitive modes of inhibition (Table 4); thus, peptides displaying the same mode of inhibition could share some features, as well as distinct peptide properties might be more relevant for each mode of action.

DPP IV is an enzyme that releases dipeptides preferentially containing proline (P) as the second residue (P_1) from the N-terminus of polypeptides. Although at slower rates, this enzyme could cleave dipeptides with, for instance, A, L, V, and G at P_1 (Power, Nongonierma et al., 2014). Diprotin A (IPI) and diprotin B (VPL) are potent DPP IV inhibitors showing an apparent competitive behavior by acting as enzyme substrates. *In vitro* evaluation of the synthesized peptide IPA, derived from β-LG by *in silico* digestion, was demonstrated to inhibit DPP IV in a competitive manner, with an IC_{50} value of 49 μM; however, the release of IPA during β-LG gastrointestinal digestion remains to be proven (Tulipano, Sibilia, Caroli, & Cocchi, 2011). Similarly, DPP IV competitive inhibition was reported for the peptide IPAVFKIDA (from bovine β-LG), which might result from its substrate-like structure (Lacroix & Li-Chan, 2014), as also reported for casein-derived peptides (Nongonierma & FitzGerald, 2013b). Similarly, it could be argued that the peptides IPAVF, and IPAVFK (Table 4) might inhibit DPP IV in a similar way. As for the inhibitory potential, their IC_{50} values demonstrate decreasing values in the order IPAVF ~ IPA < IPAVFK < IPAVFKIDA (Table 4). For IPAVFK, the presence of a K residue at the C-terminus could decrease the hydrophobicity of the peptide, affecting the binding or enzyme inhibition; analogously, residues V and F could improve this effect in IPAVF when compared to IPA (Silveira et al., 2013). Considering that the P_1 specificity of DPP IV usually decreases with increasing substrate length (Power, Nongonierma et al., 2014), a high molecular mass could be associated with increased IC_{50} values, at least for competitive substrate-like inhibitors. Regarding the peptide TPEVDDEALEK, Silveira et al. (2013) suggested that, although presenting a P residue at P_1, its higher length could have been responsible for its moderate DPP IV inhibitory activity; also, the C-terminal K residue affects peptide hydrophobicity. Peptides LAHKALCSEKL and ILDKVGINY, from α-LA, were also demonstrated to inhibit DPP IV by direct interactions with the enzyme active sites (Lacroix & Li-Chan, 2014), and Uchida et al. (2011) compared the similarity of the β-LG peptide VAGTWY with the six N-terminal amino acid residues of GLP-1 (HAEGTF) in terms of P_1, and the presence of G, T, and aromatic amino acids within the peptide sequence. In this context, amino acid residues near the P_1 position of the peptide could affect DPP IV activity (Lacroix & Li-Chan, 2012a). However, studies on the susceptibility of peptide bonds within these peptides to hydrolysis by DPP IV, and also identification and testing of hydrolysis products, are needed to clarify the actual mechanism(s) responsible for the observed DPP IV inhibition, such as substrate- or prodrug-type inhibition (Nongonierma & FitzGerald, 2014).

Peptides containing P or hydroxyl-P at position P_1' are not cleaved by DPP IV (Power, Nongonierma et al., 2014). However, the β-LG peptides LKPTPEGDL and LKPTPEGDLEIL, shown to be among the most potent DPP IV inhibitors identified from whey protein hydrolysates, acted to inhibit DPP IV activity in an un-competitive manner, that is, by potentially binding to enzyme–substrate complexes outside the active site and decreasing reaction velocity (Lacroix & Li-Chan, 2014). Interestingly, the α-LA peptide WLAHKALCSEKLDQ displayed an un-competitive inhibitor behavior, whereas WLAHKAL and LCSEKLDQ were reported to act as non-competitive inhibitors, that is, potentially binding outside the DPP IV catalytic center on either the enzyme or the enzyme–substrate complex, and LAHKALCSEKL acted as competitive inhibitor (Lacroix & Li-Chan, 2014). Therefore, structure–activity relationships and the exact means by which whey-derived peptides exert DPP IV inhibition are not completely elucidated (Power, Nongonierma et al., 2014).

