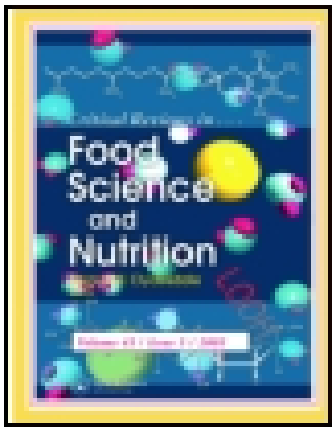

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Preparation, properties and uses of enzymatic milk protein hydrolysates

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Abstract

Enzymatic hydrolysis of milk proteins has been the subject of numerous research studies and patents. The driving force for these studies has been the increased utilization of milk proteins. The industrial uses of milk proteins are based on their unique composition, functionality and nutritive value. The diversity of milk protein fraction, the large number of proteinases and controlled hydrolysis conditions used resulted in the preparation of hydrolysates suitable for several purposes. Enzymatic hydrolysis of milk proteins modifies the technofunctional and biofunctional properties of the hydrolysates depending on the enzyme(s) and hydrolysis conditions used. Milk protein hydrolysates (MPH) are used commonly in normal and clinical nutrition and as functional food ingredient. In the present review emphasis has been made to highlight methods applied for the preparation of MPH, and the functional properties and utilization of the obtained hydrolysates.

Key words: hydrolysates, casein, whey proteins, functional properties, nutrition, bioactive peptides, sport drinks, phenylketonuria, hypoallergenic

Introduction

Protein hydrolysates are defined as ñmixtures of polypeptides, oligopeptides and amino acids that are manufactured from protein sources using partial hydrolysis" (Schaafmsa, 2009). Hydrolysis is done by using food grade proteolytic enzymes, or suitable acids and alkalis.

Enzyme hydrolysis is greatly preferred because acid and alkali hydrolysis are difficult to control. Also, acid hydrolysis oxidizes Cys and Met and partially destroys Ser and Thr and converts Gln and Asn to Glu and Asp acids and thus lowering the quality and biological value of the protein. On the other hand alkali hydrolysis causes racemization in amino acids. In addition, enzymatic hydrolysis of proteins is an affordable technology to produce high value added products. Enzyme-catalyzed hydrolysis provides several advantages including fast reaction rate and high specificity. Also, enzyme hydrolysis is carried out under mild conditions resulting in minimum changes in proteins and generates food-grade protein hydrolysates.

Proteolysis reduces the molecular weight of the hydrolyzed protein and changes its conformation and hydrophobicity. These changes depend on the specificity of the used protease, degree of hydrolysis (DH) and the protein subjected to hydrolysis.

Enzymatic hydrolysis of proteins is carried for several purposes and are discussed here in briefly category wise:

1- Human nutrition. Consumption of protein hydrolysates has been shown to result in more rapid uptake of amino acids compared to the intact protein or the free amino acid mixtures. Also, protein hydrolysates may have biologically active peptides that exhibit several physiological functions. In addition, protein hydrolysates are characterized by hypoallergenic properties compared to the intact proteins. The high availability of the

amino acids, the presence of the bioactive peptides, and the hypoallergenic characteristics have resulted in the incorporation of protein hydrolysates as a principal ingredient in medical foods such as geriatric food supplements, weight-control, sport nutrition, infant formulae and therapeutic diets or for clinical treatments in patients suffering from digestion or absorption disorders or malnutrition association with cancer, trauma and burns (Clemente, 2000). Modified protein hydrolysates have been used in the nutrition of subjects with metabolic disorders such as phenylketonuria.

2- Biotechnology applications. Traditionally protein hydrolysates have been long used to provide the nitrogen requirements for microorganisms grown in laboratory. Today, protein hydrolysates are widely used in other areas of biotechnology such as (1) animal cell cultures for the production of monoclonal antibodies, therapeutic proteins and enzymes (2) recombinant cultures for the production of therapeutic drugs and vaccine (3) tissue cultures (4) plant cell cultures for a variety of end products (5) specialized media for growing and expressing genetically modified microorganisms (Pasupuleti and Demain, 2010).

3- Food industry. Limited hydrolysis of proteins changes their functional properties (Neklyudov *et al.,* 2000). Due to their better emulsifying properties, the partially hydrolysed proteins have been used as emulsifiers in food products such as meat products.

4- Animal feeding. The productivity of livestock is largely dependent of the quality of feed particularly high quality protein. Protein hydrolysates from mixed sources can provide products of improved protein quality to be incorporated in animal feed. Protein

hydrolysates have been incorporated in animal for high milk output and meat quality (Gilbert *et al.,* 2008).

5- Fermentation industry. The use of protein hydrolysates provide microbial cultures used in the fermentation industries with their nitrogen needs in more readily accessible form to improve the performance of microbial cultures and maximize their fermentation products.
6- Cosmetic industry. Enzymatic protein hydrolysates from plant and animal sources function primarily as skin and hair conditioning agents in personal care products.

Preparation of hydrolysates from milk proteins has received much attention due to the diversity, unique functional properties and high nutritional value of milk proteins and as rich sources of bioactive peptides. Milk protein hydrolysates represent a significant segment of world protein hydrolysates. It has been estimated that the world annual market for hydrolysates derived from whey proteins, milk proteins and casein at 8000, 4000 and 3000 metric tons respectively (Schaafmsa, 2009). Milk protein hydrolysates have been used in almost all areas of protein hydrolysates applications (Fig 1)

The aim of the present article is to compile and discuss different aspects concerning the preparation and utilization of milk protein hydrolysates with special emphasis on their use in human nutrition.

2-Preparation of milk protein hydrolysates.

1-2 Methods used.

The manufacturing processes of protein hydrolysates have been dramatically improved due to the increased demand for protein hydrolysates in new and diversified areas such as animal cell

cultures. Three methods (Fig 2) can be followed in the preparation of protein hydrolysates. These are:

1- The direct addition of the selected enzyme to the protein solution to induce hydrolysis under the optimum conditions for the enzyme activity until a desired degree of hydrolysis (DH) is attained. The degree of hydrolysis is the proportion of cleaved peptide bonds in a protein hydrolysate. The reaction is then terminated by heating the solution to inactivate the enzyme. The method is the widely used process on industrial scale for the preparation of protein hydrolysates due to its simplicity. However, it has several disadvantages:
    - The enzyme is used only for one time. Due to the high cost of many enzymes, the process economy may limit its industrial application.
    - Difficulty to control the hydrolytic products. The reaction time and condition influence greatly the type and concentration of the hydrolytic products.
    - The inactivated enzyme remains in the reaction product which may add to the allergenic properties of the hydrolysates.
    - There is a risk of causing undesirable changes in the hydrosylates by the high heat treatment subjected to inactivate the enzyme.
2- Membrane bioreactor which can be used for continuous hydrolysis and removal of selected hydrolytic products. The reaction vessel is connected to a membrane system in a closed circuit. The selected enzyme is added to the protein solution in the reaction vessel. After an initial incubation time, the vessel contents are pumped in the membrane system which allows the selective separation of the hydrolytic products depending on their molecular mass and the molecular cutoff of the membrane. The enzyme, the

