

Nanoparticulated whey proteins

5 The present invention relates to a method for producing whey proteins in nanoparticulated form and to the nanoparticulated whey proteins thus obtained. Specifically, the present invention pertains to the use of these nanoparticulated whey proteins as emulsifiers, fat substitute, micellar casein substitute, whitening, foaming, texturising and/or filling agents.

10 Fat containing food material makes up a considerable proportion of the diets of many people. One of the problems encountered with the production of such products resides in that the fat must remain stabilized over the entire shelf life of the product, so that no phase separation occurs.

15 To this end, emulsifying agents are utilized, that provide a stabilization of the emulsion once formed, based on their inherent property of a lipophilic or hydrophobic part; respectively; being soluble in the non-aqueous phase and a polar or hydrophilic part being soluble in water such that said molecules are facilitate emulsifying one phase in the other phase. Additionally, the emulsifying agents also protect the once formed
20 droplets from aggregation and coalescence. As emulsifying agents naturally occurring substances are used, such as hydrocolloids, phospholipids (lecithin) or glycolipids and on the other hand synthetic agents like stearyl-2-lactylate or mono-, diacylglycerides, etc.

25 One of the major drawbacks of the agents resides in that these agents sometimes substantially add to the costs of the final product, and do not add to the nutritional value of the product. Sometimes, such kinds of materials also do not show adequate stabilizing properties because of an interfacial competition with proteins.

30 Thus, an object of the invention resides in providing alternative to the existing emulsifiers, that do not show the inherent disadvantages and is easily available.

Another object of the invention is to provide a fat substitute, whitening, foaming, texturising and/or filling agent with a high Protein Efficiency Ratio (PER).

35

To achieve this objective a method for the production of nanoparticulated whey protein is proposed that comprises the step of subjecting a solution containing whey proteins to

a specific temperature for a specific period of time and in a narrow pH range to result in the production of whey proteins aggregates having a diameter of less than 1 μm , preferably from 100 to 990 nm.

5 In particular, the present invention relates to a method for the production of nanoparticulated whey protein comprising the step of :

- i) adjusting the pH at a narrow range of an aqueous solution of whey protein, or adjusting the ionic strength of the whey protein preparation while keeping the pH constant, and
- 10 ii) subjecting the aqueous solution to a temperature of between 80° to 95°C, during a time period of from 10 seconds to 30 minutes, to obtain a liquid dispersion of spherical nanoparticulated whey protein having a particle size of less than 1 μm .

The nanoparticulated whey protein dispersion may be further dried, in particular by freeze-drying or spray-drying. It has been found that whey protein nanoparticles were observed in solution after reconstitution of the spray-dried powder. No difference of morphology and structure could be detected, confirming that whey protein nanoparticles are physically stable regarding spray drying.

20 Said nanoparticles have a Protein Efficiency Ratio of at least 100.

During the extensive experiments, leading to the present invention, the named inventors surprisingly noted that when adjusting the pH at a very precise narrow range (meaning by ± 0.2 pH unit) before heat treating an aqueous solution of whey proteins, or one or more of its major constituents, at a temperature of between about 80 to 95 °C, for a time period of between about 10 s to 30 min at the desired temperature the whey proteins thus obtained show a particulate form, with spherical shape and with a particle size having a diameter of less than 1 μm . An advantage is that the whey protein particles prepared accordingly have not been submitted to any mechanical stress leading to reduction of the particle size, contrary to the conventional process. This method induces spontaneous nanoparticulation of whey proteins during heat treatment in absence of shearing.

35 The nanoparticulated whey proteins have shown to be ideally suited for use as an emulsifier, fat substitute, substitute for micellar casein or foaming agent, since they are able to stabilize fat and/or air in an aqueous system for prolonged period. In addition, the present nanoparticulated whey proteins are still in a condition to serve as whitening

agent, so that with one compound several tasks may be fulfilled. Since whey is a material abundantly available, the use thereof reduces the cost of a product requiring an emulsifying, filling, whitening or foaming agent, while at the same time adding to its nutritional value.

Figures

In the figures:

Fig. 1 shows the result of an experiment demonstrating the effect of pH and heat treatment on the nanoparticulation of β -lactoglobulin.

Fig. 2 is showing a mean to determinate the pH of nanoparticulation for a commercial preparation (Bipro®, Batch JE032-1-420) using turbidity measurements at 500 nm.

Fig. 3 is a Transmission Electron Microscopy micrograph from nanoparticulated whey proteins (2 wt.-%, WPI 95, Lactalis) at pH 7.4. Scale bar is 200 nm.

