(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
5 June 2003 (05.06.2003)

PCT

(10) International Publication Number
WO 03/045163 A1

(51) International Patent Classification[7]: A23L 1/05, 1/00, A23J 3/00

(21) International Application Number: PCT/NZ02/00261

(22) International Filing Date:
27 November 2002 (27.11.2002)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
515752 27 November 2001 (27.11.2001) NZ

(71) Applicant *(for all designated States except US)*: **AUCKLAND UNISERVICES LIMITED** [NZ/NZ]; Level 9, 70 Symonds Street, Auckland (NZ).

(71) Applicant *(for US only)*: **SMITH, Bronwen, Geraldine** [NZ/NZ]; 315 Mt Eden Road, Mt Eden, Auckland (NZ).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **OLIVER, Christine, Maree** [NZ/NZ]; 17 Prospect Terrace, Mount Eden, Auckland (NZ). **MELTON, Laurence, David** [NZ/NZ]; 69B St Johns Road, Meadowbank, Auckland (NZ). **STANLEY, Roger, Anthony** [NZ/NZ]; 41 Kopiko Road, Titirangi, Auckland (NZ).

(74) Agent: **BALDWIN SHELSTON WATERS**; P.O. Box 852, Wellington (NZ).

(81) Designated States *(national)*: AE, AG, AL, AM, AT (utility model), AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ (utility model), CZ, DE (utility model), DE, DK (utility model), DK, DM, DZ, EC, EE (utility model), EE, ES, FI (utility model), FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SC, SD, SE, SG, SI, SK (utility model), SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

**Published:**
— *with international search report*

*For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.*

WO 03/045163 A1

(54) Title: SUGAR-PROTEIN CONJUGATES AND THEIR FORMATION

(57) Abstract: The present invention provides methods of preparing a sugar- protein conjugate comprising the step of reacting together under glycosylation conditions at a controlled relative humidity (a) an effective amount of at least one proteinaceous foodstuff; (b) an effective amount of one or more oligosaccharide sugars having between 3-30 sugar units and/or (c) an effective amount of one or more monsaccharide(s) or one or more disaccharide(s), and wherein the resulting sugar-protein conjugate products have improved functional viscosity over the combined viscosity properties of the individual reactants

# SUGAR-PROTEIN CONJUGATES AND THEIR FORMATION

This invention relates generally to a sugar-protein conjugate, having commercially useful functional properties. The invention also relates to a method of preparing one or more such sugar-protein conjugates.

## BACKGROUND

In the manufacture of a range of foodstuffs, it is common practice to include a thickening material that raises the viscosity of the foodstuff so that it sets or at least becomes more viscous. Pectins and other vegetable gums, acacia gum, xanthan, gelatine, halal gelatine, agar and carrageenan are examples of such thickeners. Methylcellulose thickeners are used in the pharmaceutical industry. Least cost functional formulation principles apply to products in this area, so permitting (for example) the dairy industry to sell more of a particular ingredient at a greater price if the functionality is higher. Another way of adding value arises if less material is required in order to achieve a desired effect. Products with improved functionality will be more readily accepted if they are based on widely available materials that are already accepted as foodstuffs in the food industry.

Combinations of casein and a selection of polysaccharides are known in the literature. However a glycosylation reaction without using non-toxic cross-linking reagents has only been described in a few publications. Most work in this area has been directed to the production of emulsifiers or foam stabilisers. Kato *et al.* (Biosci Biotech Biochem 56 (4) 567-571 (1992)) describes a conjugate of casein with dextran, or a conjugate of casein with galactomannan, which latter compound has 1.5 times improved emulsifying activity and 10 times better emulsion stability than that of casein. The material was made by heating at 60 deg C for 24 hours at a controlled humidity of 79%.

Dickinson *et al.* (Colloids and Surfaces 113 191-201 (1996)) describes a conjugate of bovine serum albumin (BSA), or lysozyme, or casein with high molecular weight dextran. The initially poor foaming properties of lysozyme were enhanced while those of beta-casein were reduced. The foaming properties of BSA were not

greatly affected. As the molecular weight (MW) of the dextran rose, the alteration was more pronounced. The material was made with a Maillard reaction, by heating 1:1 dried mixtures at 60 deg C for up to 3 weeks at controlled humidity of 40%.

Shepherd *et al* (Food Hydrocolloids 14 281-286 (2000)) describes the preparation of a conjugate of casein using maltodextrin (Maltrin 100). The chemically modified sodium casein was prepared by dry heating a 1:1 mixture of sodium casein and maltodextrin at 60 deg C for 23 - 120 hours at a controlled humidity of 79 %. The resulting chemically modified casein had better solubility at low pH, improved emulsifying activity and 10 times better emulsion stability than that of the unmodified casein.

Interestingly, Shepherd *et al.* observed a tendency for the formation of an undesirable brown colour on reaction, which is perhaps related to the Maillard reaction or to the caramelisation of sugars. They found that this tendency to brown could be abolished by the dialysis of the Maltrin 100 resulting in "pristine white powders". The tendency to brown could not be abolished by the dialysis of casein. Accordingly Shepherd *et al.* recommend that steps be taken to remove the low molecular weight carbohydrate material (believed to include 0.8% glucose and 2.8% maltose) from the reactants so that any industrial process would need less stringent control to prevent browning.

Courthaudon, J-L., Colas, B., Lorient, D.Covalent binding of glycosyl residues to bovine casein: effects on solubility and viscosity.Journal of Agricultural and Food Chemistry (1989) Vol 37, p32-36. describes methods of binding simple carbohydrates to bovine casein. The process employed toxic cross-linking agents. Additionally, the achieved increases in viscosities were low, despite using up to 10% (w/v) casein. The increase in viscosity is inefficient given the high concentration of protein required.