Considering the diversity of peptides and their modes of action, whey protein hydrolysates or mixtures of peptides therein could act synergistically, leading to a greater inhibition of DPP IV when compared to individual peptides (Lacroix & Li-Chan, 2014). Fractionation strategies are demonstrated to be suitable and feasible techniques aiming to selectively enrich the DPP IV inhibitory activity of hydrolysates. However, conventional drugs employed as DPP IV inhibitors, such as sitagliptin, are much more potent (IC_{50} in the nanomolar range) than whey protein hydrolysates and peptides (Table 4). Thus, hydrolysates and peptides might not be intended to replace available drugs.

In vitro investigations suggest the potential use of whey protein hydrolysates and peptides as natural complementary approaches, that could be implemented through dietary intervention and food–drug therapies, for the management of type 2 diabetes by inhibiting DPP IV activity and thereby increasing the half-life of incretin hormones (Lacroix & Li-Chan, 2012b; Power, Nongonierma et al., 2014). For instance, an additive effect on DPP IV inhibition was observed when whey-derived peptides and diprotin A (IPI tripeptide; IC_{50} ~ 3–7 μM) were tested in combination with sitagliptin (Nongonierma & FitzGerald, 2013a).

The in vivo availability and activity of whey protein hydrolysates and peptides remain to be determined. A major concern surrounding DPP

IV-inhibitory peptides is their bioavailability upon ingestion. Small-sized and hydrophobic peptides are candidates for intestinal absorption, allowing them to reach the circulatory system and display bioactivity (Nongonierma & FitzGerald, 2014). The components of a whey protein hydrolysate responsible for the insulinotropic effect on BRIN-BD11 cells were able to cross the barrier of an intestinal permeation model (Gaudel et al., 2011). Nevertheless, absorption might not be needed for these peptides to exert DPP IV inhibition, since this enzyme is expressed in high amounts by endothelial cells in close proximity to incretin-producing intestinal cells (Jakubowicz & Froy, 2013). Hence, if DPP IV-inhibitory peptides are present in the intestinal lumen, they could act locally without needing to reach high circulating levels (Tulipano et al., 2012). Shigemori et al. (2014) developed a β-LG-secreting *Lactococcus lactis* strain, and tryptic hydrolysates of recombinant β-LG were observed to inhibit DPP IV activity *in vitro*. Such bacterium could be potentially employed for *in situ* expression of β-LG near the intestinal mucosa, which could be hydrolyzed by intestinal enzymes to release DPP IV inhibitory peptides.

6.2. Inhibition of α-glucosidase

Another strategy to manage type 2 diabetes is the inhibition of carbohydrate-hydrolyzing enzymes, such as the membrane-bound α-glucosidase from the epithelial mucosa of the small intestine, which releases monosaccharides from complex carbohydrates, delaying the degradation of carbohydrates in the gastrointestinal tract, and thus decreasing the postprandial levels of blood glucose (Kahn et al., 2014).

The inhibition of *Saccharomyces cerevisiae* α-glucosidase by fractions of bovine WPC and β-LG hydrolysates, produced with a protease from *C. ficifolia*, was also investigated (Babij et al., 2014). Particularly, from 15 RP-HPLC fractions of WPC hydrolysates containing <3 kDa peptides, six displayed IC_{50} values below 2 mg/mL; whereas from 20 RP-HPLC fractions of WPC hydrolysates with 3–10 kDa peptides, 5 showed an IC_{50} below 4 mg/mL. As a general trend, RP-HPLC fractions from β-LG hydrolysates (<3, and 3–10 kDa) were demonstrated to be less efficient in inhibiting α-glucosidase (Babij et al., 2014). Peptic hydrolysates of bovine WPI, β-LG, and α-LA, at 2.5 mg/mL were observed to inhibit mammalian (rat) intestinal α-glucosidase by 36, 33, and 24%, respectively, whereas the non-hydrolyzed counterparts have not displayed enzyme inhibition. For WPI and β-LG hydrolysates, IC_{50} values were 4.5 and 3.5 mg/mL, respectively (Lacroix & Li-Chan, 2013).