Fig. 4 shows the result of an experiment evaluating the impact of the ionic strength (Arginine HCl) on the formation of protein nanoparticles at constant pH of 7.0.

Fig. 5 shows the volume stability (FVS) of foam stabilized by 1 wt.-% β -lactoglobulin nanoparticles (Davisco) at pH 7.0 in presence of 60 mM Arginine HCl compared to non-nanoparticulated β -lactoglobulin.

Fig. 6 shows the intensity-based size distribution of nanoparticules obtained by heat-treatment of a 1 wt% β -lactoglobulin dispersion for 15 min at 85°C at pH 4.25 (positively charged with a zeta potential around +25mV) and at pH 6.0 (negatively charged with a zeta potential around -30mV). Z-averaged hydrodynamic diameter of the nanoparticles was 229.3 nm at pH 4.25 and 227.2 nm at pH 6.0. The corresponding micrographs of the nanoparticles obtained by TEM after negative staining are shown. Scale bars are 1 μ m.

As the whey protein to be used in the present method any commercially available whey protein isolates or concentrates may be used, i.e. whey protein obtained by any process for the preparation of whey protein known in the art, as well as whey protein fractions prepared there from or proteins such as β -lactoglobulin (BLG), α -lactalbumin and serum albumin. In particular, sweet whey obtained as a by-product in cheese manufacture, and

acid whey as by-product in acid casein manufacture, native whey obtained by milk microfiltration or rennet whey as by-product in rennet casein manufacture may be used as the whey protein. Preferably, in particular under cost aspects, whey protein preparation, which has not been subjected to additional fractionation processes after its production, is preferred as starting material. The present invention is not restricted to whey isolates from bovine origin, but pertains to whey isolates from all mammalian animal species, such as from sheep, goats, horses, and camels. Also, the process according to the present invention also applies to any other demineralized or slightly mineralized globular protein, such as egg protein, soy, cereals, oilseeds, or from other vegetables or animal origin. It should be understood by "slightly mineralized", the elimination of free minerals which are dialyzable or diafiltrable, maintaining minerals associated to protein or natural mineralisation after preparation of whey protein concentrate or isolate without specific mineral enrichment.

Whey proteins may be present in aqueous solution in an amount of 0.1 wt.-% to 12 wt.-%, preferably in an amount of 0.1 wt.-% to 8 wt.-%, more preferably in an amount of 0.2 wt.-% to 7 wt.-%, even more preferably in an amount of 0.5 wt.-% to 6 wt.-%, in particular in an amount of 1 wt.-% to 4 wt.-%, each on the basis of the total weight of the solution.

The aqueous solution of the whey protein preparation as present before nanoparticulation step may also comprise additional compounds, such as by-products of the respective whey production processes, other proteins, gums or carbohydrates. The solution may also contain other food ingredients (fat, carbohydrates, plant extracts, etc).

The amount of such additional compounds preferably doesn't exceed 50 wt.-%, preferably 20 wt.-%, and more preferably 10 wt.-% of the total weight of the solution.

Whey proteins have a better protein efficiency ratio (PER) compared for example to casein 118/100.

PER = body weight growth (g) / protein weight intake (g).

Examples:	PER	% Casein
casein	3.2	100
Egg	3.8	118
Whey	3.8	118
Whole Soya	2.5	78
Wheat gluten	0.3	9

Whey proteins are an excellent source of essential amino acids (AA) (45%). Rich in AA which requirements may be increased in case of stress and in elderly: compared to casein (0.3g cysteine/100g protein) sweet whey proteins contain 7 times more cysteine and acid whey 10 times more cysteine. Cysteine is the rate limiting amino acid for glutathione (GSH) synthesis, glutathione is a tripeptide made of glutamate cysteine and glycine. Glutathione has primary important functions in the defense of the body in case of stress. Oral supplementation with whey protein increases plasma GSH levels of HIV-infected patients (Eur. J. Clin. Invest. 2001; 31, 171-178)

The nanoparticles according to the present invention have a PER of at least 100, preferably at least 118.

The whey protein, as well as the fractions and/or the main proteins thereof may be used in purified form or likewise in form of a crude product. According to a preferred embodiment the salt content of the starting material for the preparation of the nanoparticulated whey protein may be less than 2.5% in divalent cations, more preferably less than 2%.

Alternatively, if no pH adjustment step is desired, it is possible to adjust the ionic strength of the whey protein preparation while keeping the pH constant. Then, ionic strength may be adjusted by organic or inorganic ions in such a way that it allows nanoparticulation at constant pH.

The starting material is then subjected to the heat treatment. In this respect it has been found important for obtaining nanoparticulated whey protein to have the temperature in the range of from about 80 to about 95 °C, preferably of from about 82 to about 89 °C, more preferably of from about 84 to about 87 °C, most preferred at about 85 °C.

