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Producing high Fischer ratio peptides from milk protein and its application in infant formula milk powder

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REVIEW ARTICLE

Abstract

Milk protein is beneficial for the health of infants and is mainly derived from bovine, sheep, goat or camel milk. High Fischer ratio peptides usually comprise two to nine amino acid residues. The ratio is computed as a molar ratio between branched-chain amino acids (BCAAs) over aromatic amino acids (AAAs) with various claimed health-promoting functions. In this Lilliput review, the classification and nutritional functions of milk proteins are discussed, the physiological functions and preparation methods of high Fischer ratio peptides are introduced, and the application of oligopeptide with unique amino acid composition and physiological functions in infant formula milk powder are reviewed. The development status of relevant industry is also summarized with future prospects. It is concluded that application of high Fischer ratio peptides prepared from milk protein in infant formula food is of great significance and requires further studies.

Keywords: milk protein; branched-chain amino acids; aromatic amino acids; Fischer ratio; infant formula milk powder

Introduction

Fischer ratio indicates the value of molar concentration ratio of branched-chain amino acids (BCAA: leucine, isoleucine, and valine) over aromatic amino acids (AAA: tyrosine and phenylalanine) concentration in the peptides mixture (Fischer, 1984). High Fischer ratio (>20) peptides, because of their unique amino acid composition and peptide chain structure, have the following special physiological functions: (1) adjuvant treatment of hepatic encephalopathy which could correct abnormal amino acid patterns and lower the concentration of ammonia in the plasma, thus the amount of precursor AAAs of monamine neurotransmitters into the brain is reduced (Fischer, 1990); (2) promote the balance of negative nitrogen and improve the mental state of hepatic coma. Using BCAA-rich compound, amino acid preparations as nitrogen source would help reduce burden of the liver, adjust BCAA:AAA ratio in plasma, prevent and correct hepatic encephalopathy, reduce the decomposition of skeletal muscle protein, and improve protein synthesis (Moriwaki *et al.*, 2000); (3) lower ammonia in the blood, which otherwise would limit protein intake and lead to malnutrition as well as negative nitrogen balance in the organism (Pimentel *et al.*, 2014); and (4) fight fatigue, hangover, inhibit the growth of cancer cells, and other effects (Howatson *et al.*, 2012). With the continuous development of functional food industry, more and more attention is paid to high Fischer ratio peptide products.

In recent years, studies on high Fischer ratio peptides have focused on plant and/or animal proteins. For example, high Fischer ratio peptides from corn are found to have special physiological function and are easy to digest (Yu *et al.*, 2012). Zhao and Gan (2006) used konjac powder as a raw material; protein was extracted with the first step of enzymatic hydrolysis by adding alkaline protease to mainly obtain AAAs at the terminus of polypeptides chain. Pronase was then added to hydrolyze AAAs. Gel chromatography was used for separation to get high Fischer ratio peptide products. In terms of animal protein, a procedure using immobilized enzymes was developed to obtain protein hydrolysates with a high Fischer ratio from bovine casein—a fraction that represents 24% of total hydrolyzed proteins, with a Fischer ratio of 30.6 (Pedroche *et al.*, 2004). Egg white protein powder was also used as a raw material to get high Fischer ratio peptides thru hydrolysis. The optimal substrate concentration of 5.5% and denatured time of 15 min were determined by nitrogen recovery ratio testing. Alcalase was selected to hydrolyze egg white protein. The composition of analyzed results showed that the Fischer ratio of hydrolysate was 20.46 and the content of free amino acid was 8.04% (Xu *et al.*, 2011). At present, few studies are being conducted on milk protein high Fischer ratio peptides; hence, research on this aspect has important significance for enhancing the added value of dairy products. A summary of main investigations on production of high Fischer ratio peptides from various species is shown in Table 1.