Therefore a problem to be solved is to make a sugar-protein conjugate having enhanced properties over those of the raw material(s), such enhancement being for example an increase in viscosity when in solution, and to make the sugar-

protein conjugate from abundantly available raw materials in a manner which results in the sugar-protein conjugate being acceptable for use in foodstuffs.

It is therefore an object of the present invention to prepare a sugar-protein conjugate, having commercially useful viscosity properties or at least to provide the public with a useful choice.

It is to be understood that the term "sugar-protein conjugate" used throughout the specification means a sugar-protein conjugate that is obtained without using reagents that are toxic for human or animal consumption and that the sugar-protein conjugate so obtained can be consumed by humans or animals without deleterious effect.

It is also to be understood that the term "foodstuff(s)" as used throughout the specification means a non-toxic reactant(s) or product(s) that can be consumed by humans or the like without deleterious effect.

STATEMENT OF INVENTION

In a first aspect the present invention provides a method of preparing a sugar-protein conjugate comprising the step of reacting together under glycosylation conditions at a controlled relative humidity

(a) an effective amount of at least one proteinaceous foodstuff;

(b) an effective amount of one or more oligosaccharide sugars having between 3-30 sugar units and

(c) an effective amount of one or more monsaccharide(s) or one or more disaccharide(s),

wherein the reaction is conducted in the absence of further reactants intended to promote the glycosylation reaction so that the sugar-protein conjugate product is substantially devoid of materials unsuitable for use as a foodstuff and wherein the sugar- protein conjugate product has improved functional viscosity over the combined viscosity properties of the individual reactants.

Preferably, the one or more monosaccharide(s) or one or more disaccharide(s)are selected from the range of reducing sugars including fructose, glucose, ribose, deoxyribose, lactose, lactulose, maltose, galactose, and mannose.

More preferably the one or more monsaccharide(s) or one or more disaccharide(s) includes fructose and/or glucose.

Preferably, the one or more oligosaccharide sugars comprises a fructan (fructo-oligosaccharide) selected from the range including inulin (1,2 links), levan, (2,6 links) and graminans (mixed 1,2 and 2,6 links) comprising more than 2 fructose units or sugars linked together by glycosidic bonds. Alternatively, the one or more oligosaccharide sugars comprises one or more glucan oligosaccharides selected from the range dextrin (1,4 links) dextran (1,6 links) or mixed linkages comprising more than 2 glucose units or sugars linked together by glycosidic bonds or a mixture of glucan and fructan oligosaccharide sugars.

In the above aspect, it is preferred that the proteinaceous foodstuff is selected from a casein, caseinates, a whey protein, lactalbumin, bovine serum albumin, egg albumin, and soy protein.

More preferably the proteinaceous foodstuff is selected from casein or one or more caseinates.

Preferably the effective amounts of reactants are present in a gravimetric ratio of between 1 part of proteinaceous foodstuff, between 0.5 and 5 parts of one or more oligosaccharides and between 0.01 and 2 parts of one or more monsaccharide(s) or one or more disaccharide(s). It is preferred that the effective amounts of reactants are present in a gravimetric ratio of between 1 part of caseinate, between 0.5 and 5 parts of inulin and between 0.01 and 1 part of fructose. Most preferably the gravimetric ratios of the reactants is: 1 part of sodium caseinate to 1 part of inulin, and 0.2 parts of fructose.

Preferably, the controlled relative humidity is in the range of from 40 to 100 % at the temperature of the reaction. More preferably the relative humidity is about 70 to 80 % R H, most preferably 80% relative humidity.

Preferably the method includes the further steps of preparing and storing the resulting sugar-protein conjugate as a dry material.

In another related aspect the present invention provides a sugar-protein conjugate obtained according to the methods defined above.

In a further related aspect the present invention includes a food product incorporating an effective amount of the sugar-protein conjugate obtained by the methods defined above.

In a second main aspect the present invention further provides a method of preparing a sugar- protein conjugate comprising the step of reacting together under glycosylation conditions at a controlled relative humidity

(a) an effective amount of at least one proteinaceous foodstuff; and

(b) an effective amount of one or more monsaccharide(s) or one or more disaccharide(s)

wherein the reaction is conducted in the absence of further reactants intended to promote the glycosylation reaction so that the sugar-protein conjugate product is substantially devoid of materials unsuitable for use as a foodstuff and wherein the sugar- protein conjugate product has improved viscosity over the combined viscosity properties of the individual reactants.

Preferably, the one or more monsaccharide(s) or one or more disaccharide(s) are selected from the range of reducing sugars including fructose, glucose, deoxyribose, ribose, lactose, lactulose, maltose, galactose, and mannose.

More preferably the monosaccharide is fructose and/or glucose.

Preferably, the proteinaceous foodstuff is selected from a casein, caseinates, a whey protein, lactalbumin, bovine serum albumin, egg albumen, and soy protein.

Most preferably, the proteinaceous foodstuff is a casein or caseinates.

Preferably the effective amounts of reactants are present in a gravimetric ratio of between 1 part of a proteinaceous foodstuff, and between 0.01 and 2 parts of one or more monosaccharides or dissacharides. It is preferred that the effective amounts of reactants are present in a gravimetric ratio of between 1 part of sodium caseinate, and between 0.01 and 1.0 part of fructose and/or glucose. Most preferably the gravimetric ratios of the reactants is: 1 part of sodium caseinate to 0.2 parts of fructose.

Preferably, the controlled relative humidity is in the range of from 40 to 100 % relative humidity (RH) at the temperature of the reaction. More preferably the relative humidity is about 70 to 80 % R H, most preferably 70% relative humidity.

Preferably the method includes the further steps of preparing and storing the resulting sugar-protein conjugate as a dry material.

In a further aspect of the present invention provides is a sugar-protein conjugate obtained according to the methods defined above.