unhydrolysed protein and large hydrolytic products are retained by the membrane to be returned back to the reaction vessel. This enables the process efficiency and productivity exceeding those obtained by the classical methods. Casein was continuously hydrolysed with *Aspergillus oryzae* protease in a pilot scale formed-in-place membrane reactor (Chiang *et al.,* 1995). A high percentage (>99%) of trichloro acetic acid (TCA) soluble nitrogen in the hydrolysate (product) was achieved after 45 min at 37°C and pH 7. The product was completely soluble over pH range 2-9. Water sorption increased 4 to 6.5 times in the range of water activity of 0.35-0.95 as compared to intact casein. The immunologically active casein and immunologically active whey proteins in the product were reduced 99% and 97%, respectively. Long term operation showed that the membrane reactor maintained steady production of casein hydrolysate longer than 17 hr. Lin *et al.,*(1997) prepared casein hydrolysate (DH 23.2%) with low free amino acid using alcalase in a two-stage membrane bioreactor. The hydrolysate retained 2.32 and 1.09% immunologically active casein and whey protein respectively. A cyclic batch membrane bioreactor was used for the production of whey protein hydolysate of 1000 times less antigenicity than the original proteins (Prieto *et al.,* 2007). Hydrolysis was carried out using substilisin at 60ºC and pH 8.5. In order to achieve the target for reduced antigenicity, a DH of 15% was needed. The reuse of the enzyme up to five times was reported which allowed for 59% saving in the used enzyme. Casein was hydrolysed with thermolysin and substilisin in a membrane reactor (Trusek-Holownia, 2008). Thermolysin was selected as the most appropriate catalyst. At high substrate retention (about 50%) a high hydrolysis was obtained. The use of enzymatic membrane bioreactor

for the preparation of whey protein hydrolysate has been the subject of many patents. Prieto *et al.* (2010) surveyed 20 patent issued between 1989 and 2009 for the use of membrane bioreactor to prepare whey protein hydrolysates for different purposes. The DH and the molecular cut off of the membrane used for the fractionation of the hydrolysate determine mainly the composition and properties of the obtained hydrolysates.

3- Immobilization of enzymes is carried out on a solid matrix or a membrane by covalent binding and utilized in the preparation of protein hydrolysates. The immobilized enzyme is either stirred in the protein solution for a determined time and then removed by filtration or the protein solution is passed through the immobilized enzyme bed. The protein hydrolysate is obtained at the column outlet. In the case of enzymes immobilized on membranes, the hydrolytic products are selectively separated by the membrane filtration. Several proteolytic enzymes were immobilized on amino propyl-glass beads (Haque and Mozaffer, 1992a) and used in the hydrolysis of casein. Immobilized papain, trypsin and rhozyme 41 extensively hydrolyzed casein within 30 min whereas hydrolysis of casein with immobilized chymotrypsin was slow and gave few peptides.

2.2. Enzymes used and hydrolysis conditions

Selection of the proteolytic enzyme(s) for the preparation of protein hydrolysates, hydrolysis condition and DH depends on the target uses of the hydrolysates. Hydrolysates are usually classified according to DH to partial hydrolysates and extensive hydrolysates. Partial (limited) hydrolysis of proteins aims to improve the functional properties of proteins, while extensively hydrolysed proteins are mainly used in normal and clinical nutrition. Proteolytic enzymes from

animal (pepsin, trypsin, chymotrypsin and pancreatin), plant (such as papain, bromelin and ficin) and microbial (such as neutrase®, alcalase®, substilisin®, protamex®, flavourzyme® and cornolase®) sources have been singly or in combination or sequential in the preparation of protein hydrolysates. The hydrolysis conditions (pH, temperature, ionic strength, enzyme: substrate ratio, and time) are selected within the specified ranges for the enzyme action and the required DH. The use of microbial proteases is increasing due to their wide and diversified specificities and ranges of their actions (Table 1). The commercial preparation of the different proteolytic enzymes may contain traces of other proteolytic enzymes which may modify the obtained hydrolysate.

Heat denatured whey protein isolate (HDWPI) was hydrolysed with trypsin, chymotrypsin, alcalase or neutrase to 2.8, 4.3, 6.0 or 8.0% DH. Analysis of freeze-dried fractions revealed a linear increase in primary amino groups, non-protein nitrogen and ash contents. Polyacrylamide gel electrophoresis showed that high and intermediate molecular weight peptides were converted to lower molecular weights with progress of hydrolysis. Differences in proteolysis patterns were observed with different enzymes. The time required to achieve equivalent hydrolysis at 1, 2, 3 or 4% enzyme/substrate (E: S) ratio varied with the type of enzyme and DH (Mutilangi *et al.,*1995). Whey protein components were hydrolysed with corolase 7092 (peptidases from *Aspergillus* strains), pepsin and corolase PP (a mixture of pancreatic enzymes), either individually or in combination, to eliminate protein allergenicity (Ena *et al.,* 1995). Enzyme specificity rather than DH or molecular mass distribution of hydrolysates determined the residual antigenicity of the whey proteins in the hydrolysates. Ultrafiltration was performed to remove large peptides the

permeate containing the small peptides was used to prepare the hypoallergenic whey protein hydrolysates.

In order to obtain casein hydrolysates containing high dipeptides and tripeptides contents, substilisin and trypsin were used separately or in mixture or simultaneously (Morato *et al.,* 2000). The hydrolysate with the highest small peptide was obtained when substilisin was only used at E:S 4%. Using substilisin first for 5 min and then trypsin gave hydolysate with the lowest large peptide content.

Hydrolysis of native and heated whey protein concentrate (WPC) with pepsin followed by trypsin revealed greater hydrolysis of WPC (Kim *et al.,* 2007a). The highest hydrolysis and the lowest antigenicity were obtained when heated WPC was incubated with 1% pepsin followed by 1% trypsin for 120 min. Heat treatment altered the rate of whey protein hydrolysis and the peptides released from the different whey proteins (OôLoghlin *et al.,*2013). Native and heat denatured WPI were subjected to hydrolysis (DH 5%) with corolase PP. Hydrolysis of denatured WPI favoured the generation of higher levels of free essential amino acids and increased the number of peptides derived from Ŭ-lactalbumin and glycomacropeptide.

The use of combinations of different enzyme was more effective in reducing the antigenicity of whey proteins than the use of single enzyme (Nakamura *et al.,* 1993). Hydrolysis of whey proteins with alcalase followed by treatment with papain was highly effective in reducing the immune reactivity and improving the sensory properties of the hydrolysate (Wröblewska *et al.,*2004). Two enzyme (1:1) combinations of trypsin (T), papain (P), nutrase (N) and protease S (S) were used for the hydrolysis of whey proteins (Shin *et al.,*2007). The lowest antigenicity was observed in hydrolysates prepared with the use of trypsin-containing combinations and that the

TP and TN mixtures were the most effective in removing of ß-lactoglobulin from the hydrolysate. The newly acid proteinases MPiAP1 and MPiAP2 produced by *Monascus pilosus* were found effective in complete digestion of Ŭ-lactalbumin (Lakshman *et al.,* 2011). Complete digestion of WPC proteins was achieved with the combination of *Monascus pilosus* proteinases and trypsin. The obtained hydrolysates were characterized by the lowest antigenicity compared to hydrolysates prepared by the use of trypsin and *Monascus pilosus* proteinases separately.

Changing the hydrolysis conditions can be used to regulate the antigenicity of whey protein hydrolysate. Treatment of whey protein concentrate with alcalase at different pH, temperature and enzyme to substrate ration resulted in different rates of antigenicity reduction (Zheng *et al.,* 2008). Combinations of these parameters can be used to regulate the antigenicity of whey protein hydrolysate. The anti-Ŭ-lactalbumin and IgG binding inhibition was negatively related to the DH, while the anti-ß-lactoglobulin binding inhibition was not related to DH. Combined microwave treatment and peptic hydrolysis reduced the immune reactive binding properties of whey proteins better than the combined heat treatment and enzymatic hydrolysis (El-Mecherfi *et al.,* 2011).