Once the desired temperature has been reached, it will be kept there for a minimum of 10 seconds and a maximum of 30 minutes (at the desired temperature). Preferably the time period, during which the aqueous whey protein solution is kept at the desired temperature ranges of from 12 to 25 minutes, more preferably of from 12 to 20 minutes, or more preferred about 15 minutes.

By nanoparticles, in the present description, we understand particles with a diameter of less than 1 µm, preferably between 100 and 700 nm. The mean diameter of the nanoparticles can be determined using Transmission Electron Microscopy (TEM). In

this case, the liquid nanoparticulated samples were encapsulated in agar gel tubes. Fixation was achieved by immersion in a solution of 2.5% glutaraldehyde in 0.1M, pH 7.4 cacodylate buffer and post-fixation with 2% Osmium tetroxide in the same buffer, both solutions containing 0.04% Ruthenium red. After dehydration in a graded ethanol series (70, 80, 90, 96, 100% ethanol), the samples were embedded in Spurr resin (Spurr/ethanol 1:1, 2:1, 100%). After polymerization of the resin (70°C, 48 hours), semi-thin and ultra-thin sections were cut with a Leica ultracut UCT ultra-microtome. Ultra-thin sections, stained with aqueous uranyl-acetate and lead citrate, were examined in transmission electron microscopy (Philips CM12, 80 kV).

According to the present finding, the pH and the ionic strength are important factors in the present method. Thus, for extensively dialyzed samples which are virtually devoid of or depleted in free cations like Ca, K, Na, Mg, it has been found that when performing the heat treatment during the time period indicated at a pH below 5.4, curd would be obtained, while at a pH exceeding 6.8, soluble whey protein will result. Thus, only in this rather narrow pH window whey protein in the particulate form with a size having a diameter of less than 1µm will be obtained. The same particulate form is obtained symmetrically below the isoelectrical pH, i.e from 3.5 to 5.0.

According to a preferred embodiment, to obtain negatively charged nanoparticles, the pH is adjusted in a range of from 5.6 to 6.4, more preferably from 5.8 to 6.0 for a low content (below 0.2g for 100g of initial whey protein powder) in divalent cations. The pH may be increased up to 8.4 depending on mineral content of whey protein source (concentrate or isolate). In particular, the pH may be between 7.5 to 8.4, preferably 7.6 to 8.0 to obtain negatively charged nanoparticles in presence of large amount of free minerals and the pH may be between 6.4 to 7.4, preferably 6.6 to 7.2 to obtain negatively charged nanoparticles in presence of moderate content of free minerals. The pH is generally adjusted by the addition of acid, which is preferably food grade, such as e.g. hydrochloric acid, phosphoric acid, acetic acid, citric acid, gluconic acid or lactic acid. When mineral content is high the pH is generally adjusted by the addition of alkaline solution, which is preferably food grade, such as sodium hydroxide, potassium hydroxide or ammonium hydroxide.

According to another preferred embodiment, to obtain positively charged nanoparticles, nanoparticulation of whey proteins is done in a salt free solution at pH adjusted between 3.5 and 5.0 depending on mineral content of protein source.

According to a preferred embodiment the pH is adjusted in a range of from 6.3 to 9.0, for a content in divalent cations comprised between 0.2% and 2.5% in whey protein powder.

5 According to another embodiment a buffer may be added to the aqueous solution of whey protein so as to avoid a substantial change of the pH value during heat treatment of the whey protein. In principle, the buffer may be selected from any food-grade buffer system, i.e. acetic acid and its salts, such as e.g. sodium acetate or potassium acetate, phosphoric acid and its salts thereof, e.g. NaH_2PO_4 , Na_2HPO_4 , KH_2PO_4 , K_2HPO_4 , or
10 citric acid and its salts etc.

The nanoparticulated whey proteins obtained according to the present method shall have a size with a diameter of less than $1\mu\text{m}$, preferably of from 100 to 990 nm, more preferably from 100 to 700 nm, while depending on desired application the proportion
15 of nanoparticles is of at least 80% and residual soluble aggregates or soluble protein below 20%. The average nanoparticle size is characterised by a polydispersity index below 0.200. In consequence, the white suspension obtained by the present invention is stable and has a milky appearance in a large range of pH 3-8.

20 The turbidity measured by absorbance at 500nm is at least 3 absorbance units for 1% protein solution but can reach 16 absorbance units when the yield of nanoparticulation is above 80%.

