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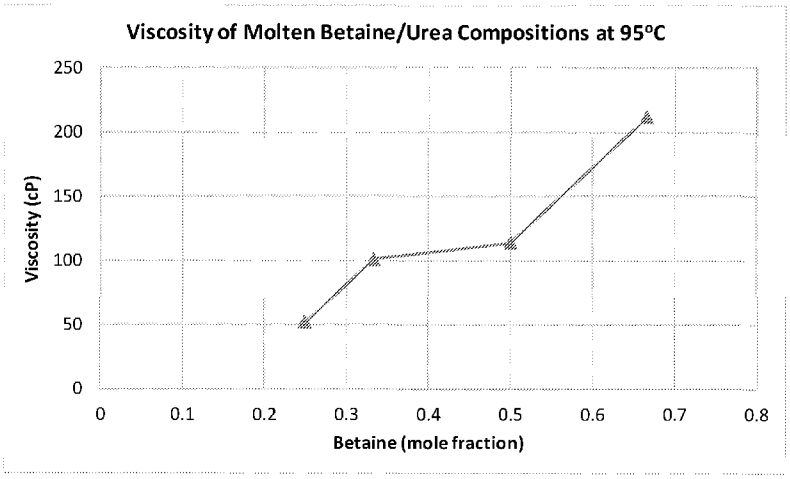


FIG. 1

(57) Abstract: The present disclosure is generally related to freezing point depressed solid matrix (FPDSM) systems, methods thereof and formulations thereof, wherein the FPDSM system stabilizes solid enzyme compositions thereof. In certain embodiments, the disclosure is related to methods for inhibiting the degradation of solid enzyme compositions formulated with one or more FPDSM systems of the disclosure.

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# FREEZING POINT DEPRESSED SOLID MATRIX COMPOSITIONS FOR MELT GRANULATION OF ENZYMES

## FIELD OF THE DISCLOSURE

**[0001]** The present disclosure is generally related to the fields of protein chemistry, formulation chemistry, enzymology, rheology, physical chemistry and the like. In certain embodiments, the disclosure is related to stabilized protein compositions and formulations thereof. In certain other embodiments, the disclosure is related to storage stable protein compositions and formulations thereof. In other embodiments, the disclosure is related to one or more freezing point depressed solid matrix (FPDSM) systems, melt granulation methods thereof and formulations thereof, wherein the FPDSM system encapsulates and stabilizes the protein compositions thereof, during the melt granulation process and subsequent storage.

## BACKGROUND

**[0002]** Although proteins such as enzymes, transport proteins, structural proteins, defense proteins, regulatory proteins, receptor proteins, and the like have many remarkable biological properties, they are all subject to denaturation and degradation at elevated temperatures, and loss of activity over time at ambient temperature. For example, proteins (*e.g.*, enzymes) can lose activity as result of proteolysis, unfolding, misfolding, aggregation, thermal instability, thermal stress, mechanical stress, sub-optimal buffer conditions, pH and the like. One particular factor that limits the lifetime (activity) of a protein (*e.g.*, an enzyme) is its susceptibility to enzymatic and non-enzymatic chemical reactions which degrade the protein.

**[0003]** For example, proteins (*e.g.*, enzymes) are subject to various forms of non-enzymatic denaturation and degradation including, but not limited to, unfolding, oxidative degradation of cysteine and methionine sulfur atoms, the deamidation of glutamine and asparagine residues, peptide-bond hydrolysis, cross-linking reactions between the protein amino groups and natural reducing sugars, and the like. Other factors influencing protein degradation and/or protein conformational stability include, but are not limited to, conditions such as temperature, pH, salt concentration, water activity, oxygen concentration, $\Delta G$ of folded versus unfolded protein, solvent/buffer systems, pressure, electromagnetic radiation (*e.g.*, *uv* light), molecular contaminants, microbial contaminants, and the like.

**[0004]** Thus, as stated briefly above, protein denaturation, degradation, protein stability and/or protein activity (which are often inextricable) are significantly influenced by the temperatures at which the protein is expressed, produced, formulated, recovered, stored, used and the like. For example, the net free energy ($\Delta G$) stabilizing a folded *versus* unfolded protein in an aqueous solution at 25°C is generally about 5-15 kcal/mol, which is approximately the amount energy needed to dissociate (break) about one to three non-

covalent protein (intramolecular) interactions (*e.g.*, electrostatic, Van der Walls, H-bonding). More particularly, even slight increases (*e.g.*, 1-5°C) in the temperature at which a particular enzyme is expressed, produced, formulated, recovered, used and/or stored can negatively impact the conformational stability (*e.g.*, protein unfolding/instability and aggregation) and increase the rate of protein degradation.