The proteolytic effects of pancreatin and proteases from *Bacillus licheniformis, Aspergillus oryzae* and *Aspergillus sojae* on whey protein concentrate (WPC) at enzyme: substrate ratios (E:S) of 0.5:100, 1:100, 2:100,3:100, 4:100 and 8:100 were evaluated (Morais *et al.,* 2013). Protease from *Bacillus licheniformis* at E:S 8:100 and pancreatin at E:S 2:100,3:100, 4:100 and 8:100 respectively yielded WPC hydrolysates with the highest sums of dipeptides and tripeptides and free amino acid contents and the lowest amounts of large peptides compared to other proteases used (Morais *et al.,*2013).

ɓ-Casein (ɓ-CN) and ɓ-lactoglobulin (ɓ-Lg), were hydrolysed with alcalase, neutrase and bromelain at different time points. Hydrolysates were analyzed by SDS-PAGE and RP-HPLC. Selected samples were tested against 6 patientsô IgE sera by inhibition ELISA respectively (Herranz *et al.,* 2013). Higher values of IC50 (lower allergenicity) for ɓ-CN and ɓ-Lg hydrolysates were obtained at higher incubation times when alcalase or neutrase were employed as enzymes. ɓ-CN was hydrolyzed more efficiently showing higher IC50 values than ɓ-Lg, at similar incubation times. Between the two enzymes, neutrase produced hydrolysates with lower allergenicity. On the other hand, bromelain had no influence on ɓ-Lg hydrolysis or allergenicity, but it showed a discrete effect on ɓ-CN hydrolysis with a moderate influence on its allergenicity (Herranz *et al.,* 2013).

2.3. Debittering of milk protein hydrolysates

The release of bitter tasting properties is a negative aspect associated with most protein hydolysates. Bitterness has been attributed to the release of peptides containing hydrophobic amino acid residues (Ney, 1971). Hydrophobic peptides are usually characterized by bitter taste. Compared to casein, whey proteins develop less bitterness on hydrolysis due to their low contents of hydrophobic amino acids. Therefore, peptides found in whey protein hydrolysates are less hydrophobic than that found in casein hydrolysates. Removal of bitter peptides from protein hydrolysates using different matrices or extraction has been proposed but these methods suffer from losses in some amino acids from the hydrolysates. The reaction conditions during the preparation of the hydrolysates may play a highly significant role in the development of bitterness. Increasing the total solids during the preparation of whey protein hydrolysates (WPH) significantly decrease its bitterness (Spellman *et al.,* 2005). The bitterness of WPC hydrolysates

was affected by the type of enzyme used (Spellman *et al.,* 2009). Comparing the bitterness of WPH generated by three commercially available *Bacillus* proteinases revealed that alcalase 2.4L hydrolysates were more bitter than those obtained from the action of prolyve 1000 and corolase 7089 on WPC. These differences were attributed to the presence of a glutamyl endopeptidase activity in the alcalase preparation but not found in the other two enzymes (Spellman *et al.,* 2009). Debittering can be mediated using exopeptidases including amino and carboxypeptidases with special emphasis on proline exopeptidases (Rakasakulthai and Haard, 2003). Thus, bitterness of tryptic casein hydrolysate was removed by treatment with Procine exopeptidases immobilized on chitin film (Ge and Zhang, 1996).

The bitter taste and hygroscopic property of WPH limit its direct utilization as food ingredient. Encapsulation of WPH by spray drying using maltodextrin/b-cyclodextrin mixture as wall materials attenuated the bitter taste and enhanced the stability of whey protein hydrolysate (Yang *et al,* 2012).

Masking bitterness has been proposed as an alternative for its removal. FitzGerald and O'Cuinn (2006) reported that bitterness can be masked in hydrolysates by the addition of polyphosphate or specific amino acids such as aspartic and glutamic acids. Several bitter taste inhibitors have been found effective ($P<0.05$) in masking the bitter taste of WPH (low and high DH) and in beverages supplemented with WPH (Laksrisompong *et al.,* 2012). Sweeteners (sucralose, fructose and sucrose) effectively inhibited bitterness of WPH and enhanced flavour of vanilla and chocolate beverages containing these hydrolysates.

Encapsulation of WPH with whey protein concentrate (WPC) or with mixture of WPC and sodium alginate was beneficial for reducing the bitter taste and hygroscopicity without impairing

the immunoregulatory activity of WPC (Ma *et al.,* 2014). Spray drying or freeze drying of non-encapsulated or encapsulated WPH did not exert any negative effect on the immunomodulatory activity of WPH.

3. Functionality of milk protein hydrolysates

Dramatic changes in the functional properties of milk proteins occur upon proteolysis due to changes in size, structure and hydrophobicity of the hydrolytic products. These changes depend of the degree of protein hydrolysis. An increase in solubility and decrease in viscosity are generally expected as hydrolysis progressed. Also, proteolysis reduces gel formation, increases foam volume, decreases the foam stability and enhances thermal stability of protein hydrolysates. Emulsifying activity may be altered depending on the nature of the protein substrate and the type of the enzyme used and extent of hydrolysis.

The extent to which a protein is hydrolyzed (DH) and the specificities of the enzymes used for hydrolysis were found to be extremely important in determining the change in functionality (Abert and Kneifel, 1992). Casein hydrolysates prepared using 15 proteolytic enzymes were screened for improvement of functional properties. Apart from type of enzyme used, the relative changes in functional properties also depended on factors such as preheat treatment of milk, pH value, and the degree of hydrolysis (Abert and Kneifel, 1992).

When casein was hydrolyzed by a single or a combination of enzymes, hydrolysates obtained in the low DH range (1 to 4) showed improvements in functional properties (Svenning *et al.,* 1992). The use of commercial enzyme preparations, corolase (*Aspergillus* spp.) PS, and a combination of corolase and neutrase 257 (*Bacillus subtilis* protease) improved emulsifying activity, whereas corolase L 10 (papaya) and naxatase (*Bacillus licheniformis* protease) and Neutrase did not. On

the other hand, hydrolysis of casein by protease from *Staphylococcus aureus* V8 to 2% or 6.7% DH, decreased the emulsifying activity of casein (Chobert *et al.,* 1988b). The V8 protease hydrolyses only peptide bonds in which glutamic acid provides the carbonyl group. However, when casein was hydrolyzed with trypsin to 4.3%, 8.0%, or 9.9% DH, the emulsifying activity increased with DH up to an optimum and then decreased with further hydrolysis (Chobert *et al.,* 1988a). Also, hydrolysis of $\breve{U}_{s1}$-casein with trypsin to 3.2%, 5.0%, 5.8%, and 7.4% DH resulted in hydrolysates of higher emulsifying activity index (EAI) than unmodified $\breve{U}_{s1}$-casein but the emulsions made with the use of the hydrolysates were less stable (Chobert *et al.,* 1989). An increase in the EAI and emulsion stability (Haque and Mozaffer, 1992b) was found when casein was hydrolyzed with immobilized trypsin up to an optimum DH and then decreased with further hydrolysis. The existence of an optimum DH for improvement of emulsifying properties was also reported for the hydrolysis of casein by alcalase and neutrase (Mietsch *et al.,* 1989). Hydrolysis up to 4% DH improved emulsifying activity and then decreased with further hydrolysis. Protomax hydrolysate (DH 0.5 and 1%) of casein showed higher EAI at pH 2.0 as compared with the intact casein (Slattery and FitzGerald, 1998). However, at higher DH of 9.0 and 15% hydrolysates exhibited lower EAI than the caseinate. However, few studies have been done to isolate, identify, and characterize the peptides responsible for improved functionality.