25 The purity of nanoparticulated whey protein produced according to the method of the present invention can be obtained by determining the amount of residual soluble proteins. Nanoparticles are eliminated by centrifugation at 20 °C and 26900 g for 15 min. The supernatant is used to determine the protein amount in quartz cuvettes at 280nm. Values are expressed as a percentage of the initial value before heat treatment.

30 Proportion of nanoparticles = (Amount of initial proteins – amount of soluble proteins) / Amount of initial proteins

Without wishing to be bound by any theory it is presently believed that the method as described results in the formation of very small aggregates of whey proteins, i.e.
35 aggregates having the size indicated, and being in a particular denatured status resulting from an electrostatic balance between repulsive and attractive forces present at the surface of the proteins, that produces the properties observed. In particular, since the

nanoparticulated whey proteins have perfect emulsifying and foaming properties, the denatured status of the protein seems to allow interaction with a hydrophobic phase, e.g. a fat droplet or air, and a hydrophilic phase, the aqueous solution.

5 Thus, according to another embodiment, the present invention also relates to the use of the nanoparticulated whey proteins as an emulsifying agent, for which the material is ideally suited, since it has a neutral taste, i.e. no off-flavor is created by the use of such material. They may also be used as micellar casein substitute.

10 The nanoparticulated whey proteins obtained according to the method of the present invention can be used for the preparation of any kind of food product requiring stabilization of an emulsion or a foam, such as e.g. present in mousse or ice cream, in coffee creamers, or also in low fat or essentially fat free dairy products, or also where it finds application, as a micellar casein substitute. Examples for products, where the
15 present nanoparticulated whey proteins may find application are exemplarily, pasteurized UHT milk, sweet condensed milk, yoghurt, fermented milks, milk-based fermented products, milk chocolate, mousses, foams, emulsions, ice creams, fermented cereal based products, milk based powders, infant formula, diet fortifications, pet food, tablets, liquid bacterial suspensions, dried oral supplement, wet oral supplement.

20 In particular, the present nanoparticulated whey proteins may be used either alone or together with other active materials, such as polysaccharides (e.g. acacia gum or carrageenans) to stabilize matrices and for example milky foam matrices. Due to their neutral taste, whitening power and stability subsequent to heat treatment, the present
25 nanoparticulated whey proteins may be used to increase skimmed milk whiteness and mouthfeel.

Apart from increasing the whitening power of dairy systems for the same total protein content, at the same time the fat content in a food matrix may be reduced. This feature
30 represents a particular advantage of the present nanoparticulated whey proteins, since it allows e.g. adding a milk creamer without adding additional fat derived from the milk as such.

Hence, according to another embodiment the present invention also encompasses a food
35 product, a food supplement, a nutritional and/or pharmaceutical composition containing nanoparticulated whey proteins as described herein.

The following examples illustrate the present invention without limiting it thereto.

Examples

The invention is further defined by reference to the following examples describing in detail the preparation of the nanoparticles of the present invention. The invention described and claimed herein is not to be limited in scope by the specific embodiments herein disclosed, since these embodiments are intended as illustrations of several aspects of the invention. Any equivalent embodiments are intended to be within the scope of this invention. Indeed, various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description. Such modifications are also intended to fall within the scope of the appended claims.

Example 1: Nanoparticulation of β -Lactoglobulin

β -Lactoglobulin (lot JE002-8-922, 13-12-2000) was obtained from Davisco (Le Sueur, MN, USA). The protein was purified from sweet whey by ultra-filtration and ion exchange chromatography. The composition of the powder is 89.7 % protein, 8.85 % moisture, 1.36% ash (0.079 % Ca^{2+} , 0.013 % Mg^{2+} , 0.097 % K^{+} , 0.576 % Na^{+} , 0.050 % Cl^{-}). All other reagents used were of analytical grade (Merck Darmstadt, Germany).

The protein solution was prepared at 0.2% concentration by solvation of β -lactoglobulin in MilliQ® water (Millipore), and stirring at 20 °C for 2 h. Then pH of aliquots was adjusted to 5.0, 5.2, 5.4, 5.6, 5.8, 6.0, 6.2, 6.4, 6.6, 6.8, 7.0 by HCl addition. The solutions were filled in 20 ml glass vials (Agilent Technologies) and sealed with aluminum capsules containing a silicon/PTFE sealing. The solutions were heated at 85 °C for 15 min (time to reach the temperature 2.30 – 3.00 min). After the heat treatment, the samples were cooled in ice water to 20 °C.

The visual aspect of products (Figure 1) indicates that the optimal pH of nanoparticulation is 5.8.

Example 2: Nanoparticulation of whey protein isolate

Whey protein isolate (WPI) (Bipro®, Batch JE032-1-420) was obtained from Davisco (Le Sueur, MN, USA). The composition of the powder is reported in table 1.