In a further related aspect the present invention includes a food product incorporating an effective amount of the sugar-protein conjugate obtained by the methods defined above.

In a further aspect the present invention provides a method for manufacturing a sugar-casein conjugate the method including the steps of:

(a) mixing an effective amount of casein and/or an effective amount of a caseinate and an effective amount of at least one reactant capable of forming a conjugate with the casein and/or the caseinate in a desired ratio, and in the presence of a controlled amount of water,

(b) evaporating the resultant admixture to about 3-10% water content by weight,

(c) heated under controlled relative humidity.

Preferably the mixing step (a) is carried out in solution, more preferably an aqueous solution.

Preferably, step (b) is carried out to produce a powder.

Preferably, step (c) is carried out at temperatures of between 40 degrees to 80 degrees, more preferably at a temperature of 60 degrees.

Preferably step (c) is carried out for periods of time from between 3-120 hours, more preferably between 24 – 48 hours.

Alternatively step (c) is carried out at a temperature of 80-120 degrees for 0.1 - 3 hours.

Preferably, the caseinate is selected from sodium, calcium or potassium caseinates, most preferably the caseinate is sodium caseinate.

Preferably, the at least one reactant capable of conjugating casein and/or the caseinate is selected from one or more of the following sugars including fructose, glucose, deoxyribose, ribose, lactose, lactulose, maltose, galactose, mannose; one or more fructans (fructo-oligosaccharide) selected from the range including inulin (1,2 links), levan, (2,6 links) and graminans (mixed 1,2 and 2,6 links) comprising more than 2 fructose units or sugars linked together by glycosidic bonds; and one or more glucan oligosaccharides selected from the range dextrin (1,4 links) dextran (1,6 links) or mixed linkages comprising more than 2 glucose units or sugars linked together by glycosidic bonds or a mixture of glucan and fructan oligosaccharide sugars.

Preferably, where the at least one reactant capable of conjugating casein and/or the caseinate is selected from one or more of the sugars defined above, the effective amount of the sugars is selected so as to provide a change in the viscosity of the sugar-casein/caseinate conjugate over the individual viscosity properties of the one or more sugars and the casein/caseinate conjugate.

In a preferred embodiment preferably the effective amounts of the casein and/or a caseinate and the at least one reactant capable of conjugating casein and/or the caseinate are present in a gravimetric ratio of between 1 part of sodium caseinate, between 0.5 and 5 parts of inulin, and between 0.01 and 2 parts of fructose and/or

glucose. Most preferably, the gravimetric ratios are 1 part of sodium caseinate, 1 part of inulin, and 0.2 parts of fructose.

In the alternative, preferably the effective amounts of the casein and/or a caseinate and the at least one reactant capable of conjugating casein and/or the caseinate are present in a gravimetric ratio of between 1 part of a proteinaceous foodstuff, and between 0.01 and 2 parts of one or more monosaccharides or dissacharides. It is preferred that the effective amounts of reactants are present in a gravimetric ratio of between 1 part of a caseinate, and between 0.01 and 1.0 part of fructose and/or glucose. Most preferably the gravimetric ratios of the reactants is: 1 part of sodium caseinate to 0.2 parts of fructose.

Preferably the controlled relative humidity in step (c) of the reaction corresponds to a relative humidity of between 40 % and 100%, more preferably of between 70 % and 80 % and most preferably of about 70 %.

In a preferred embodiment an industrial scale synthesis of the substance includes the steps of:

(a) thorough mixing of a finely divided effective amount of casein and/or an effective amount of a caseinate and a finely divided effective amount of at least one reactant capable of forming a conjugate with the casein and/or the caseinate in a desired ratio, and in the presence of a controlled amount of water sufficient to solublise the sugar components of the mixture,

(b) evaporating the resultant admixture to about 3-10% water content by weight by evaporating the admixture obtained in step (a) in either a batch process or a continuous process, to create a powder

(c) heating the powder with regulation of the relative humidity to within specified limits by means of humidity sensors and water evaporators connected to process control devices, and

(d) testing the resulting viscosity of the casein/caseinate conjugate then bagging and storage as a substantially dry powder.

Steps (a) and (b) can be combined by careful control of the evaporation and heating to result in a powder of the desired water activity.

Further aspects of the present invention will become apparent from the following detailed description given by way of example only.

DETAILED DESCRIPTION

This invention involves the conjugation/glycosylation of a protein source, such as a casein/caseinate with a combination of a monosaccharide or disaccharide and a large sugar molecule (between 3 to 30 sugar units), such as the monosaccharide fructose, with the fructan (oligosaccharide) inulin) in a dry heat-promoted reaction to completion under controlled humidity produces a more usefully modified sugar-protein conjugate product having enhanced viscosity properties.

Surprisingly, the inventors have also established that the glycosylation/conjugation of a monosaccharide or a disaccharide, with a protein source such as a casein/caseinate in a dry heat-promoted reaction under controlled humidity produces a more usefully modified sugar-protein conjugate product having enhanced viscosity properties.

The inventors sought to eliminate the need to use a chemical oxidising and reducing agent to catalyse and then stabilise a reaction involving a casein or a salt thereof in order to modify the physical properties of the casein.

The following preparations, examples and results outline some of the preferred embodiments of the present invention.

**General Methods of Examples**

**Sample Preparation**

**Materials:** Sodium caseinate (Alanate 180, New Zealand Dairy Ingredients Ltd.); Maltodextrin (Dridex 10, NZ Starch); Inulin (Frutafit HD,) average chain length 9-12 monomers, ≤ 10 % monomers, fructose, glucose and ribose were supplied by Sigma Chemical Company, St Louis, Missouri, U.S.A.