Casein was hydrolysed by trypsin to 5% degree of hydrolysis, and the hydrolysate was fractionated by ultrafiltration. The retentate fraction was fractionated by ammonium sulphate precipitation, ion exchange, and preparative HPLC. The fraction precipitated by 30% ammonium sulphate saturation yielded 3 fractions on anion exchange chromatography. Four peptides with good emulsifying properties were separated by using preparative reverse-phase HPLC. Three

peptides were identified as originating from $\breve{U}_{S1}$-casein (f167-208), ß-casein (f48-63), and (f129-184) but the 4th peptide could not be conclusively identified (Panyam and Kilara, 2004). Casein was enzymatically hydrolyzed at different hydrolysis time (HT) intervals. Extensive antigenicity loss occurred during the first 10% of HT and relatively small changes occurred during the remaining 90% of HT. Hydrophobicity and emulsifying activity decreased, while the magnitude of the net charge increased ($P < 0.01$) with increasing degree of hydrolysis. Strong correlations (r^{*2} 0.89) were found among hydrophobicity, emulsifying activity and zeta potential (Mahmoud *et al.,* 1992).

Peptides with superior foaming properties obtained from the hydrolysis of casein by a fungal alkaline protease were identified by systematic fractionation and purification (Patel, 1994). Enzymatic hydrolysis of casein with protomax (DH 0.5 and 1%) displayed increased foam expansion at pH 2.8 and 10 as compared with unhydrolysed caseinate (Slattary and FitzGerald, 1998).

Comparing the proteolytic effects of papain, trypsin and *Bacillus subtilus* protease revealed that trypsin was the most efficient agent to solubilize the heat denatured whey proteins (Monti and Jost, 1978). The foaming and emulsifying properties of whey protein were improved by limited proteolysis with papain followed by heating under acidic conditions (Lieske and Konrad, 1996). The optimum foaming and emulsifying properties were obtained at about 3.0% degree of hydrolysis. Liberated small peptides increased foaming whereas the emulsifying properties were further improved when these peptides were removed by ultrafiltration. Specificity of the enzyme used and DH influenced the functional properties of hydrolysates from heat denatured whey proteins (Mutilangi *et al.,* 1996). Hydrolysates generated by trypsin at different degrees of

hydrolysis had higher solubility, emulsifying and foaming properties than hydrolysates obtained with the use of nutrase, alcalase and chymotrypsin. Limited proteolysis of whey proteins with immobilized trypsin yielded a fraction (ß-Lactoglobulin residue 41-100 with a disulphide bond to residue 149-162) which showed emulsifying properties 2-3 fold higher than the native protein at pH 3.0 to 9.0 (Chen *et al.,*1994; Huang *et al.,* 1996). Also, limited hydrolysis of heated whey protein isolates (WPI) exhibited gelation characteristics that were different from that obtained from unheated WPI. Values for all textural parameters were significantly greater for gels prepared from partially hydrolysed WPI (Huang *et al.,* 1999). Gels were formed from heat denatured whey proteins partially hydrolysed by enzymatic treatment with trypsin, papain, pronase and *Streptomyces griseus* protease, whereas treatment with pepsin failed to induce gelation in partially heat denatured whey proteins (Sato *et al.,* 1995). The gel strength was dependent on the enzyme used and increased with increasing DH. Treatment with *Bacillus liceniformis* protease partially hydrolysed the major whey proteins and induced gelation in 12% whey protein isolate solution (Otte *et al.,* 1996). The formed gels were characterized by particulate microstructure and that aggregates forming the gel were held together by non-covalent interactions. Peptide aggregation was found to increase with the increase in DH (up to 6.8%) of whey protein concentrate using *Bacillus liceniformis* protease (Creusot, 2006). The dominant aggregating peptides were identified as ß-lactoglobulin (f1-45), (f 90-108) and (f 135-157/158). *Bacillus liceniformis* protease was found to break down the hydrophilic segments in the protein leaving the hydrophobic segments to aggregate once exposed to aqueous solution (Creusot, 2006). Hydrolysis of whey protein concentrate solution (10%) with pepsin (up to 2.5% DH) and trypsin (1% DH) ameliorated the gelling ability of the hydrolysate (Roche *et al.,* 2009).

Addition of small amount (0.1-0.3%) of locust bean gum in the presence of salt enhanced markedly the gel strength of the hydrolysate.

Maximum emulsifying capacity was obtained with WPH of 10 to 20% DH (Singh and Dalglish, 1998) and the formed emulsions were stable at 90ºC for 30 min. Higher hydrolysis resulted in too short peptides to act as emulsifiers. Peptides in the WPH emulsions formed weaker and looser mobile interfacial structures than that formed with intact whey proteins (Singh, 2006). The droplet size of WPH emulsions increased during retort treatment at 121ºC for 16 min indicating coalescence, which was enhanced considerably with the addition of polysaccharides (Singh, 2006).

Whey peptides, with potential functionalities including promoting iron absorption, have been widely studied over several years. Kim *et al.* (2007b) have proven that alcalase had noticeable effectiveness in producing iron-binding peptides from whey protein concentrate. With prominently high lysine, alanine, and phenylalanine content, these peptides could be separated by ion-exchange chromatography and reversed-phase HPLC. ϐ-lactoglobulin hydrolysates obtained with alcalase after hydrolysis for 6h possessed the highest iron-binding capacity (Zhou *et al.,* 2012). The highest yield of complexes was obtained when the mass ratio between ϐ-lactoglobulin hydrolysate and Fe^{3+} reached 40:1, with the optimal pH value of 7.0. All of the spectra indicated that some sites such as amido bonds transformed during chelation, and nitrogen atoms could chelate with Fe^{3+} to form coordinate bonds by offering electron pairs.

Limited enzymatic hydrolysis of ß-lactoglobulin reduced its binding properties towards hydrophobic compounds (Sponton *et al.,* 2014). Immobilized Ŭ-chymotrypsin was used to

hydrolyse (DH 1-5%) ß-lactoglobulin. The binding capacity of the obtained WPH for linoleic acid was less than the intact protein.

5- Applications of milk protein hydrolysates in food products.

The use of milk protein hydrolysates as food texture-modifier has been described in several areas. Casein was hydrolysed with pepsin , the complete hydrolysate (CH) and supernatant obtained by centrifugation (S) were freeze dried (Kwak *et al.,* 2002). Both CH and S were used to replace, partially, the phosphate salt used in the preparation of processed cheese. The casein hydrolysate and its soluble fraction showed good emulsifying properties, meltability, microstructure and textural properties in processed cheese except with high use of the S fraction. The addition of casein hydrolysates and its fraction had no adverse effect on the organoleptic properties of the processed cheese. Whey protein concentrate (34% protein) was hydrolysed with a metalloprotease from *Bacillus amyloliquefacians*, hydrolysate was homogenized and then added to increase the whey protein content to 23-32% of total proteins of milk used for the production of Mozzarella (pizza cheese). It was claimed that the addition of the prepared WPH in cheese manufacture improved the yield and meltability of pizza cheese (Sorensen *et al.,* 2003).

Whey protein hydrolysates have been prepared and used for improving backing, modifying the water retention, softness and organoleptic properties of bread and dough (Souppe and Prodhomme, 2006). Also, whey protein hydrolysates (DH 20%) was fractionated by ultrafiltration. The retentate was used in the manufacture of nutrition bars as high-water activity peptides to avoid the formation of hard crystalline sugars in the bars (Gautam *et al.,* 2006).

Dressings were prepared with WPH (DH 1% with corolase PN-L) and compared to dressing produced with WPC or ß-lactoglobulin (ß-LG). WPH and ß-LG formed mainly creamy dressing

whereas WPC produced mainly thin dressings (Christiansen *et al.,* 2004). The dressings became stable when WPH or ß-LG concentrations were increased from 2 to 4% and oil from 5 to 30% while similar WPC emulsions became less stable. Emulsions made with 4% WPH and 30% oil at pH 4 showed high stability at 75ºC. The microstructures of dressing prepared with the different protein preparations were special and identifiable and dressings showed highly ordered microstructure.