Milk proteins contain rich bioactive peptides, obtained by hydrolysis, which are not only cheap and safe but also easy to be produced on a large scale. At present, dozens of active peptides have been isolated from different milk protein hydrolyzed products (Saadi *et al.*, 2015). Therefore, high Fischer ratio peptides prepared from milk as the source of high-quality protein could partly replace the infant formula of larger milk protein molecules with peptides or free amino acids obtained from hydrolysis to avoid infant protein allergy and cater the patients with

Table 1 Fischer ratio studies for plant or animal proteins.

Protein sources	Protein hydrolyzing enzymes	Methods used for removing AAA	Peptides molecular weight	Original Fischer ratio	Peptides Fischer ratio	References
Antarctic krill	Alcalase, flavorzyme	Activated carbon adsorption	779.9 Da	2.22	21.12	Lan *et al.*, 2019
Corn	α-chymotrypsin, carboxypeptidase A	Idem	180–1,000 Da	2.56	41.87	Li *et al.*, 2019
Corn	Alcalase, neutrase, flavorzyme	Idem	<7 kDa	Not available	>20	Wang *et al.*, 2019a
Skipjack	Pepsin, flavorzyme	Idem	600–1,300 Da	Not available	37.52	Wang *et al.*, 2019b
Rice	Chymotrypsin, carboxypeptidases A	Chitosan-based biosorbent cross-linked with phenethylamine adsorption	Not available	0.86	21.2	Jiang *et al.*, 2017
Whitmania pigra	Alcalase, carboxypeptidase A	Activated carbon adsorption or ion-exchange chromatography	<874.0 Da	3.62	31.92	Ren *et al.*, 2016
Corn	Alkaline protease, neutral protease, papain	Gel filtration chromatography	231–2,392 Da	Not available	28.40	Jiang *et al.*, 2014
Corn	Alcalase	Not available	<5 kDa	3.0	3.1	Yu *et al.*, 2012
Egg white	Alcalase, protease II	Activated carbon adsorption chromatography	350–600 Da	Not available	20.46	Xu *et al.*, 2011
Flaxseed	Thermolysin, pronase	Activated carbon adsorption	<4 kDa	1.59	23.65	Udenigwe and Aluko, 2010
Pearl oyster	Pancreatin	Idem	300–1,800 Da	3.05	24.58	Zheng *et al.*, 2009
Casein	Trypsin, chymotrypsin, carboxypeptidase A	Gel filtration	500–1,400 Da	2.0	30.6	Pedroche *et al.*, 2004
Sunflower	Chymotrypsin, carboxypeptidase-A	Size exclusion chromatography	750–3,500 Da	1.8	75	Bautista *et al.*, 2000
Sunflower	Kerase	Idem	<5 kDa	5.52	20.47	Bautista *et al.*, 1996
Corn	Alkalophilic proteinase, actinase	Idem	Not available	Not available	20.0	Tanimoto *et al.*, 1991

phenylketonuria (Pedroche *et al.*, 2004). This literature review mainly introduced the classification of milk proteins, preparation and purification of high Fischer ratio peptides from milk sources as well as the potential of their application in infant milk powder formula. This may lay foundation for the future research to produce high Fischer ratio peptides from milk protein and the related products.

Milk Protein

Milk protein has high nutritional value and contains all essential amino acids required by a human body. Essential amino acids are the ones that a human body needs but cannot synthesize on its own or cannot synthesize fast enough to meet its needs, and must be obtained from food products. There are nine essential amino acids required by a human body: lysine, tryptophan, phenylalanine, methionine, threonine, isoleucine, leucine, valine, and histidine. Milk protein is rich in the above-mentioned nine amino acids, but there are some differences between different proteins types. The quantity of essential amino acids contained in casein is 45.1 g/100 g, and whey protein has 50.9 g/100 g amino acids (Nimse and Pal, 2015). Milk protein is considered as one of the most nutritious proteins because the content and proportion of amino acids are basically the same as the amount and proportion required by human synthesis. Milk protein can promote the growth and development of human body as well as various metabolic activities whose biological valence reaches 85, and is easy to be absorbed by the body and beneficial for human health. Casein and whey are the two main protein types found in milk in addition of milk fat globular membrane proteins (Hellwig, 2019). The determination of protein levels in various matrices is mostly done by Kjeldahl method of multiplying a conversion factor to the nitrogen content in milk; summing constituent amino acids could be an alternative measurement (Elgar *et al.*, 2020).