As few non-saccharide inhibitors of α-glucosidase are reported, investigations should focus on the identification of the peptides responsible for enzyme inhibition, also aiming to gain insight on the inhibitory mechanism and in vivo significance (Lacroix & Li-Chan, 2013; Yu et al., 2011).

7. Miscellaneous activities

In addition to the biological activities described in the previous sections, a number of other properties have been also associated with peptides derived from hydrolysis of whey proteins. Hydrolysates, peptide fractions, and/or isolated peptides are described to possess immunomodulatory, antiproliferative, opioid, and other biological activities (Hernández-Ledesma, Ramos, & Gómez-Ruiz, 2011; Madureira, Tavares, Gomes, Pintado, & Malcata, 2010).

A number of immunomodulatory peptides have been associated with whey-derived products (Gauthier, Pouliot, & Saint-Sauveur, 2006; Szwajkowska, Wolanciuk, Barlowska, Król, & Litwinczuk, 2011). Peptides derived from WPI hydrolysis with trypsin/chymotrypsin seem to modulate immune parameters in vivo using non-infected and *E. coli* infected mice model. In particular, the basic F3 peptide fraction showed promising results, stimulating serum TGF-β1 secretion, which coincided with a significant increase in IgA levels (Saint-Sauveur, Gauthier, Boutin, Montoni, & Fliss, 2009). Eriksen, Vegarud, Langsrud, Almaas, and Lea (2008) investigated if whey-derived products with different immunological responses may be generated by using different enzymes. Samples of cow and goat whey were hydrolyzed with either commercial enzymes pepsin and Corolase PP, or by using human gastric (HG) and duodenal (HD) juices. Whey protein samples from both goat and cow showed dose-dependent inhibition of peripheral blood mononuclear cell proliferation *in vitro*. This effect is suggested to be associated with intact or hydrolyzed components in whey samples that affect the generation of important activating signals, thus inhibiting further lymphocyte proliferation.

Eleven synthetic peptides derived from theoretical release from β-LG and α-LA by hydrolysis with trypsin or chymotrypsin were evaluated for their immunomodulatory properties. The peptides β-LG f(15–20), f(55–60), f(84–91), f(92–105), f(139–148), f(142–148) and α-LA f(10–16) stimulated proliferation to different extents, whereas β-LG f(15–20), f(55–60) and f(139–148) also induced various inhibiting and/or stimulating effects on cytokine secretion (Jacquot, Gauthier, Drouin, & Boutin, 2010). These results confirm that hydrolysis of α-LA and β-LG by digestive enzymes may result in peptides that have the potential to influence the specific immune response through the modulation of splenocyte proliferation and cytokine secretion. Recent reports indicate that the addition of whey peptides has a positive effect in development of immune-modulating diets in both murine (Yanagawa et al., 2013) and rat models (Kume, Okazaki, Takahashi & Yamaji, 2014).

Opioid activity of peptides derived from hydrolysis of β-LG or α-LA hydrolysis with digestive enzymes has been described earlier (Antila et al., 1991). Opioid-like sequences are encrypted in the primary structure of major whey proteins: human and bovine α-LA f(50–53) and bovine β-LG f(102–15), which have been termed α- and β-lactorphins, respectively (Pihlanto-Leppälä, 2001). The whey protein-derived peptide, α-lactorphin, with proved opioid activity, although with lower affinity towards μ-opioid receptors than β-casomorphin 7, can induce mucin secretion and mucin gene expression in human colonic goblet-like cells (Martínez-Maqueda et al., 2012). Similar effect was observed for a trypsin β-LG hydrolysate and β-lactorphin, probably operating through an opioid pathway (Martínez-Maqueda, Miralles, Ramos, & Recio, 2013). Whey protein hydrolysates with the ability to modulate mucin production could be promising for improving gastrointestinal protection.