**Sample production:** Samples were prepared by dissolving the reactants in Milli-Q water according to the Tables in the Examples. Prepared solutions were frozen and subsequently freeze-dried. Freeze-dried samples were pre-equilibrated to the selected relative humidity (RH) at room temperature (~22 °C) by being placed within a desiccator maintaining a controlled RH. Various saturated salt solutions were used to achieve the specified RH (Smith, 1971, refer Table 1 below). The actual RH was recorded using a thermohygrometer. In each case 100 ml of the saturated salt solution was placed inside a 500 ml desiccator and equilibrated at room temperature (18-22 °C) at least 24 h before introducing the samples. The dry specimens were stirred daily to encourage uniform equilibration. After 3 days the samples had equilibrated, as determined by measuring water activity of samples in a preliminary trial. Following pre-equilibration the bottled samples were capped and sealed outside with parafilm until placed in the heating desiccator. Samples were subsequently heated within a desiccator (pre-equilibrated at 80 % RH, 60 °C) for the desired time.

Alternatively freeze-dried samples from Example 2 were not pre-equilibrated but immediately placed in a temperature and humidity controlled chamber with a fan to circulate the atmosphere across the freeze-dried material.

**Table 1: Equilibrium Relative Humidity of selected saturated salt solutions.**

| Salt | RH at 22 °C |
|---|---|
| Sodium Bromide | 50 % |
| Cupric Chloride | 67 % |
| Barium Chloride | 80 % |

**Viscosity property determination**

**Equipment:** Viscosity measurements were acquired using a cone and plate Paar Physica Rheometer UDS-200 (USA).

**Method of testing :** Dry specimens were weighed out and Milli-Q water added to give 3 % w/v caseinate content plus any carbohydrate additive. Thus all samples

were compared at the same protein concentration. Samples were left at room temperature (18-22 °C) overnight (approximately 16 h), with intermittent stirring, to allow full hydration. Aliquots of the 3 % w/v caseinate stock solution were diluted to give conjugates at 2 % and 1% w/v caseinate. A constant volume of sample (200 µl) was pipetted onto the rheometer Peltier plate, equilibrated at 20 °C, to form a small circular shape about 1 cm diameter. The sample was left to temperature equilibrate (1 min). The probe (plate, 25 cm diameter) was then moved down onto the sample compressing it until a gap of 0.3 mm remained between the probe and the Peltier plate. Viscosities were compared at 108/s shear rate.

**Soluble protein determination:**

The soluble protein content of caseinate conjugates was determined pH according to a modified method of Bradford (1976) after centrifugation in buffers of different pH.

**Example 1**

Samples were prepared according to the reagent ratios in the Table and heated for 48 h at 60 °C, 80 % RH. The viscosity of the preparations was determined and values are the average of duplicate measurements ± standard deviation.

| Sample (protein:sugar w/w) | Protein concentration (% w/v) | Viscosity at 20 °C 108/s (cP) |
|---|---|---|
| Caseinate | 3 % | 12.3 ± 1.63 |
| Caseinate/inulin 1:1 | 3 % | 13.8 ± 0.64 |
| Caseinate/inulin 1:1.5 | 3 % | 15.1 ± 0.71 |
| Caseinate/inulin 1:2 | 3 % | 16.7 ± 1.06 |
| Caseinate/inulin/fructose 1:1:0.2 | 3 % | 180.5 ± 4.95 |
| Caseinate/inulin/fructose 1:1:0.2 | 2 % | 101.4 ± 13.6 |
| Caseinate/inulin/fructose 1:1:0.2 | 1 % | 42.5 ± 8.84 |
| Caseinate/inulin/fructose 1:2:0.2 | 3 % | 298.0 ± 4.24 |

| | | |
|---|---|---|
| Caseinate/inulin/fructose 1:2:0.2 | 2 % | 137.5 ± 0.71 |
| Caseinate/inulin/fructose 1:2:0.2 | 1 % | 41.8 ± 3.82 |
| Caseinate/inulin/fructose 1:5:0.2 | 3 % | 325.0 ± 4.24 |
| Caseinate/inulin/fructose 1:5:0.2 | 2 % | 108.0 ± 14.14 |
| Caseinate/inulin/fructose 1:5:0.2 | 1 % | 54.9 ± 2.47 |

As can be seen from the results the combination of the oligosaccharide inulin and caseinate alone did not substantially improve the viscosity. However conjugates formed at 1:1:0.2 caseinate/inulin/fructose ratio formed highly viscous suspensions having 15x the viscosity of unmodified caseinate. The viscosity of caseinate/inulin/fructose increased linearly with protein concentration.

**Example 2**

Samples were prepared according to the ratios in the following Table and heated for up to 96 hours at 60 °C, 70 % RH. Samples were taken at 24, 32, 48 and 96 hours. The viscosity of the preparations was determined and values are the average of duplicate measurements ± standard deviation.

| **Sample (protein:sugar w/w)** | **Viscosity at 20 °C, 108/s, of caseinate glycoconjugates at 3 % w/v protein (cP)** | | | |
|---|---|---|---|---|
| | **Heating time** | | | |
| | **24 h** | **32 h** | **48 h** | **96 h** |
| Caseinate | 11.0 ± 0.71 | | 10.6 ± 0.71 | 11.6 ± 0.92 |
| Caseinate/maltodextrin 1:1 | | | | 12.2±0.42 |
| Caseinate/inulin 1:1 | | | | 16.5 ± 1.77 |
| Caseinate/inulin/fructose 1:1:0.2 | 10.2 ± 0.61 | | 11.6 ± 0.14 | 40.9±0.49 |
| Caseinate/fructose 1:0.2 | | 180.5 ± 2.12 | | Gelled particles |
| Caseinate/glucose 1:0.2 | 202.0 ± 14.14 | | Gelled particles | Gelled particles |

As can be seen from the Table the preparations containing protein, and a monosaccharide reacted to form a product that made a viscous solution at 3% w/w protein concentration faster than equivalent preparations containing protein, oligosaccharide and monosaccharide. The presence of the oligosaccharide modified the reaction preventing the product from overreacting to insoluble but hydrated gelled particles. Preparations of protein and glucose monosaccharide reacted fastest forming product that after, only 48 hours, made insoluble gelled particles. Neither caseinate nor caseinate inulin nor caseinate maltodextrin products incubated for 96 hours formed 3% solutions with increased viscosity.