Casein

Casein, which is the main component of milk, is the general term for a large group of proteins found in milk (Castro *et al.*, 2009). Casein contains eight essential amino acids, and its protein efficiency ratio is 2.5. As a main protein found in the milk of mammals, including cows, sheep and humans, casein accounts for 80–82% of the total protein content of bovine milk. Human breast milk contains about 29% casein, and sheep's milk contains 75% of casein. Casein consists of αS1-casein, αS2-casein, β–casein, and K-casein, with a mass ratio of about 4:1:4:1 and confers a protein-stable structure (Donato and Guyomarc'h, 2009).

Casein has a unique micelles structure that contributes tremendously to our understanding of milk water-holding capacity by nanoclusters, and coagulation thru acidification of milk-clotting enzymes (Dalgleish, 2011). Lots of studies have been conducted and are still ongoing but there are much unsolved questions about the structure, size, and distribution of casein (Bouchoux *et al.*, 2010). Holt (2016) compares the amino acid sequences of casein in 20 species and finds a large divergence existing mostly in disorder-promoting residues. All caseins belong to a secreted, calcium phosphate-binding protein group encoded with genes developed from ODontogenic AMeloblast (ODAM)-associated protein (Rehan *et al.*, 2019).

Whey protein

Whey protein is a general term for various protein components of supernatant after removal of casein precipitation from skimmed milk, and it accounts for 20% of total proteins found in bovine milk (Almeida *et al.*, 2016). It consists of α-lactalbumin (α-LA), β-lactoglobulin (β-LG), glycomacropeptide, and lactoferrin (Zapata *et al.*, 2017). α-LA and β-LG account for around 70% of whey protein, and provide good physiological benefits as they are good source of essential amino acids, especially BCAA (Almeida *et al.*, 2016).

When milk is processed into cheese, casein is removed, and whey, lactose, and phospholipids are retained (Chevallier *et al.*, 2016). Whey protein is easy to be digested and a good source of calcium with high bioavailability (Hoffman and Falvo, 2004). A large number of *in vitro* and *in vivo* studies have shown that whey protein has the following multiple biological effects: (i) *Immunomodulatory and antibacterial effects*. Whey contains several constituents with broad-spectrum properties. Immunoglobulin, which accounts for 1% of whey protein weight, has antimicrobial property in the gut (Brody, 2000). (ii) *Anticancer effect*. A study has shown that whey protein concentrate supplementation makes tumor cells more sensitive to chemotherapy by depleting glutathione (Tsai *et al.*, 2000). (iii) *Promoting effect on oral health*. Whey protein has been shown to reduce tooth demineralization, and acts as an anti-caries agent (Warner *et al.*, 2001).

Other types

Milk fat globular membrane proteins are tiny spherical objects with a diameter of 0.2–15.0 μm, and are surrounded by a membrane of milk fat globules. They are membrane-binding proteins accounting for 1–2% of milk protein (Michalski *et al.*, 2004). In addition to the above-mentioned different proteins, milk also contains a small amount of alcohol-soluble protein and protein similar to fibrin.

Protein Types in Milk of Different Sources

Human milk

Human milk contains all the nutrients and varieties of bioactive ingredients that body needs. To a large extent, it is a natural, ideal food and affects the growth and development of infants (Klein *et al.*, 2013). Human milk has complete range of essential amino acids. Moreover, protein clots are smaller and easier to be digested and metabolized by the stomach, and this gives higher protein quality (Lönnerdal, 2014).