The protein solution was prepared at 3.4% protein by solvation of whey protein powder in MilliQ® water (Millipore), and stirring at 20 °C for 2 h. The initial pH was 7.2. Then pH of aliquots was adjusted at 5.6, 5.8, 6.0, 6.2, 6.4 and 6.6 by HCl 0.1N addition.

The solutions were filled in 20 ml glass vials (Agilent Technologies) and sealed with aluminum capsules containing a silicon/PTFE sealing. The solutions were heated at 85 °C for 15 min (time to reach the temperature 2.30 – 2.50 min). After the heat treatment, samples were cooled in ice water to 20 °C.

The turbidity of heated whey proteins has been determined at 500 nm and 25°C, samples were diluted to allow the measurement in the range of 0.1-3 Abs unit (Spectrophotometer Uvikon 810, Kontron Instrument). Values were calculated for the initial protein concentration 3.4%.

The pH of nanoparticulation was considered to be reached upon stability (less than 5% variation of the initial value) of the absorbance measured at 500 nm within an interval of 10 minutes for the same sample as illustrated by the figure 2. For this product the optimal pH for nanoparticulation was 6.0 to 6.2. For this pH adjusted before heat treatment stable turbidity was 21 and residual soluble protein evaluated by absorbance at 280 nm after centrifugation was 1.9%. We can conclude that 45% of initial proteins were transformed in nanoparticles at pH 6.0.

Table 1: Composition of WPI and sample characteristics after nanoparticulation

Supplier	Davisco
Product name	Bipro
Batch number	JE 032-1-420
Composition (mg/100 g)	
Sodium	650
Potassium	44
Chloride*10 if ≤ 40	10
Calcium	82
Phosphorus	49
Magnesium	6
Initial pH	7.2
pH nanoparticulation	6.0
Turbidity (500 nm) for 3.4% protein in solution	21
Residual Soluble protein (%) by absorbance at 280 nm	1.9

Example 3: Microscopic observation of nanoparticles

Production of nanoparticles:

Protein solution was prepared at 2% protein by solvation of whey protein powder (WPI 90 batch 989/2, Lactalis, Retier, France) in MilliQ® water (Millipore), and stirred at 20 °C for 2 h. Then pHs of aliquots were adjusted using HCl 0.1N or NaOH 0.1N.

The solutions were filled in 20 ml glass vials (Agilent Technologies) and sealed with aluminum capsules containing a silicon/PTFE sealing. The solutions were heated at 85°C for 15 min (time to reach the temperature 2.30-2.50 min). After the heat treatment, the samples were cooled in ice water to 20 °C. For this product the optimal pH for nanoparticulation was 7.4.

Microscopic observations:

Liquid nanoparticulated samples were encapsulated in agar gel tubes. Fixation was achieved by immersion in a solution of 2.5% glutaraldehyde in 0.1M, pH 7.4 cacodylate buffer and post-fixation with 2% Osmium tetroxide in the same buffer, both solutions containing 0.04% Ruthenium red. After dehydration in a graded ethanol series (70, 80, 90, 96, 100% ethanol), the samples were embedded in Spurr resin (Spurr/ethanol 1:1, 2:1, 100%). After polymerization of the resin (70°C, 48 hours), semi-thin and ultra-thin sections were cut with a Leica ultracut UCT ultra-microtome. Ultra-thin sections, stained with aqueous uranyl-acetate and lead citrate, were examined in transmission electron microscopy (Philips CM12, 80 kV).

TEM micrograph is presented in figure 3. Obtained nanoparticles are presenting a spherical shape with a diameter of 200 nm.

Particle size distribution

The intensity-based size distributions of nanoparticles were measured for nanoparticles obtained by heat-treatment of a 1 wt % β -lactoglobulin dispersion for 15 min at 85°C at pH 4.25 (positively charged with a zeta potential around +25mV) and at pH 6.0 (negatively charged with a zeta potential around -30mV). Z-averaged hydrodynamic diameter of the nanoparticles was 229.3 nm at pH 4.25 and 227.2 at pH 6.0. β -LG and whey protein aggregations were followed using dynamic light scattering. A Nanosizer ZS apparatus (Malvern Instruments, UK) equipped with a laser emitting at 633 nm and with 4.0 mW power was used. The instrument was used in the backscattering configuration, where detection is done at a scattering angle of 173°. This allows considerable reduction of the multiple scattering signals found in turbid samples. Samples were placed in a squared quartz cell (Hellma, pathlength 1 cm). The pathlength

of the light beam was automatically set by the apparatus, depending on the sample turbidity (attenuation). The autocorrelation function was calculated from the fluctuation of the scattered intensity). The results are presented in figure 6. It shows that the average particle is characterized by a very narrow polydispersity index (<0.200). In consequence, the white suspension obtained by the present invention is stable and has a milky appearance in a large range of pH 3-8.