**Example 3**

Samples were prepared according to the following Tables and heated for 48 h at 60 °C, 67 % RH. The viscosity of the preparations was determined and values are the average of duplicate measurements ± standard deviation.

| Sample (protein:sugar w/w) | Protein concentration (% w/v) | Viscosity at 20 °C, 108/s (cP) |
|---|---|---|
| Caseinate | 3 % | 11.0 ± 0.00 |
| Caseinate/fructose 1:0.02 | 3 % | 25.7 ± 0.99 |
| Caseinate/fructose 1:0.02 | 2 % | 19.5 ± 1.77 |
| Caseinate/fructose 1:0.02 | 1 % | 14.0 ± 1.34 |
| Caseinate/fructose 1:0.04 | 3 % | 47.3 ± 1.06 |
| Caseinate/fructose 1:0.04 | 2 % | 34.0 ± 4.95 |
| Caseinate/fructose 1:0.04 | 1 % | 18.7 ± 0.92 |
| Caseinate/fructose 1:0.2 | 3 % | 261.5 ± 16.26 |
| Caseinate/fructose 1:0.2 | 2 % | 153.5 ± 9.19 |
| Caseinate/fructose 1:0.2 | 1 % | 101.4 ± 3.68 |
| Caseinate/fructose 1:0.4 | 3 % | 81.4 ± 6.79 |
| Caseinate/fructose 1:0.4 | 2 % | 38.8 ± 4.81 |
| Caseinate/fructose 1:0.4 | 1 % | 23.3 ± 0.85 |
| Caseinate/fructose 1:0.8 | 3 % | 10.7 ± 0.42 |

As can be seen from the Table increasing ratios of the monosaccharide fructose to protein showed a marked increase in viscosity compared to that of the unmodified protein. A maximum viscosity improvement, approximately 20x that of unmodified caseinate, was observed at 1:0.2 caseinate:fructose after which the viscosity was observed to fall. The viscosity of the caseinate/fructose conjugates increased linearly with concentration.

**Example 4**

Samples were prepared according to the following Tables and heated for 24 h (*) or 48 h at 60 °C, 67 % RH. The viscosity of the preparations was determined and values are the average of duplicate measurements ± standard deviation.

| Sample (protein:sugar w/w) | Protein concentration (% w/v) | Viscosity at 20 °C, 108/s (cP) |
|---|---|---|
| Caseinate/fructose 1:0.2 | 3 % | 261.5 ± 16.26 |
| Caseinate/fructose 1:0.2 | 2 % | 153.5 ± 9.19 |
| Caseinate/fructose 1:0.2 | 1 % | 101.4 ± 3.68 |
| Caseinate/lactose 1:0.2 | 3 % | 189.0 ± 7.07 |
| Caseinate/lactose 1:0.2 | 2 % | 144.0 ± 4.24 |
| Caseinate/lactose 1:0.2 | 1 % | 66.8 ± 9.40 |
| Caseinate/glucose 1:0.2* | 3 % | 303.5 ± 9.19 |
| Caseinate/glucose 1:0.2* | 2 % | 190.0 ± 1.41 |
| Caseinate/glucose 1:0.2* | 1 % | 98.7 ± 7.50 |
| Caseinate/glucose 1:0.02 | 3 % | 123.0 ± 1.41 |
| Caseinate/glucose 1:0.02 | 2 % | 77.6 ± 7.71 |
| Caseinate/glucose 1:0.02 | 1 % | 45.4 ± 14.0 |
| Caseinate/ribose 1:0.02* | 3 % | 245.0 ± 31.11 |
| Caseinate/ribose 1:0.02* | 2 % | 142.0 ± 2.83 |
| Caseinate/ribose 1:0.02* | 1 % | 65.2 ± 8.77 |

As can be seen from the Table the monosaccharides glucose-, fructose-, ribose- and lactose-caseinate glycoconjugates all showed significant viscosity that was

10-25x that of unmodified caseinate. Glucose at 1:0.2 protein:sugar and ribose containing reactions proceeded faster and were stopped at 24 hours. Ribose formed viscous solutions even at protein:saccharide ratios as low as 1:0.02.

**Example 5**

Samples were prepared according to the following Tables and heated at 60 °C for the times as indicated the relative humidities as indicated. The viscosity of the preparations was determined and values are the average of duplicate measurements ± standard deviation.

| Heating time | Sample (protein:sugar w/w) | 50 % RH | 67 % RH | 80 % RH |
|---|---|---|---|---|
| Unheated | Caseinate | 10.2± 0.00 | 10.2± 0.00 | 10.2± 0.00 |
| Unheated | Caseinate/fructose 1:0.2 | 10.1± 0.07 | 10.1± 0.07 | 10.1± 0.07 |
| 24h | Caseinate/fructose 1:0.2 | 9.36± 0.13 | 10.7± 0.28 | Gelled particles |
| 48h | Caseinate/fructose 1:0.2 | 13.0± 0.28 | 24.5± 0.71 | Gelled particles |
| 72h | Caseinate/fructose 1:0.2 | 21.0± 1.48 | 46.1± 3.75 | Gelled particles |
| 96 h | Caseinate/fructose 1:0.2 | 36.2± 2.83 | Gelled particles | Gelled particles |
| 120h | Caseinate/fructose 1:0.2 | 31.8± 6.86 | Gelled particles | Gelled particles |

The results Table indicates that the viscosity of the product made by reacting fructose monosaccharide and caseinate increased up to 3x with 4-5 days heating at 50% RH. However at higher RH levels the reaction proceeded faster as a function of RH proceeding to form products that were more viscous and then would not redissolve properly.