Addition of milk protein hydrolysates had beneficial effects on the survival of probiotics in yoghurt and the quality of the product (Lucas *et al.,* 2004; Sodini *et al.,* 2005; Zhao *et al.,* 2006). Addition of casein hydrolysate or whey protein hydrolysates enhanced the acidification and decreased the coagulation time of yoghurt (Lucas *et al.,* 2004; Zhao *et al.,* 2006), decreased post-acidification during storage (Zhao *et al.,* 2006) improved the survival of probiotics (Lucas *et al.,* 2004; Sodini *et al.,* 2005; Zhao *et al.,* 2006), improved the organoleptic properties and adhesiveness of yoghurt (Zhao *et al.,* 2006) and yoghurt was characterized by open texture and decreased graininess (Sodini *et al.,* 2005).

Whey protein hydrolysates were found to increase the lactose conversion and yield of lactic acid in fermentation of whey permeate (Fitzpatrik and OÕKeeffe, 2001). Whey protein concentrate was hydrolysed (DH 20%) with *Bacillus licheniformis* protease. Addition of 3-4% of the WPH to the whey permeate batch fermented with *Lactobacillus helveticus* for 30-40 hr resulted in complete conversion of lactose to maximum yield of lactic acid. The amount of WPH needed represent 36-47% of WPC separated from whey.

6. Applications of milk protein hydrolysates in clinical nutrition.

6.1. Whey Protein hydrolysates for milk and soy allergic patients.

Food allergy is defined as "an adverse health effect mediated by the immune system which occurs on exposure to a particular food" (Taylor and Hefle, 2006). Cow milk is the most allergent food for infants and children. Global incidence of cow milk allergy (CMA) ranges from 2-3% of new born babies. Once newborn babies get foreign protein either in cowôs milk or commercial human milk substitutes they are sensitized. CMA symptoms can involve gastrointestinal, cutaneous, respiratory or general symptoms (Taylor and Hefle, 2006).

Hydrolysates have long been used for the treatment of food allergies and intolerance and more recently for prevention of atopic dermatitis in high risk infants (Hays and Wood, 2005). Hydrolysates for milk allergic patients must be free from immunogenic and antigenic peptides and proteins. Controversial results have been reported on the immunogenicity and allergenicity of peptides of molecular weight less than 3000 Da. Peptides less than 2500 Da have been considered to be not immunogenic (Cordle, 1994), while Kinghts and Manes (1987) stated that non-antigenic peptides should be smaller than 1500 Dalton. Van Berosteijn *et al.,* (1994) hydrolysed WPC with corolase 7092, separated peptides less than 3000 Da from the hydrolysate by ultrafiltration. They found that these peptides were not allergenic. Peptides of molecular weight > 2600 Da from whey protein hydrolysates were reported to be allergenic whereas peptides below 1400 Da were not allergenic (Van Hoyveld *et al.,* 1998). Puerta *et al.,* (2006) fractionated peptides from hypoallergenic formula and found that peptides of molecular weight less than 3000 Da were antigenic and probably allergenic. It is of interest to note that partially hydrolysed protein can induce tolerance to specific antigen. Consumption of partially hydrolysed whey proteins were able to induce specific oral tolerance to ß-lactoglobulin whereas extensively hydrolysed WPH did not (Fritschè *et al.,* 1997).

The hydrolysates formulas are generally classified into eHfs (extensively hydrolysed formulas) and pHf (partially hydrolysed fomulas) based on the degree of hydrolysis. The pHf formulas are more palatable and less expensive than most eHf formulas and they may induce oral tolerance without sensitization (Exl, 2001). Hays and Wood (2005) evaluated results of studies carried on human subjects between 1985-2005 on the role of protein hydrolysates in prevention of allergy. They concluded that extensively casein hydrolysates and partially hydrolysed whey proteins formulas can be considered as appropriate alternative for breast milk in allergy prevention in infants at risk. Consumption of partial WPH has been widely recommended to prevent the development of allergic disease in early childhood (Heine and Tang, 2008). However, a randomized trial failed to get evidence to support the recommendation for the use of partially hydrolysed whey proteins at weaning for the prevention of allergic disease in high risk infants (Lowe *et al.,* 2011).

Alexander *et al.* (2010) evaluated clinical studies that reported reduced risk of atopic dermatitis (AD) among infants fed with 100% whey partially hydrolysed infant formula (pHf-w) compared with intact cowôs milk formula. They concluded that that the incidence of AD was significantly lower among infants over three years of follow up in the pHf-w group compared with the cowôs milk group.

6.2. Low phenyl alanine milk protein hydrolysates.

Phenylketonuria (PKU) is one of the most prevalent genetically inborn errors of metabolism. It is characterized by high levels of Phe in the blood and massive excretion of its metabolites due to the deficiency of the hepatic enzyme phenylalanine hydroxylase which convert Phe to Tyr. It is estimated that PKU is prevalent at the ratio of 1/15,000. Prenatal infants are protected by

placenta so they are born normal. Rise in blood Phe starts within 24 hr of birth and unless treated it causes brain damage. Irreversible neurological damage occurs in untreated PKU subjects and manifested clinically as mental retardation (Lara *et al.,* 2005). Dietary restriction of consumed Phe is the effective treatment for PKU subjects when done early in infancy (Smith, 1994). However, diets should contain small and controlled amounts of Phe to maintain the normal development of PKU subjects since Phe is an essential amino acid for the integrity of the neuro system (Smith, 1994). Several low Phe preparations (Table 2) have been developed by enzymatic hydrolysis of milk protein fractions and subsequent removal of free Phe from the hydrolysate by adsorption using different matrixes or by nanofiltration.

Glycomacropeptide (GMP) , is a 64-amino acid glycosylated peptide results from the action of chymosin on ə-casein during cheese manufacture. The use of GMP as an alternative protein source for PKU diet has been the subject of an excellent review (Calcar and Ney, 2012). GMP is currently isolated from cheese whey on industrial scale and available commercially as food ingredient and has excellent safety record (Calcar and Ney, 2012). GMP contains no aromatic amino acids including Phe. Therefore, it is uniquely suited to PKU nutrition and several commercial GMP foods including beverages, pudding, puffed cereals, crackers, salad dressing and a snack bar are available now in the market (Calcar and Ney, 2012). However, supplementation with other essential amino acids missing from GMP particularly tryptophan is needed to produce a balanced diet. GMP products have been reported to have better tasting and overall acceptability compared with the PKU amino acid formulas (Calcar and Ney, 2012).

In addition, GMP exhibited several interesting biological activities (Brody, 2000) including inhibition of *E.coli* and *Vibrio chlorea* toxins, promoting the growth of *Bifidobacteria*,

modulation of immune response, attenuating colitis in rats, increasing zinc absorption and promoting satiety. Recently (Badawy, 2013) novel GMP formulations have been proposed for nutritional treatment of maniac and psychotic disorders.

7. Hydrolysates rich in bioactive peptides.

Hydrolysis of proteins set free bioactive peptides encrypted in the structure of the intact protein. Controlling the hydrolysis and fractionation of the hydrolysate can results in hydrolysate fractions rich in bioactive peptides of specific physiological effect. This area has gain increased interest as a natural source for potential medication. Electromembrane separation is a process combining an electric force as a driving force to porous membrane separation. The method has been developed as a powerful tool for the separation of peptides from the complex mixture of protein hydrolysates (Bozinet and Firdous, 2009). These methods can separate complex protein hydrolysate according to molecular weight and charge into three fractions, the basic and acidic small peptide fractions and the intact protein and large peptide fraction retained by the membrane. Also, membrane filtration processes have been used in the fractionation of milk protein hydrolysates (Muro *et al.,* 2013). The combination of ultrafiltration (UF) and nanofiltration (NF) is often used to fractionate protein hydrolysates according to molecular weight. In the first step, the protein hydrolysates are ultrafiltered to remove the intact protein and large polypeptides. In the second step, permeate from the 1st step is then fractionated by NF into small peptides fraction (<1kDa) passed in the permeate and large peptides concentrated in the NF retentate. The NF separation can be changed by changing the pH of the permeate.