Bovine milk

Bovine milk is the most commonly used animal milk, accounting for 95% of dairy consumption. It mainly comprises water, protein, fat, lactose, and minerals, and provides the body with essential nutrients (Mestawet *et al.*, 2012). Milk is rich in minerals like calcium, iron, zinc, copper, manganese, and molybdenum, and the ratio of calcium and phosphorus is similar to that found in human milk; therefore, it is conducive for the absorption of calcium by the human body (Kawada, 2017). Bovine milk contains about 3.5% protein, and casein accounts for the largest proportion, followed by lactoalbumin, lactoglobulin, other serum albumins, immunoglobulin, and enzymes. Milk protein contains all the essential amino acids required by the human body, and its relative content is similar to egg protein but with higher digestibility (Liao *et al.*, 2011).

Camel milk

Camel milk is also a high-quality protein source which contains 18 kinds of amino acids, including a variety of essential and non-essential amino acids, other than alanine, isoleucine, glycine, aspartic acid, valine, and cystine. The contents of all other amino acids are higher than those of bovine milk. The amount of total amino acids is also higher than bovine milk. Glutamic acid is the highest and cystine is the lowest amino acid found in both camel and bovine milk (Pihlanto and Korhonen, 2003).

Goat milk

Goat milk mainly provides protein, fat, minerals, and vitamins. It is very close to human milk in terms of compositional structure or nutrient element ratio. Goat's dairy products are widely favored in western countries because of its nutrition value and taste. Goat milk formula has become of particular interest to many families to feed their infants and the elderly (Ceballos *et al.*, 2009). In common with bovine milk, both provide a large amount of nutrients and bioactive substances (Haenlein, 2004). The difference is that goat milk has the advantage of possessing smaller fat globules, which are similar to human milk, and are easy to be absorbed (Clark and García, 2017). Goat milk contains lower levels of casein than bovine milk and is richer in mineral elements like calcium, phosphorus, magnesium and copper, (Ceballos *et al.*, 2009).

Interestingly, peptides derived from casein seems to be particularly suitable for diet with low AAA, which lays a good rationale for developing high Fischer ratio peptides from milk proteins (Kruger *et al.*, 2019). To give an overview of the potential of being used for raising Fischer ratio, the content and composition of main amino acids found in milk proteins from different sources are given in Table 2.

Preparation of High Fischer Ratio Peptides from Milk

Protein hydrolyzation

There are two commonly used methods to prepare oligopeptides: enzymatic and microbial fermentation methods. The main proteases used in enzymatic preparation are given in Table 1, some of them (e.g. alcalase and pepsin) are used to cut polypeptide chains and expose AAAs at chain terminus, while others (e.g. flavorzyme and papain) cleave them into free amino acids. The enzymatic reaction conditions are usually mild and the catalytic location is specific. However, this approach has many by-products and low yield (Le Maux *et al.*, 2016). Microbial fermentation method uses some special microorganisms to break down proteins and get polypeptides after separation and purification (Wei *et al.*, 2014). Compared with enzymatic hydrolysis, this method has the advantage of producing enzyme, and enzymatic hydrolysis in one step which saves the purification and separation of enzyme; therefore, it greatly reduces the preparation time of polypeptide and improves production efficiency (Kim *et al.*, 2001). In addition, microorganisms can also produce endopeptidase, which not only modifies the bitter peptide produced during the enzymatic hydrolysis but also presents a fermented fragrance (Ranathunga *et al.*, 2006). A procedure using immobilized enzymes has been developed to obtain protein hydrolysates from bovine casein with a high Fischer ratio. Pre-digestion with trypsin is followed by treatment with chymotrypsin, which

Table 2 Comparison of protein and essential amino acids in milk from different sources.