Example 4: Nanoparticulation of a β -lactoglobulin at a constant pH

The method described in example 1 was repeated with the proviso of using an aqueous solution of 2 % β -lactoglobulin. The pH of this solution has been adjusted to 7.0 after adding Arginine HCl solutions to obtain a final salt concentration ranging from 5 to 200 mM and a final β -lactoglobulin concentration of 1%. Subsequent heat treatment (80 °C, 10 min, about 2 min heating up) was carried out to produce nanoparticles.

The results are shown in Fig. 4 and clearly indicate that only in the ionic strength range of from about 50 to 70 mM a substantial turbidity, indicating the presence of nanoparticulated whey proteins, has been observed.

Example 5: Preparing a whitening agent

Native whey proteins (WPI 95 batch 848, Lactalis; 8 wt-% aqueous solution) were treated according to example 2. The resulting product lightness (L) was measured in trans-reflectance mode using a MacBeth CE-XTH D65 10° SCE apparatus equipped with a 2 mm measuring cell. The resulting lightness was $L = 74.8$, that could be compared to the value of $L = 74.5$ for full-fat milk.

Example 6: Preparing a coffee creamer

Native whey proteins (Bipro®, lot JE 032-1-420, 0.5 wt-% aqueous solution) were mixed at 50°C with 10 wt.-% partially hydrogenated palm oil, 14 wt.-% maltodextrin (DE 21) and in presence of 50 mM phosphate-citrate buffer adjusted to the nanoparticulation pH of 6.0 for this Bipro®. The mixture was homogenized under 400/50 bars using a Rannie homogeniser and subsequently heat-treated for 15 minutes at 85°C.

The emulsion obtained showed a high stability over a time period of at least one month at the conditions of storage at 4 °C and gave a whiteness of $L = 78$ compared to a reference liquid creamer (Crème à Café, Emmi, Switzerland) having a fat content of 15% and a lightness of $L = 75.9$.

5

Example 7: Preparing an aqueous foam

Native β -lactoglobulin (Biopure, Davisco, lot JE 002-8-922, 2 wt-% aqueous solution) was mixed with 120 mM Arginine HCl solution so that the final β -lactoglobulin concentration was 1 wt.-% and Arginine HCl 60 mM. The pH was then adjusted to 7.0 by addition of 1N HCl. The mixture was then heat treated at 80°C for 10 minutes so that 90% of initial β -lactoglobulin was converted into nanoparticles having a z-averaged diameter of 130 nm. In this case, the diameter of the nanoparticles was determined using a Nanosizer ZS apparatus (Malvern Instruments, UK). The sample was poured in a quartz cuvette and variations of the scattered light were recorded automatically. The obtained autocorrelation function was fitted using the cumulants method so that the diffusion coefficient of the particles could be calculated and thereafter the z-averaged hydrodynamic diameter using the Stokes-Einstein law. For this measurement, the refractive index of the solvent was taken as 1.33 and that of the nanoparticles 1.45. A volume of 50 mL of the resulting dispersion of β -lactoglobulin nanoparticles is then foamed by nitrogen sparging through a glass frit generating bubbles of 12-16 μm to produce a foam volume of 180 cm^3 using the standardised Foamsan™ (ITConcept) apparatus. The volume stability of the foam was then followed with time at 26°C using image analysis and compared to the stability of the foam obtained with β -lactoglobulin treated in the same conditions, but without Arginine HCl, where no nanoparticles were formed. Fig. 5 shows that the foam volume stability is greatly improved by the presence of β -lactoglobulin nanoparticles.

Example 8: Whey based Fermented dairy product – fermentation trials

30

Material

Whey protein isolate (WPI) (Bipro®) was obtained from Davisco (Le Sueur, MN, USA) (protein concentration 92.7%).

Spray dried whey permeate (Variolac 836): Lactose concentration: 83 % -Minerals: 8%

35

Lactic Acid 50 %

Edible Lactose (Lactalis)

De-ionized water

Method

The Bipro® powder was dissolved in de-ionized water in order to have a protein concentration of 4.6 %, i.e. for 3 liters of solution 154.5 g of WPI powder and 2845.5 g of water. The hydration time was 3 hours. After hydration, this solution has been divided in samples of 200 ml to prepare the different trials:

Trial	Whey permeate (%)	Lactose (%)	pH adjustment	Heating 85°C / 15 min
1	2.9	2.5	6.5	+
2	0	5	6	+
3	0	5	6.7	-
4	0	5	6.7	+
5	0	5	6.1	+
6	0	0	6	+
7	0	5 (added after pH adjustment)	6	-
8	0	5 (added after pH adjustment)	6	+

Table 2.