Different casein fractions have clusters of adjacent phosphate groups. Enzymatic hydrolysis of casein set free these clusters in the form of phosphopeptides (CPP). CPP can be simply prepared

by casein digestion with different proteinases, removal of the insoluble materials from the hydrolysates and recovering of CPP from the soluble fraction by aggregation with Ca (McDonagh and FitzGerald, 1998). CPP may enhance the solubility of calcium in the intestinal lumen, thereby increasing mineral availability for absorption in the small intestine (FitzGerald, 1998). Therefore, CPP can be used as an additive for healthy foods or for dietetic of pharmaceutical compositions to increase the absorption of calcium and other minerals (FizGerald, 1998; Pinto *et al.,* 2012). The CPP are prepared now on industrial scale in the form of casein hydrolysate rich in CPP or in the form of CPP bound to amorphous calcium phosphate (ACP) (Pinto *et al.,* 2012). CPP-ACP displayed anticariogenic effect when added to dentifrices or oral care products by localizing calcium and phosphate ions at the tooth surface. Also, chewing gums or other confectionery products containing CPP-ACP and sodium bicarbonate as active ingredient can provide dental health benefits (Pinto *et al.,* 2012). CPP-ACP nanocomplexes incorporated in mouth rinses and sugar-free gums have been proven experimentally on humans to be potential anticariogenic agent (Reynolds *et al.,* 2003). Consumption of milk supplemented with CPP-ACP increased remineralization of enamel (Walker *et al.,* 2009). This increase was reported to be dose dependent with 0.2 and 0.3% CPP-ACP added to milk increased the mineral content by 81 and 164% respectively relative to the normal milk. Recently (Kosalková *et al.,* 2012), addition of CPP in cell cultures was reported to cause the reprogramming of cellular metabolism, which leads to massive secretion of extracellular proteins.

The antihypertensive peptides derived from milk protein hydrolysates have received much attention. Not all peptides which exhibit ACE- inhibitory effect have antihypertensive activity,

only those resisting intestinal digestion. A patent (Roos *et al.,* 2008) was issued to describe a process for the production of ACE-inhibitory tripeptide Ile-Pro-Pro. The process comprises the hydrolysis of the casein glycomacropeptide (GMP) by a proline specific endopeptidase to liberate at least 40% of the Ile-Pro-Pro- sequence in the GMP into the tripeptide Ile-Pro-Pro. Bonte *et al.,* (2010) used a special whey protein concentrate (WPC) rich in ß-lactoglobulin in the preparation a potent ACE-inhibitory peptide. The WPC was first hydrolysed (DH 5) with neutrase, followed by hydrolysis with Thermolysin. Boolsma and Kloek (2010) compared commercial milk protein hydrolysate (MPH) harbouring bioactive peptides on blood pressure (BP) viz., MPH1 (containing Ile-Pro-Pro) and MPH2 (containing Met-Ala-Pro and Lue-Pro-Pro) and concluded that MPH1 exerted BP lowering effects on human subjects with stage 1 hypertention. Henle *et al.* (2011) patented the preparation of WPH possessing ACE-inhibiting and antihypertensive action and physiologically active quantities of peptide(s) containing tryptophan (Try-Leu, Ile-Try). Casein hydrolysis was scaled up to produce a hydrolysate fraction rich in the antihypertensive peptides $\breve{U}_{s1}$-CN (f 90-94) and (f 143-149). The product showed potent ACE-inhibitory activity *in vitro* and significantly decreased the systolic blood pressure of spontaneously hypertensive rats after oral administration (Contreras *et al.*, 2011). Peptides were stable during the process of spray drying, homogenization and pasteurization. In addition, when it was incorporated in liquid yoghurt, no reduction of either peptide was observed during the shelf life of the product. Whey protein concentrate enriched in Ŭ-lactalbumin was extensively hydrolysed (>50% less than 4kDa) first with trypsin followed by further hydrolysis with alcalase and flavourzyme. The product can be used in pharmaceutical antihypertensive agents, food supplement and functional foods. Whey protein isolate solutions (10%) were heat treated

(80ºC/10 min) and then hydrolysed (DH 5% and 10%) on pilot scale using corolase PP (OðLoghlin *et al.,* 2014b). The hydrolysates were membrane fractionated using a series of membranes of 0.14µm pore size and 30,10,5 and 1 kDa cut-off membranes. The most potent ACE-inhibitory fraction (IC_{50}=0.23 g L^{-1}) was the 1 kDa permeate of 10% DH hydrolysate. This fraction was further fractionated with ultrafiltration and isoelectric focusing and an enriched ACE- inhibitory (IC_{50}=0.17 g L^{-1}) product was obtained (OðLoghlin *et al.,* 2014a). Also, a strong negative correlation was found between the molecular mass and the antioxidant activity of the test sample. Ion-exchange membrane adsorption chromatography and cross-flow electro membrane filtration were successfully developed for the fractionation of ß-LG and ß-casein hydrolysates (Leeb *et al.,*2014). The ACE-inhibitory peptides were enriched in individual fractions, which exhibited 3- to 6-fold higher activity compared with the hydrolysates.

Whey protein isolates were hydrolysed (DH 10%) using trypsin and chymotrypsin mixture (1:1), followed by fractionation of the hydrolysate by liquid phase isoelectric focusing (Gauthier *et al.,* 2009). Three fractions derived from ß-lactoglobulin were obtained which differed in their isoelectric point; F1 (pH <4.5), F2 (pH>4.5<7.0) and F3 (>7.0). The basic F3 fraction showed promising immunomodulatory effect. Oral administration of this fraction in infected mice with *E.coli* O7:157 increased the total serum IgA and other components of the immune response.

Dipeptidylpeptidase IV (DPP-IV) is a multifunctional trans membrane glycoprotein that contains N-terminal peptidase activity. DPP-IV is implicated in cellular processes involving immune, inflammatory and endocrine functions. Protein hydrolysate fraction enriched in DPP-IV inhibitory peptides was prepared from casein hydrolysates (Boots, 2013). Casein was hydrolysed with single or mixture of proteases and hydrolysate was fractionated by nanofiltration. The

permeate contained small peptides (2-8 amino acid residues with at least one proline residue) and exhibited DPP-IV inhibitory effect. The product was reported to be effective for prophylaxis and/or treatment of DPP-IV mediated conditions particularly groups with obesity, diabetes type 2, and immunological disorders. The product can be formulated in capsules, or as functional ingredient in chocolate caramel bar, yoghurt and soft drinks.

Weight loss can be achieved by a modulation of appetite and satiety through the central nervous system (CNS) involving increased serotonin (5-hydroxytryptamine) neurotransmission. In addition, it has been established that central regulation of appetite can be achieved through 5-HT_{2C} serotonin receptor agonism . Expression of the 5-HT_{2C} serotonin receptors has been linked with suppression of appetite, reinforcing its role in body weight management. Milk protein hydrolysates generated with different starting substrates, including sodium caseinate (NaCN), acid casein (Acid CN), skim milk powder (SMP) and glycomacropeptide (GMP) were demonstrated to behave as serotonin 2C (5-HT_{2C}) receptor agonists (Nongonierma *et al.,* 2013).