	Total protein (%)	Casein (%)	Whey protein (%)	Lysine (mg/g)	Tryptophan (mg/g)	Phenylalanine (mg/g)	Methionine (mg/g)	Threonine (mg/g)	Isoleucine (mg/g)	Leucine (mg/g)	Valine (mg/g)	Histidine (mg/g)
Human milk (Yang *et al.*, 2013)	2.01	40	60	56.1	17	29.5	12.2	31.6	40.9	83.9	48.1	17.5
Bovine milk (Xu *et al.*, 2011)	3.39	80	20	72.8	14	38.5	20.9	32.5	44.7	94.3	46.1	23.8
Goat milk (Moriwaki *et al.*, 2000)	3.84	75	25	76.3	28	39.9	20.9	39.4	45.0	99.3	54.0	24.5
Camel milk (Xu *et al.*, 2011)	4.31	72	28	28.7	11.8	17.4	10.2	17.4	19.8	36.8	22.1	10.8
Soy bean milk (Liu *et al.*, 2012)	3	60	40	63	13	90	26	38	49	82	50	26

generates a hydrolysate enriched in peptides with AAA at the carboxyl end. Carboxypeptidase A is then used to remove these AAA (Pedroche *et al.*, 2004). Wang *et al.* (2019a) found that oligopeptides with high Fischer ratios depended on sequences in corn protein hydrolysates by differing approaches of enzyme hydrolysis, especially BCAA–AAA-containing oligopeptides. The protease isolated from *Bacillus methanolicus* LB-1 (protease LB-1) is a microbial protease proved to possess a high milk-clotting activity and could be potentially used in functional cheese processing and development of bioactive peptides of dairy origin (Yang *et al.*, 2020).

Hydrolysate purification

The components in hydrolysate are very complex, including incompletely hydrolyzed protein, polypeptide, oligopeptide, and a large number of free amino acids and salt molecules. If the oligopeptide mixtures with high purity and activity are to be obtained, the proteolytic substances need to be isolated and purified.

The adsorption and removal of AAAs

There are many methods to remove AAAs from hydrolysate of proteins, among which the activated carbon adsorption method is simple and easy to use (Gineys *et al.*, 2016). The adsorption strength of activated carbon to various amino acids is different and the adsorption capacity of phenylalanine and tyrosine should be strong. Through activated carbon, oligopeptides can be separated from free AAAs. Activated carbon has a high affinity for hydrophobic compounds (Cermakova *et al.*, 2017), and both AAAs and BCAAs are hydrophobic amino acids which can be decreased simultaneously by using activated carbon. Therefore, the selection of activated carbon and the optimization of experimental conditions are the main technical key points.

Li *et al.* (2019) directly performed sequential digestion with α-chymotrypsin and carboxypeptidase A to hydrolyze corn peptides, with the additional step of adsorption of AAAs by activated carbon, and corn oligopeptides with a Fischer ratio of 41.87 and low bitterness were prepared. Directed hydrolysis of corn crude peptides is performed by sequential digestion with the additional step of adsorption of AAAs by activated carbon, corn oligopeptides with a Fischer ratio of 41.87 and low bitterness are obtained (Li *et al.*, 2019). Activated carbon there played the role of decreasing AAA-containing oligopeptide contents.

Ultrafiltration

Ultrafiltration membrane separation takes pressure difference as the driving force, allows the material pass through microporous membrane with different pore diameters, and separates the material according to molecular weight (Yu *et al.*, 2012). During ultrafiltration, molecules which are smaller than the membrane aperture size pass through the membrane with the solvent, while larger molecules are left behind and expelled with the water. Ultrafiltration separation does not change the composition and activity of substances and there is no need to add other additives and performing any pretreatment. Therefore, it is a simple operation with low cost, high recovery rate, time-saving, and is widely used in the separation and purification of substances (Li *et al.*, 2011). Puchalska *et al.* (2014) used 3-, 5-, and 10-ku molecular weight interception filter to ultrafilter infant formula and identified the sequence structures of endogenous antioxidant peptides and angiotensin-converting enzyme (ACE) inhibitory peptides by further isolation and purification.