For each solution, lactic acid at 50 % has been added to adjust the pH before heating. Samples were heated with the double boiler up to 85°C and maintain at this temperature during 15 minutes. After heating, solutions were cooled at 40°C and inoculated with *Lactobacillus bulgaricus* and *Streptococcus thermophilus*. Samples stayed 5h30 in a steam room at 41°C before to be placed in a cold room at 6°C.

The results are presented in Table 3.

Trial	Whey permeate	Lactose	pH	Heating	pH after 5h30	Aspect
1	+	+	6.5	+	4.68	Very firm
2	-	+	6	+	4.7	Firm
3	-	+	6.7	-	5.78	Liquid
4	-	+	6.7	+	4.81	Very firm
5	-	+	6.1	+	4.59	Very firm
6	-	-	6	+	4.99	Very firm
7	-	- added after pH adjustment	6	-	4.87	Liquid with white speckles
8	-	- added after pH adjustment	6	+	4.77	Firm

Table 3.

Exemple 9: Whey protein boosted ice cream with reduced fat contentMaterial

- 5 Whey protein isolate (WPI, Prolacta90® from Lactalis, Rétiers, France) with a protein content of 90%
Skim milk powder with 35% protein content
Sucrose
Maltodextrins DE39
10 Anhydrous milk fat
Emulsifier
De-ionised water
Edible hydrochloric acid 1M

15 Method

- Using a double-jacketed 80 L tank, the Prolacta90® powder was dispersed at 50°C in de-ionized water at a protein concentration of 9.67 wt% under gentle stirring in order to avoid foam formation, i.e. 3.3 kg of Prolacta90® were dispersed in 31.05 kg of de-ionised water. After 1 hour of dispersion, the pH of the dispersion was adjusted to the
20 nanoparticulation pH by addition of HCl. The temperature of the dispersion was raised to 85°C and maintained for 15 minutes in order to generate the whey protein nanoparticles. After 15 minutes, the temperature was decreased to 50°C and the additional ingredients were sequentially added to the nanoparticles dispersion (i.e. skim milk powder, maltodextrins DE39, sucrose, emulsifier and anhydrous milk fat). The
25 final amount of mix was 50 kg with total solids content of 39.5% and a fat content of 5 wt%. After 30 minutes of hydration, the mix was two-step homogenised (80/20 bars) and pasteurised (86°C/30s) before ageing during overnight.
The day after, the ice-cream mix was frozen at an overrun of 100% using a Hoyer MF50 apparatus and hardened at -40°C before storage at -20°C. The final ice cream contained
30 8wt% proteins (20% caseins, 80% whey proteins) and 5 wt% fat on the ice cream mix basis.

Exemple 10: Powdered whey protein nanoparticles obtained by spray drying35 Material

- Whey protein isolate (WPI, Prolacta90® from Lactalis, Rétiers, France) with a protein content of 90%

Edible lactose
Maltodextrins DE39
De-ionised water
Edible hydrochloric acid 1M

5

Method

Using a double-jacketed 100 L tank, the Prolacta90® powder was dispersed at 50°C in de-ionized water at a protein concentration of 10 wt% under gentle stirring in order to avoid foam formation, i.e. 11 kg of Prolacta90® were dispersed in 89 kg of de-ionised water. After 1 hour of dispersion, the pH of the dispersion was adjusted to the nanoparticulation pH (around 6.3 in that case) by addition of HCl. The temperature of the dispersion was raised to 85°C and maintained for 15 minutes in order to generate the whey protein nanoparticles. After 15 minutes, the temperature was decreased to 50°C and the 10 wt% whey protein nanoparticles dispersion was split in two batches of 50 kg. In a first trial, 20 kg of lactose were dispersed in 50 kg of nanoparticles dispersion at 50°C and stirred for 30 min. Similarly, 20 kg of maltodextrins DE39 were added to the remaining 50 kg of whey protein nanoparticles dispersion.

The two mixtures were then spray dried into a NIRO SD6.3N tower at a flow rate of 15 L/h. The air input temperature was 140°C and the air output temperature was 80°C. The water content of the obtained powders was lower than 5%.