**Example 6**

Samples were prepared according to the following Table and heated at 60 °C, 80 % RH, unless otherwise indicated for 48 h. The solubility of the protein in the preparations was determined and values (mg/ml) are the average of duplicate measurements ± standard deviation.

| Sample (protein:sugars w/w) | pH | | | | | | |
|---|---|---|---|---|---|---|---|
| | 3.0 | 3.6 | 4.0 | 4.6 | 5.0 | 5.6 | 6.0 |
| Caseinate | 1.90±0.19 | 0.04±0.16 | 0.00±0.04 | 0.06±0.14 | 2.23±0.15 | 2.29±0.25 | 2.20±0.40 |
| Caseinate/fructose (67 % RH)1:0.2 | 0.51±0.06 | 0.05±0.00 | 0.03±0.04 | 0.44±0.24 | 0.74±0.14 | 0.69±0.23 | 0.81±0.07 |
| Caseinate/inulin1:1 | 1.87±0.14 | 0.03±0.00 | 0.01±0.04 | 0.18±0.21 | 2.23±0.26 | 2.15±0.31 | 2.19±0.19 |
| Caseinate/inulin/fructose1:1:0.2 | 0.91±0.22 | 0.03±0.07 | 0.03±0.03 | 0.60±0.21 | 0.88±0.16 | 0.89±0.22 | 0.92±0.03 |
| Caseinate/maltodextrin1:1 | 1.89±0.37 | 1.80±0.84 | 1.68±0.01 | 1.96±0.61 | 1.98±0.40 | 2.10±0.45 | 2.11±0.16 |
| Caseinate/maltodextrin/fructose1:1:0.2 | 1.94±0.07 | 0.27±0.02 | 0.08±0.07 | 2.10±0.66 | 1.95±0.12 | 2.15±0.26 | 2.06±0.02 |

As can be seen from the Table caseinate/fructose, and caseinate/inulin/fructose conjugates had low solubility over the entire pH range 3.0-6.0. However, close to the isoelectric point of casein, pH 4.6, their solubility was greater than unmodified caseinate. The solubility of caseinate/inulin glycoconjugates was comparable to unmodified caseinate at all pH values 3.0-6.0. Only caseinate/maltodextrin glycoconjugates had high solubility at all pH values. Moreover, between pHs 3.6-4.6, solubility was considerably greater for caseinate/maltodextrin conjugates than unmodified caseinate, as reported by Shepherd et al (Food Hydrocolloids 14 281-286 (2000)) indicating that the products of the present invention are distinctly different.

It is to be appreciated that this invention can be applied to many of the abundant food proteins and is not limited to the materials used in the examples. For example it is envisaged that one could utilise soy proteins, casein, caseinates, whey protein, lactalbumin, bovine serum albumin, egg albumin, or soy protein, all being abundant food proteins. Caseinate was selected as a convenient example and because of its use in industrial applications.

While inulin and fructose appear to be particularly appropriate in the embodiments of the invention, other sugars may be used, such as another inulin family, with for example one or more of glucose, ribose, lactose, lactulose, maltose, galactose, and mannose.

Inulin, having a fructan structure is a selected representative of the oligosaccharides. Other molecules which could be substituted in part or completely for the inulin used in the invention may be selected from the levan family, or the graminan family or the like. Some oligosaccharides may be branched, as found in nature.

Fructose, a preferred monosaccharide, is a member of the ketose family. It is to be appreciated that other "analogues" of fructose could be substituted in part or completely for the fructose used in the invention.

Other than fructans, possibilities include fructose, glucose polymers and short glucose chains. Glucose and fructose are similarly reactive with caseins.

Some applications to which the present invention could be put include the following

1 The invention could make known by-products of the dairy industry (or the Soya bean industry for example) more versatile by increasing their viscosity.

2 The invention could find application as food thickening agents in the dairy industry

3 The invention could find application in the manufacture of non fat or low fat salad dressings or the like.

Finally, it will be understood that the scope of this invention as described herein is not limited to the specified embodiments. Those of skill will appreciate that various modifications, additions, known equivalents, and substitutions are possible without departing from the scope and spirit of the invention as set forth.

CLAIMS

1 A method of preparing a sugar- protein conjugate comprising the step of reacting together under glycosylation conditions at controlled relative humidity,

(a) an effective amount of at least one proteinaceous foodstuff;

(b) an effective amount of one or more oligosaccharide sugars having between 3-30 sugar units and

(c) an effective amount of one or more monsaccharide(s) or one or more disaccharide(s)

wherein the reaction is conducted in the absence of further reactants intended to promote the glycosylation reaction so that the sugar-protein conjugate product is substantially devoid of materials unsuitable for use as a foodstuff and wherein the sugar- protein conjugate product has improved functional viscosity over the combined viscosity properties of the individual reactants.

2 The method according to claim 1 wherein the one or more monsaccharide(s) or one or more disaccharide(s) are selected from the range of reducing sugars including fructose, glucose, deoxyribose, ribose, lactose, lactulose, maltose, galactose, and mannose.

3 The method according to claim 2 wherein the one or more monsaccharide(s) is fructose.

4 The method according to any one of claims 1 to 3 wherein the one or more oligosaccharide sugars comprises a fructan (fructo-oligosaccharide) selected from the range including inulin (1,2 links), levan, (2,6 links) and graminans (mixed 1,2 and 2,6 links) comprising more than 2 fructose units or sugars linked together by glycosidic bonds.

5 The method according to any one of claims 1 to 3 wherein the one or more oligosaccharide sugars comprises a glucan oligosaccharide selected from the range including dextrin (1,4 links) dextran (1,6 links) or mixed linkages comprising

more than 2 glucose units or sugars linked together by glycosidic bonds or a mixture of glucan and fructan oligosaccharide sugars.