Other commonly used methods of peptide purification

Macroporous resin has a porous structure and no exchange functional group. The physical adsorption of molecules is realized by hydrophobic groups or intermolecular forces and elution of adsorbed components under the action of eluent so as to achieve separation and purification (Zhang *et al.*, 2008). Macroporous resin has the advantages of high recovery rate, high efficiency, low operating cost, easy regeneration, good selectivity, and mild separation conditions. Moreover, it has a large specific surface area, ideal pore structure, and unique adsorption property, so is widely used in the separation and purification of substances (Cheng *et al.*, 2011). Kumar *et al.* (2017) used DA201-C macroporous resin for the separation of protease hydrolysates from wheat germ; the results showed that it was feasible to separate enzymatic hydrolysates with different concentrations of ethanol according to their hydrophobicity, and the enrichment of hydrophobic amino acids significantly enhanced the antioxidant properties of enzymatic hydrolysates by gradient elution. In order to develop brevilaterin's efficient extraction technology, first, macroporous resin XAD-7HP with good adsorption and desorption effect was screened by investigating the adsorption and desorption capacity of macroporous resin to brevilaterin process, and the optimal technological conditions were obtained through optimization (Ning *et al.*, 2020).

Gel chromatography is a method that uses the network structure of dextran gel to separate substances according to the difference of molecular size in mixture. In the process of separation, molecules greater than the diameter of the gel particle are eluted first because they cannot enter the interior of the gel particle, while smaller molecules enter the interior of the gel particle and are retained for a longer time due to adsorption by the gel (Zhou *et al.*, 2011). Gel chromatographic separation method is widely used in the fields of biotechnology and medicine because of its simplicity and the absence of organic solvent. Three hydrolases (Protex 6L, Protex 7L, and papain) were used to hydrolyze corn gluten meal; oligopeptide mixture was acquired by ultrafiltration then was separated and purified by Sephadex G-15. The relative molecular weight and antioxidant activity of the oligopeptide mixture and separated fractions were determined (Jiang *et al.*, 2014).

Reverse-phase high-performance liquid chromatography (RP-HPLC) is a modern technology that has a series of advantages such as convenient operation, high sensitivity, and good separation effect. Chen *et al.* (2012) separated and purified the enzymatic hydrolysate of walnut by ultrafiltration, gel chromatography, and RP-HPLC, and finally obtained a peptide fragment with high antioxidant activity. Wu *et al.* (2007) studied and optimized optimal conditions for the separation of blood pressure-lowering peptide AHP by RP-HPLC: Agilent 1100, reverse-phase column, 4.6 × 250 mm (238TP54), TFA concentration 0.03% (v/v), time 10.30 min, elution velocity 0.8 mL/min, and ACN concentration 12% (v/v).

The preparation process of high Fischer ratio peptides is summarized in Figure 1.