8. Milk protein hydrolysates in sports nutrition

Depletion of glycogen stores is known to be associated with fatigue during sprint and endurance exercise. Dietary carbohydrates are the sources of tissue glycogen. However, combinations of carbohydrates and proteins were found to be more effective than carbohydrate alone to replenish muscle glycogen after exercise. Post-exercise feeding rats combination of carbohydrates and WPH caused significant increase in skeletal muscle glycogen compared to combinations of carbohydrates with the intact whey protein, branched chain amino acids or casein hydrolysates (Morifuji *et al.,* 2010). Also, pre-exercise feeding of carbohydrate and WPH attenuated the skeletal muscle glycogen compared to glucose or water only (Morifuji *et al.,* 2011). Long term

feeding of WPH was more effective for increasing glycogen content in skeletal muscle, and improving exercise performance than other protein sources (Kanda *et al.,* 2010). The branched chain amino acid (BCAA) containing dipeptide in WPH was found to be responsible for the increased glycogen content in skeletal muscle (Morifugi *et al.,* 2009). The BCAA containing dipeptides Ile-Val, Leu-Val, Val-Leu, Ile-Ile, Ile-Leu and Leu-Leu were detected in WPH by Lc/MS/MS. These dipeptides significantly stimulated glucose rate uptake in L6 myotube. Also, the main dipeptide in WPH, Ile-Leu, stimulated glucose uptake in isolated skeletal muscle and increased glycogen content in skeletal muscles.

Several studies showed that protein hydrolysates containing mostly dipeptides and tripeptides are absorbed faster than free amino acids and much more rapidly than the intact proteins. This is an advantage to maximize the delivery of amino acids to muscles of athletes. The greater absorption rate of dipeptides and tripeptides than amino acid mixtures appears to be the result of uptake by a system that has a greater transport capacity than the amino acid carrier system thus minimizing competition between its substrates (Manninen, 2009).

The synthesis of muscle proteins is depressed for several hours following exercise. In the mean time, the rate of protein degradation is increased for several days. This results in a net loss of total protein and a delay in restoration of muscle function (Rennie and Tipton, 2000, Bosse and Dixon, 2012). Depletion of glutamine was found to occur after intense and prolonged exercise. Therefore, increasing the level of glutamine is required for muscle restoration. Compared with amino acid formula, consumption of whey protein hydrolysate (WPH) led to higher N retention and plasma and muscle glutamine concentration (Boza and Moënnez, 2000). Also, the WPH formula showed significantly higher protein efficiency and biological value than the amino acid

formula. Consumption of WPH was reported to increase plasma and muscle stores of glutamine (Castell and Newshelme, 1998). In this respect protein hydrolysates may be useful for enhancing the recovery from exercise particularly muscle damage induced by exhaustive exercise. Consumption of whey protein hydrolysates or soy protein induced mixed muscle protein synthesis (MPS) greater than casein both before and after resistance exercise (Tang *et al.,* 2009). However, WPH stimulated MPS to a greater degree than soy after resistance exercise.

Whey protein hydrolysates were found to accelerate recovery from exercise induced muscle damage (Buckley *et al.,* 2010). Compared to non-hydrolysed whey protein isolate, WPH from the same WPI gave more rapid recovery from muscle damage. WPH achieved complete recovery of muscle-force generation capacity after 6 supplementations. Compared to carbohydrate supplementation, whey protein hydrolysates resulted in an attenuation of the exercise-reduced force reduction during the recovery period (Cooke *et al.,* 2010). This was attributed to an increase in protein synthesis and reduction in the extent of muscle damage. Hydrolysed whey proteins were reported to reduce muscle damage indicators in soccer players while the intact whey proteins or maltodextrin had no effect (Lollo *et al.,* 2014).

In comparison with WPI, acute ingestion of a novel WPH based supplement resulted in a higher transient leucine response with a sequential increase in insulin. Furthermore, chronic ingestion of the tested WPH supplement was reported to be safe (Toedebusch *et al*., 2012).

A process was patented for the preparation of a sport drink from hydrolyzed casein and intact whey proteins (Edans and De Rees*,* 2005). Sodium caseinate was first hydrolysed with thermolysin, followed by debittering of the hydrolysate using a proline specific endoprotease from *Aspergillus niger.* The hydrolysate was then mixed with twice concentrated whey.

9. Future trends.

Remarkable progress has been achieved in the production and utilization of milk protein hydrolysates (MPH). The extensive research done in this area led to better understanding of nutritional significance of MPH, development of several new products and uses for MPH. However, several points need further studies along this line.

Hydrolysis of milk proteins using microbial proteinases has been useful in diversifying the composition and functional properties of the obtained MPH. However, most of these enzymes have wide range of specificities being difficult to control, standardize and use in making of products for specific purposes. Search for microbial proteinases with narrow specificities can allow the production of tailored MPHs characterized by standardized composition and functions. Also, production of MPH with maximum dipeptides and tripeptides contents and minimum amino acid contents would improve the nutritive value of MPH for special groups.

The area of separation and concentration of MPH fractions rich in bioactive peptides need further development. Apart from CPP and GMP the available methods are lagging to achieve this purpose, for peptides with other biological activities, on industrial scale. The membrane separation processes offer promising tools for better fractionation of MPH into more simple fraction up to the level of single peptide which needs further development in the existing technologies and/or fabrication of new powerful tools. For example development of membranes with controlled and uniform pore sizes may improve the capabilities of the membrane separation processes for better fractionation of MPH.

Formulation and delivery of MPH particularly the bioactive rich hydrolysates is an important area for improving their biological functions. Nanoencapsulation has proven useful in improving

the biological value of several bioactive materials. Therefore, nanoencapsulation of the bioactive peptides rich MPH singly or in combination with other synergistic compounds is a promising area that need further investigations.

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Table 1: Microbial proteases used in the preparation of whey protein hydrolysates*

Trade name	Microorganism	pH	Temperature	Specificity (residues P1)*
Alcalase 2.4L®	*Bacillus licheniformis*	6-10	10-80	F,W,Y,E,M,L,A,S,K
Subtilisin carlesberg	"	6-10	10-80	F,W,Y,M,L,A,S,K
Esperase	*Bacillus lentus*	7-12	10-80	Broad
Nutrase	*Bacillus amyloliquifacus*	6-8	10-65	L,V,F
Protamex	*Bacillus spp.*	6-8	10-65	Not specified
Therolysin/ Therolase	*Bacillus thermoproteolyticus*	5-9.5	10-80	I,L,V,F
Flavouzyme	*Aspergillus oryzae*	4-8	10-55	Not specified
Protease N	*Bacillus subtilis*			Not specified
Bacillus licheniformis protease	*Bacillus licheniformis*			E, D

*P1 residue from carboxyl side

Table 2: Preparation of low phenylalanine milk protein hydrolysates

Protein	Enzyme	Method of production	Referene
Skim milk/caseinate WPC WPC Skim milk Whey WPC	*Aspergillus oryza* protease followed by papain Pancreatin Pancreatin Papain/pepsin separately or with *Aspergillus oryzea* protease Pancreatin/papain immobilized on active carbon/alumina Immobilized chymotrypsin, followed by Immobilized Carboxypeptidase A (CPA)	Enzymatic hydrolysis (5 h +21 h)Ÿ removal of Phe with active carbonŸ homogenization with milk fatŸ add in formulation Enzymatic hydrolysis (7 h)Ÿ ultrafiltration (Mol cut off 20,000) Ÿ permeate Ÿ concentration/dryingŸ 20% solution Ÿ adsorption resin XAD-16 elution with waterŸ concentration/drying (powder 0.5 Phe) Enzymatic hydrolysis (27 h)Ÿ gel filtration (Sephadex G-25/acetic acid as eluant)Ÿ removal of 74% of Phe from hydrolysate Enzyme hydrolysisŸ removal of Phe with active carbon (mixed papain and AO gave highest Phe removal 97-98%) 84-97% removal of Phe with pacreatin on active carbon, 45-70% and 63-78% losses of Tyr and Try respectively. High oligopeptides (40) and low amino acids (2%) WPC (5%)Ÿ Chymotrypsin hydrolysis Ÿ Enzyme hydrolysis (CPA)Ÿ ultrafiltration (1kDa) Ÿ Permeate (Phe) Ź WPH (low in Phe)	Lopez-Bajanero *et al.,* 1991 Outinen *et al.,*1996 Lara *et al.,* 2005 Soares *et al.,* 2006 Silva *et al.,* 2007 Cabrora-Paolilla *et al.,*2009