6 The method according to any one of claims 1 to 5 wherein the proteinaceous foodstuff is selected from a casein, caseinates, a whey protein, lactalbumin, bovine serum albumin, egg albumin, and soy protein.

7 The method according to any one of claims 1 to 6 wherein the proteinaceous foodstuff is selected from casein or one or more caseinates.

8 The method according to any one of claims 1 to 7 wherein the effective amounts of reactants (a), (b) and (c) are present in a gravimetric ratio of between 1 part of a proteinaceous foodstuff, between 0.5 and 5 parts of one or more oligosaccharides and between 0.01 and 2 parts of one or more monsaccharide(s) or one or more disaccharide(s).

9 The method according to any one of claims 1 to 8 wherein the effective amounts of reactants (a), (b) and (c) are present in a gravimetric ratio of 1 part of a caseinate, between 0.5 and 2 parts of inulin and between 0.01 and 1 parts of fructose.

10 The method according to any one of claims 1 to 9 wherein the effective amounts of reactants (a), (b) and (c) are present in a gravimetric ratio of: 1 part of sodium caseinate to 1 part of inulin, and 0.2 parts of fructose.

11 The method according to any one of claims 1 to 10 wherein the controlled relative humidity is in the range of from 40 to 100 % relative humidity.

12 The method according to claim 11 wherein the relative humidity is between 70-80%.

13 The method according to claim 11 or claim 12 wherein the relative humidity is 80%.

14 The method according to any one of claims 1 to 13 further including the steps of preparing and storing the resulting sugar-protein conjugate as a dry material.

15 A sugar-protein conjugate obtained according to the methods defined in any one of claims 1 to 14.

16 A food product incorporating an effective amount of the sugar-protein conjugate obtained according to the methods defined in any one of claims 1 to 14.

17 A method of preparing a sugar-protein conjugate comprising the step of reacting together under glycosylation conditions at a controlled relative humidity

(a) an effective amount of at least one proteinaceous foodstuff; and

(b) an effective amount of one or more monsaccharide(s) or one or more disaccharide(s)

wherein the reaction is conducted in the absence of further reactants intended to promote the glycosylation reaction so that the sugar-protein conjugate product is substantially devoid of materials unsuitable for use as a foodstuff and wherein the sugar- protein conjugate product has improved functional viscosity over the combined viscosity properties of the individual reactants.

18 The method according to claim 17 wherein the one or more monsaccharide(s) or one or more disaccharide(s) are selected from the range of reducing sugars including fructose, glucose, deoxyribose, ribose, lactose, lactulose, maltose, galactose, and mannose.

19 The method according to claim 17 or claim 18 wherein the monosaccharide is fructose and/or glucose.

20 The method according to any one of claims 17 to 19 wherein the proteinaceous foodstuff is selected from a casein, caseinates, a whey protein, lactalbumin, bovine serum albumin, egg albumin, and soy protein.

21 The method according to any one of claims 17 to 20 wherein the proteinaceous foodstuff is selected from casein or one or more caseinates.

22 The method according to any one of claims 17 to 20 wherein the effective amounts of reactants are present in a gravimetric ratio of between 1 part of a

proteinaceous foodstuff, and between 0.01 and 2 parts of the one or more monsaccharide(s) or one or more disaccharide(s).

23 The method according to claim 22 wherein the effective amounts of reactants are present in a gravimetric ratio of between 1 part of a caseinate, and between 0.01 and 2 parts of fructose and/or glucose.

24 The method according to claim 22 or claim 23 wherein the gravimetric ratios of the reactants are: 1 part of sodium caseinate to 0.2 parts of fructose and/or glucose.

25 The method according to any one of claims 17 to 24 wherein, the controlled relative humidity is between from 40 to 100 %.

26 The method according to claim 25 wherein the relative humidity is between 70-80%.

27 The method according to claim 25 or claim 26 wherein the relative humidity is 70%.

28 The method according to any one of claims 17 to 27 wherein the method includes the further steps of preparing and storing the resulting sugar-protein conjugate as a dry material.

29 A sugar-protein conjugate obtained according to the methods defined in any one of claims 18 to 28.

30 A food product incorporating an effective amount of the sugar-protein conjugate obtained by the methods defined in any one of claims 18 to 28.

31 A method for manufacturing a sugar-protein conjugate the method including the steps of:

(a) mixing an effective amount of casein and/or an effective amount of a caseinate and an effective amount of at least one reactant capable of forming a conjugate with the casein and/or the caseinate in a desired ratio, and at a controlled relative humidity,

(b) evaporating the resultant admixture to about 3-10% water content by weight, and

(c) heating under controlled relative humidity.

32 A method according to claim 31 wherein the mixing step (a) is carried out in solution.

33 The method according to claim 32 wherein the mixing step is carried out in an aqueous solution.

34 The method according to any one of claims 31 to 33 wherein step (b) is carried out to produce a powder.

35 The method according to any one of claims 31 to 34 wherein step (c) is carried out at temperatures of between 40 degrees to 80 degrees.

36 The method according to any one of claims 31 to 35, wherein step (c) is carried out at a temperature of 60 degrees.

37 The method according to any one of claims 31 to 36, wherein step (c) is carried out for periods of time from between 3 -120 hours.

38 The method according to any one of claims 31 to 37, wherein step (c) is carried out for periods of time from between 24 – 48 hours.

39 The method according to any one of claims 31 to 38 wherein step (c) is carried out at a temperature of 80-120 degrees for 0.1 - 3 hours.

40 The method according to any one of claims 31 to 39 wherein the caseinate is selected from sodium, calcium or potassium caseinates.