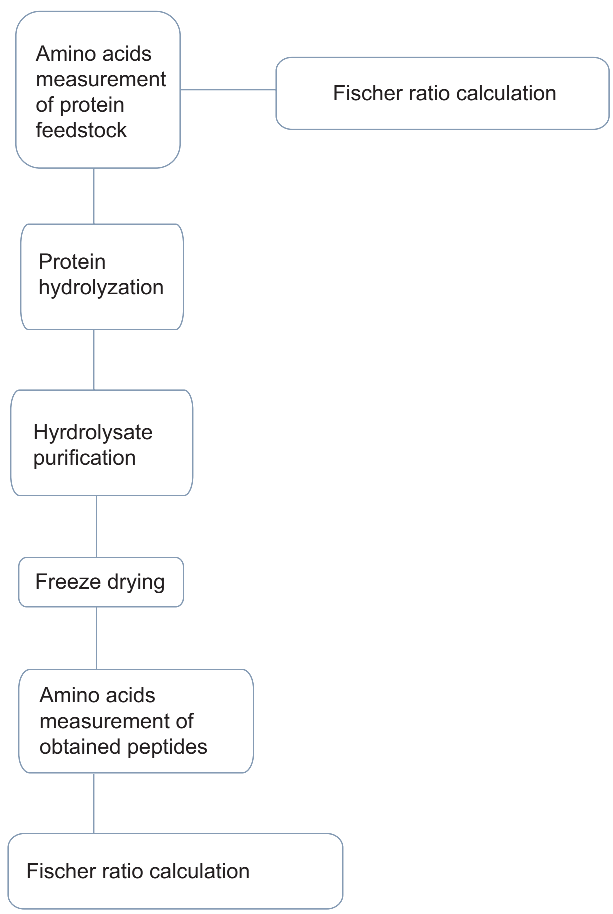

Figure 1. Flow chart of preparation of high Fischer ratio peptides.

Application of High Fischer Ratio Peptides in Infant Formula Milk Powder

With the rapid development of food technology in the 20th century and the continuous progress of nutrition, biochemistry, pharmacy, toxicology, and immunology, infant formula continues to develop in the direction of imitating breast milk (Martin *et al.*, 2016). Since the 1990s, the study of infant formula milk powder has been toward improving the digestion and absorption characteristics and strengthening its necessary nutrition and functional components in order to become closer to breast milk (Knip *et al.*, 2010). At present, infant formula milk powder's emulation of human milk is by no means a simple simulation of protein composition; milk metabolism, biological availability, and activity should also be considered. Lacking bioactive protein is one of the main problems to be solved in the process of infant formula milk powder protein development (Owen *et al.*, 2005).

More specifically, owing to different physiology patterns between infant and adult, incorporating partially hydrolyzed protein (to prevent atopic dermatitis), highly hydrolyzed protein, and free amino acids (to circumvent allergy) in the formula has become very necessary to cater for infant needs (Nutten, 2016). High Fischer ratio peptides can be made from hydrolyzed milk protein and then adsorbed. This cannot only meet the protein requirements of infant formula milk powder but also make full use of the special physiological function of high Fischer ratio peptides to help in counteracting certain pediatric symptoms (e.g. phenylketonuria). Although studies done to raise Fischer ratio are quite extensive, its application in infant formula milk powder is still lacking to our knowledge, as loss of nutrients by heating process and bitterness of peptides might be the limiting factors. Pires *et al.* (2020) demonstrated that Ohmic heating contributed to the maintenance of essential nutrients and reduction in formation of Maillard reaction by-products in infant milk formulas. Li *et al.* (2019) obtained less bitter, high Fischer ratio corn peptides by using α-chymotrypsin and carboxypeptidase A followed by activated carbon adsorption of bitterness-causing hydrophobic AAAs, both of which could be the future applying directions for food industry.

Conclusions and Prospects

To sum up, a large number of studies have been conducted for preparing high Fischer ratio peptides, and a relatively mature preparation system has been established for the selection of protein raw materials and extraction process. Scholars have conducted comprehensive research on the preparation technology of high Fischer ratio peptides and invested more energy in the treatment of oligopeptide solution to study the conditions of using activated carbon to remove AAAs. In addition, the bioactivity of high Fischer ratio peptides in anti-fatigue, antioxidation, and auxiliary treatment of liver protection has been explored actively. In recent years, the preparation of high Fischer ratio peptides from plant proteins, such as corn protein powder and soybean protein, has been studied in depth, but there are relatively less studies on animal proteins. Using milk protein as a raw material could have important practical guiding significance for promoting the deep development of dairy industry and further increasing the added value of infant formula products by improving the absorption rate of nutrients present in milk as well as helping to alleviate phenylketonuria syndrome.

Conflict of interest

None

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Compliance with ethical guidelines

Authors confirm that the research met the ethical guidelines.

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