The size of the whey protein nanoparticles was determined in presence of lactose and maltodextrin (DE39) in water using dynamic light scattering before and after spray drying. The total protein concentration was set to 0.4 wt% by dilution of the dispersion before spray drying or reconstitution of the powder in order to be in the dilute regime of viscosity for whey protein nanoparticles. A Nanosizer ZS apparatus (Malvern Instruments) was used and nanoparticle diameter was averaged from 20 measurements.

The particle diameter determined for whey protein nanoparticles in presence of lactose and maltodextrins (DE39) was 310.4 nm and 306.6, respectively. After reconstitution of the powders, the respective diameters were found to be 265.3 nm and 268.5, respectively. These measurements confirm that whey protein nanoparticles were physically stable regarding spray drying. The results were corroborated by TEM microscopy observations of 0.1 wt% whey protein nanoparticles dispersions in water using negative staining in presence of 1% phosphotungstic acid at pH 7. A Philips CM12 transmission electron microscope operating at 80 kV was used. Whey protein nanoparticles were observed in solution before spray drying and after reconstitution of the spray-dried powder. No difference of morphology and structure could be detected.

Claims

1. A method for the production of nanoparticulated whey protein comprising the
5 step of :
 - i) adjusting the pH at a very precise narrow range of an aqueous solution of whey protein, or adjusting the ionic strength of the whey protein preparation while keeping the pH constant, and
 - ii) subjecting the aqueous solution to a temperature of between 80° to 95°C, during a
10 time period of from 10 seconds to 30 minutes, to obtain a liquid dispersion of spherical nanoparticulated whey protein having a particle size of less than 1 µm,
2. The method according to claim 1, inducing spontaneous nanoparticulation of whey proteins during heat treatment in absence of shearing, in which the proportion of
15 nanoparticles in the liquid dispersion is of at least 20% and residual soluble aggregates or soluble protein below 80%.
3. The method according to claim 1 or 2, in which the average nanoparticle size is characterised by a polydispersity index below 0.200
20
4. The method according to claim 1, wherein the time period is 15 minutes and the temperature 85°C
5. The method according to one of claims 1 to 4, wherein :
25 - the pH is 5.6 to 6.4, preferably 5.8 to 6.0 to obtain negatively charged nanoparticules or,
- the pH is 7.5 to 8.4, preferably 7.6 to 8.0 to obtain negatively charged nanoparticules in presence of large amount of free minerals or,
- the pH is 6.4 to 7.4, preferably 6.6 to 7.2 to obtain negatively charged nanoparticules
30 in presence of moderate content of free minerals.
6. The method according to one of claims 1 to 4, wherein the pH is 3.5 to 5.0, to obtain positively charged nanoparticules.
7. The method according to one of claims 1 to 6, in which the dispersion of
35 nanoparticles is subjected to drying.

8. The method according to any of the preceding claims, wherein the aqueous whey protein solution is essentially salt free or where ionic strength has been adjusted by organic or inorganic ions in such a way that it allows nanoparticulation at constant pH.

5

9. The method according to any of the preceding claims, wherein the whey proteins are present in the aqueous solution in an amount of 0.1 wt.-% to 12 wt.-%, preferably 0.1 wt.-% to 8 wt.-%, more preferably 0.2 wt.-% to 7 wt.-%, even more preferably 1 wt.-% to 4 wt.-%, on the basis of the total weight of the solution.

10

10. The method according to any of the preceding claims, in which the whey is replaced by any other demineralized or slightly mineralized globular protein, in particular from egg, soy, cereals, oilseeds, or from other vegetable or animal origin.

15

11. Nanoparticulated whey protein obtainable by a method according to any of claims 1 to 10.

12. Nanoparticulated whey protein according to claim 11, having a Protein Efficiency of at least 118.

20

13. Nanoparticulated protein obtainable by a method according to claim 11, having a Protein Efficiency Ratio of at least 100

14. Use of a nanoparticulated whey protein according to any of the claims 11 to 13 for the preparation of a food product, a food supplement, a nutritional and/or pharmaceutical composition.

25

15. Use according to claim 14, for low fat products.

30

16. The use according to claim 14 or 15, for coffee creamers, yoghurt, pasteurized, UHT milk, sweet condensed milk, fermented milks, milk-based fermented products, **milk chocolate**, mousses, foams, emulsions, ice cream, milk based powders, infant formulae, diet fortifications, pet food, or tablets, dried oral supplement, wet oral supplement.

35

17. Food product, food supplement, nutritional and/or pharmaceutical composition, containing a nanoparticulated whey protein according to any of the claims 11 to 13.

18. A composition according to claim 17, which is coffee creamers, yoghurt, fermented milks, milk-based fermented products, mousses, ice creams, milk based powders, infant formula, diet fortifications, pet food, or tablets, dried oral supplement, wet oral supplement.
- 5