41 The method according to any one of claims 31 to 40 wherein the caseinate is sodium caseinate.

42 The method according to any one of claims 31 to 41 wherein the at least one reactant capable of conjugating casein and/or the caseinate is selected from

one or more of the following sugars including fructose, glucose, ribose, lactose, lactulose, maltose, galactose, mannose; fructan (fructo-oligosaccharide) selected from the range including inulin (1,2 links), levan, (2,6 links) and graminans (mixed 1,2 and 2,6 links) comprising more than 2 fructose units or sugars linked together by glycosidic bonds; and one or more glucan oligosaccharides selected from the range dextrin (1,4 links) dextran (1,6 links) or mixed linkages comprising more than 2 glucose units or sugars linked together by glycosidic bonds or a mixture of glucan and fructan oligosaccharide sugars.

43 The method according to any one of claims 31 to 42 wherein the at least one reactant capable of conjugating casein and/or the caseinate is selected from one or more of the sugars defined in claim 42, the effective amount of the sugars is selected so as to provide a change in the viscosity of the sugar casein/caseinate conjugate over the individual viscosity properties of the one or more sugars and the casein/caseinate conjugate.

44 The method according to any one of claims 31 to 43 wherein the effective amounts of the casein and/or a caseinate and the at least one reactant capable of conjugating casein and/or the caseinate are present in a gravimetric ratio of between 1 part of sodium caseinate, between 0.5 and 2 parts of inulin, and between 0.01 and 2 parts of fructose.

45 The method according to claim 44 wherein the effective amounts of the casein and/or a caseinate and the at least one reactant capable of conjugating casein and/or the caseinate are present in a gravimetric ratio of; 1 part of sodium caseinate, 1 part of inulin, and 0.2 parts of fructose.

46 The method according to any one of claims 31 to 43 wherein the effective amounts of the casein and/or a caseinate and the at least one reactant capable of conjugating casein and/or the caseinate are present in a gravimetric ratio of between 1 part of sodium caseinate, and between 0.01 and 2 parts of fructose and/or glucose.

47 The method according to claim 46 wherein the effective amounts of the casein and/or a caseinate and the at least one reactant capable of conjugating

casein and/or the caseinate are present in a gravimetric ratio of; 1 part of sodium caseinate, and 0.2 parts of fructose.

48 A method according to any one of claims 31 to 47 wherein the relative humidity in step (a) of the reaction corresponds to a relative humidity of between 40 % and 100 %.

49 The method according to claim 48 wherein the relative humidity is between 70-80%.

50 The method according to claim 48 or claim 49 wherein the relative humidity is 80%.

51 The method according to any one of claims 31 to 50 an industrial scale synthesis of the substance includes the steps of:

(e) thorough mechanical mixing of a finely divided effective amount of casein and/or an effective amount of a caseinate and a finely divided effective amount of at least one reactant capable of forming a conjugate with the casein and/or the caseinate in a desired ratio, and in the presence of a controlled amount of water expressed in terms of relative humidity,

(f) drying the resultant admixture to about 3-10% water content by weight by heating the admixture obtained in step (a) in either a batch process or a continuous process, together with regulation of the relative humidity to within specified limits by means of humidity sensors and water evaporators connected to process control devices, and

(g) testing the viscosity of the resulting casein/caseinate conjugate then bagging and storage as a substantially dry powder.

**INTERNATIONAL SEARCH REPORT**

International application No.
**PCT/NZ02/00261**

**A. CLASSIFICATION OF SUBJECT MATTER**

Int. Cl. [7]: A23L 1/05, A23L 1/00, A23J 3/00

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
WPIDS, CA, FSTA: protein, peptide, polypeptide, albumin, casein, lactoalbumin, sugar saccharide, monosaccharide, disaccharide, glucose, fructose, lactose, mannose, galactose, lactulose, maltose, ribose, deoxyribose, glycosylate, conjugate, glycoconjugate, viscosity, humidity

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | Yen G-C *et al*, "Effect of Maillard Browning Reaction on the Chemical Properties of Various Proteins", *Food Science and Technology*, 1989, 29(*Protein Quality and the Effects of Processing*): 273-289<br>Materials and Methods, pages 274-275 | 17-22, 25-30 |
| X | Aoki T, "Improvement of Functional Properties of Food Proteins by Conjugation of Glucose-6-Phosphate", *ACS Symposium Series*, 1996, 650(*Macromolecular Interactions in Food Technology*):230-242<br>Materials and Methods, pages 231-232 | 17-22, 25-30 |
| X | Kato A *et al*, "Functional Casein-Polysaccharide Conjugates Prepared by Controlled Dry Heating", *Bioscience, Biotechnology and Biochemistry*, 1992, 56(4):567-571<br>whole of document | 31-38, 42, 48-50 |

[X] Further documents are listed in the continuation of Box C [ ] See patent family annex

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 30 January 2003 | -4 FEB 2003 |
| Name and mailing address of the ISA/AU<br>AUSTRALIAN PATENT OFFICE<br>PO BOX 200, WODEN ACT 2606, AUSTRALIA<br>E-mail address: pct@ipaustralia.gov.au<br>Facsimile No. (02) 6285 3929 | Authorized officer<br>**GARETH COOK**<br>Telephone No : (02) 6283 2541 |

INTERNATIONAL SEARCH REPORT

International application No.
**PCT/NZ02/00261**

**C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT**

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| A | Courthaudon J-L *et al*, "Covalent Binding of Glycosyl Residues to Bovine Casein: Effects on Solubility and Viscosity", *Journal of Agricultural and Food Chemistry*, 1989, 37:32-36 whole of document | |
| A | Tainturier G *et al*, "Electroassisted Glycosylation of Bovine Casein: An Alternative to the Use of Reducing Chemicals in N-Alkylation of Proteins", *Journal of Agricultural and Food Chemistry*, 1992, 40:760-763 whole of document | |