Studies on animal models suggest that certain peptides and amino acids derived from dietary proteins may influence carcinogenesis. These studies, usually for colon and mammary tumorigenesis, indicate that whey protein is superior to other dietary proteins for suppression of tumor development (Parodi, 2007). This benefit is accredited to its high content of cystine/cysteine and γ-glutamylcysteine dipeptides, which are efficient substrates for the synthesis of glutathione. Lactoferrin, a minor whey protein, has received most attention since it inhibits intestinal tumors and possibly other tumors. The lactoferrin-derived peptide lactoferricin acts by induction of apoptosis, modulation of carcinogen metabolizing enzymes and perhaps acting as an iron scavenger (Gilford, Hunter, & Vogel, 2005; Mader, Salsman, Conrad, & Hoskin, 2005).

Peptide concentrates obtained from whey hydrolysis with proteases of *C. cardunculus* displayed antiulcerogenic activity. Both the peptide concentrate and its fraction containing peptides with molecular mass below 3 kDa were both effective against ulcerative lesions of the gastric mucosa induced by oral administration of ethanol in a rat model. Gastric cytoprotection by low molecular mass peptides appears to depend on sulfhydryl-containing moieties, whereas peptide concentrate likely protects the gastric mucosa *via* the prostaglandin cycle and production of nitric oxide (Tavares, Contreras et al., 2011; Tavares, Monteiro et al., 2011). The hypocholesterolemic peptide IIAEK was identified from tryptic hydrolysates of β-LG. Studies on Caco-2 cells and animal model indicate that serum and liver cholesterol levels were significantly decreased in rats fed β-LG hydrolysates, and the inhibition of micellar solubility of cholesterol seems to cause the suppression of cholesterol absorption (Nagaoka et al., 2001). Lastly, the inhibitory activity of

xanthine oxidase by dipeptides Val-Trp or Trp-Val, and the lactoferrin hydrolysate obtained by gastric and pancreatic enzymes was recently described (Nongonierma & FitzGerald, 2012). In vivo, xanthine oxidase is involved in the oxidation of the aldehyde groups in xanthine or hypoxanthine, producing uric acid. Thus, lactoferrin hydrolysates and dipeptides could have potential as a natural alternative to synthetic drugs for xanthine oxidase inhibition, eliminating the side effects associated with drugs such as Allopurinol.

8. Conclusion and perspectives

The dairy industry generates substantial amounts of whey, which should be adequately managed not only to attend to environmental concerns, but also to generate added valued products. The conversion of whey into useful products can be achieved by enzymatic catalysis. Intensive research has been devoted to investigate the ability of different enzymes and conditions to generate bioactive peptides from whey. Several proteolytic enzymes have been used to produce whey-derived products showing improved biological activities, such as antimicrobial, antioxidant, antihypertensive, and antidiabetic, among others. The association of this variety of biological activities with diverse peptide sequences derived from whey proteins indicates that whey is an important source of bioactive peptides. However, additional research is necessary regarding cytotoxicity studies to ensure safety and the absence of adverse effects *in vivo*. The importance and necessity of confirming the already established effects by human trials, evaluating different aspects such as dose, toxicity, and possible side effects should be also considered. Detailed investigation is required to provide a better knowledge about the maintenance of bioactivity during gastrointestinal transit, mechanisms of peptide absorption, and their fate and site(s) of action in vivo. The mechanism of action of many whey-derived bioactive peptides needs to be completely elucidated. As these peptides (or hydrolysates) would be intended to be ingested in food formulations, the interaction with other components in the food matrix should be investigated. In addition, their incorporation as free or encapsulated ingredients is also an interesting topic for future research. The cost of peptide isolation or synthesis leads to the necessity of large-scale production methodologies to warrant sufficient material for in vivo studies and food formulations. Therefore, further technological studies are required to establish experimental conditions to reach higher yields of these bioactive molecules.

Acknowledgments

AB is research awardee of CNPq and APFC received a post-doctoral fellowship of CAPES (Brasília, Brazil).

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