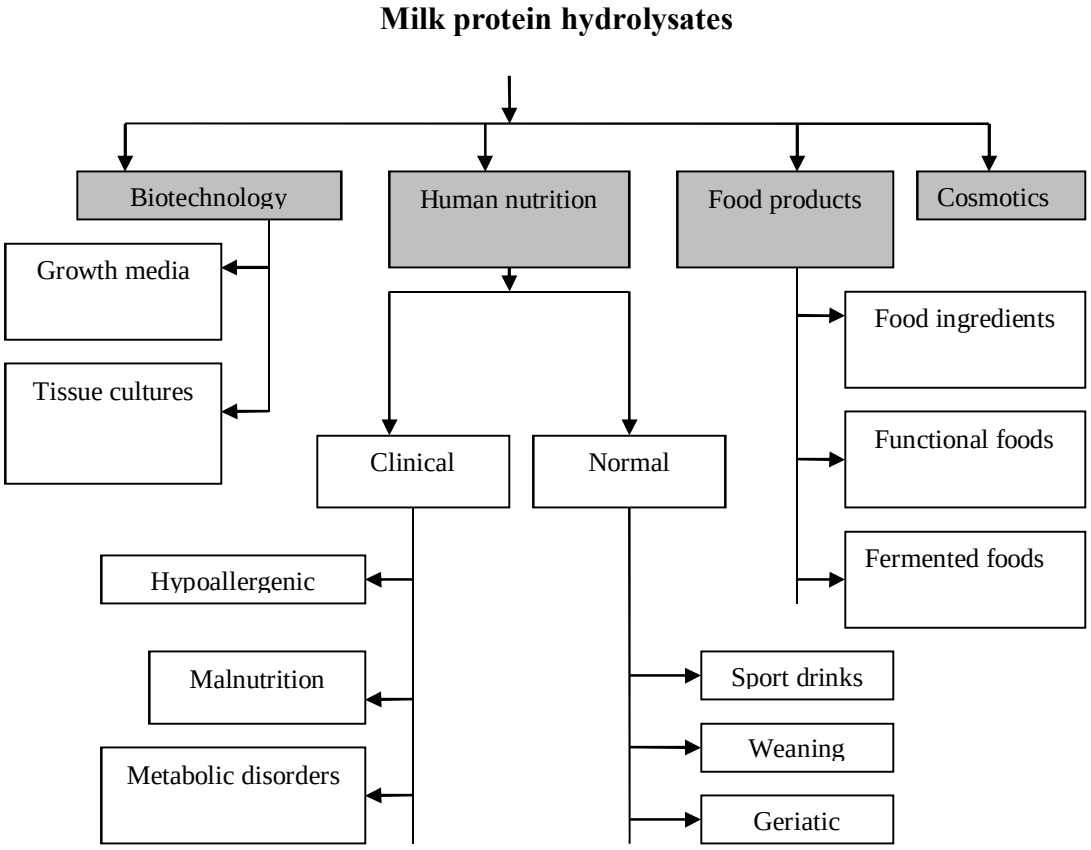

Fig 1: Uses of milk protein hydrolysates

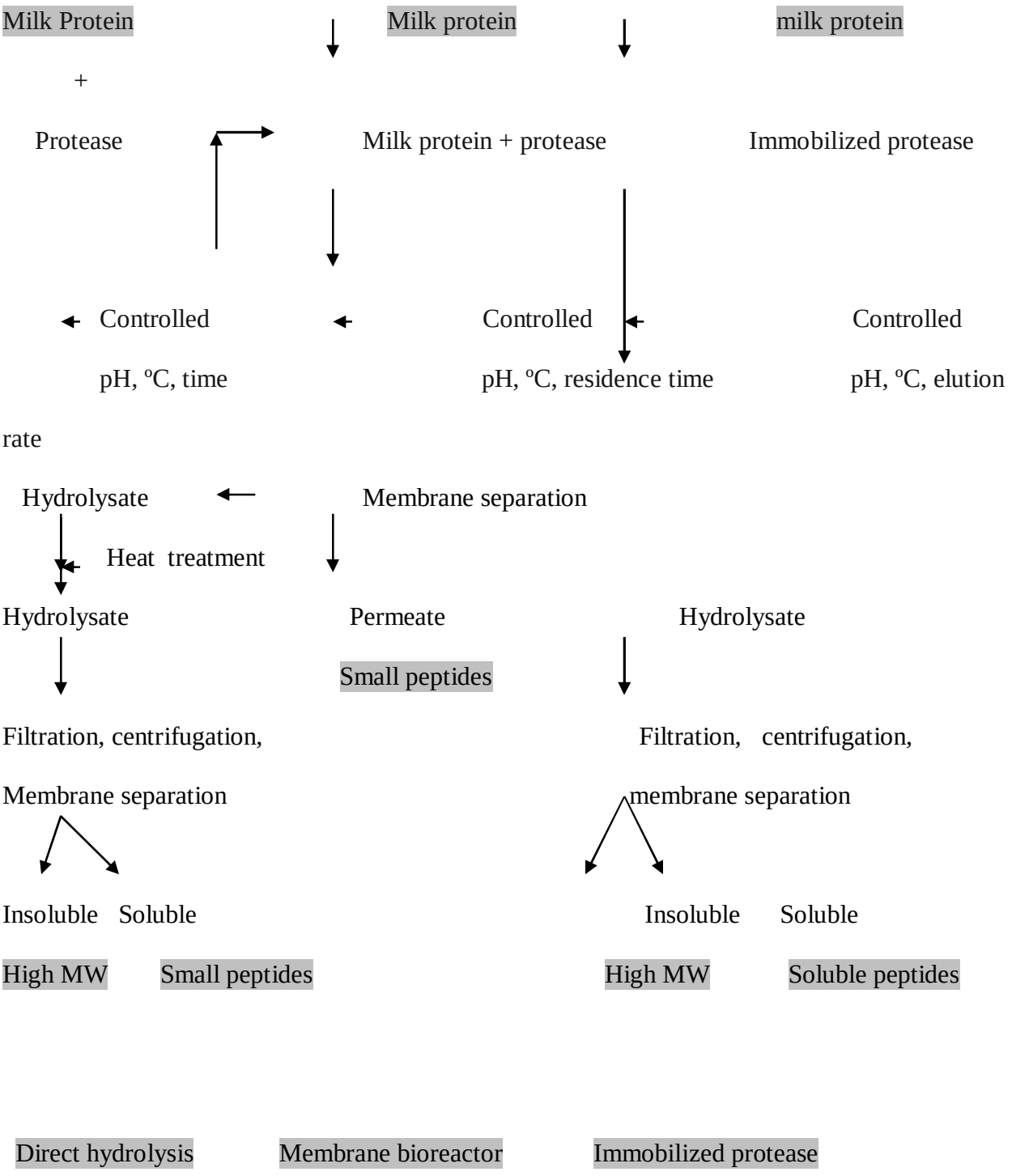

Fig 2: Methods for the preparation of milk protein hydrolysates