**[0005]** For example, the stability (and activity) of a typical industrial enzyme such as a protease or amylase (*e.g.*, for use in a laundry or dish cleaning) relates to the entire life cycle of the enzyme, which includes production (*e.g.*, fermentation, downstream processing and formulation), distribution (*e.g.*, transport and storage) and the final application/end-use (*e.g.*, *see*, Misset, 1992; Chotani *et al.*, 2017; Garske *et al.*, 2017). The production phase of an enzyme can generally be subdivided into three processing steps, which include but are not limited to, (i) fermentation of the production organism (host cell) wherein the enzyme is either secreted into the culture medium or the host cells lysed to recover the enzyme, (ii) downstream processing of the fermentation broth (*e.g.*, protein purification methods such as precipitation, ultrafiltration, chromatography, spray drying, *etc.*) to yield either a liquid or solid enzyme composition, and (iii) formulation of the enzyme composition to yield optimal performance and maximal stability of the final enzyme product. Following the above described production process, the formulated enzyme product is (iv) distributed by transportation and often stored at temperatures around 20°-39°C for a period time before (v) the final end use/application. It therefore becomes evident that such industrial enzymes are subjected to numerous environmental insults throughout their chain of production, formulation and final storage thereafter.

**[0006]** Thus, there remains an ongoing and unmet need in the art for methods, formulations and compositions thereof which mitigate or prevent protein degradation, protein instability and/or protein inactivation, particularly during exposure to high temperatures, and most particularly for purposes of preparing solid formulations thereof, for example by processing conditions that expose enzymes or other proteins to high temperatures, in melt granulation processes, wherein the protein(*e.g.*, an enzyme) is suspended in a hot molten carrier that is subsequently cooled to form a solid matrix entrapping the protein. Likewise, such ongoing and unmet needs in the art further apply to methods, formulations and compositions thereof which mitigate or prevent degradation, instability and/or inactivity of enzyme compositions which are stored for an amount of time prior to their end use.

**[0007]** The use of multi-component meltable solid solvent systems suitable for stabilizing proteins and other enzymes in non-aqueous liquid formulations for purposes of extraction and biocatalysis has been described by Smith *et al.* (2014) and others (Wang *et al.*, 2016 and Xu *et al.*, 2015). Similarly, non-aqueous molten solvent systems for extracting water-insoluble flavonoid compounds has been described in PCT International Publication No. WO2011/155829, wherein the solvent systems are liquid at ambient temperatures, and wherein the water-insoluble flavonoid compounds are dissolved and extracted therefrom.

The use of molten solvent mixtures for performing enzymatic reactions has been described in U.S. Patent Publication No. US2009/0117628, wherein the enzymatic activity of different lipases was tested at 60°C in various mixtures, wherein the mixtures were liquid (*i.e.*, above the freezing or solidification point of the solvent) at 60°C.

**[0008]** Similarly, Huang *et al.* (2014) describe the use of aqueous molten solutions which can activate and stabilize *Penicillium expansum* lipase (PEL), wherein the enzymatic activity of the PEL was assayed at 60°C. The use of molten solvent mixtures for stabilizing and preserving RNA, DNA, proteins, whole cells and tissues has been described in PCT International Publication No. WO2014/131906, wherein the solvent system is a liquid at room temperature. For example, WO2014/131906 provides Example 17, "Protein stabilization in animal tissue samples", wherein mouse liver was added to molten choline chloride/trifluoroacetamide (1:2) or PBS buffer (control) and incubated at 24°C for up to 18 days prior to protein extraction.

**[0009]** In general, the above described processes and applications of liquid molten solvent systems, the components are added in fixed molar ratios so as to minimize the melting or solidification point. In addition, these liquid molten systems described are typically formulated without the addition of water. Thus, these liquid molten solvent systems do not address the need to prepare and provide protein formulations wherein such formulations remain solid at ambient conditions (*e.g.* for use in applications requiring granulated enzymes).

**[0010]** In particular contrast to the aforementioned references described above, the present disclosure is directed to stable solid protein compositions, formulations thereof and melt granulation methods and compositions thereof, wherein the solid protein of interest (POI) is first dispersed in a molten (FPDSM) solvent system of the disclosure at an elevated temperature (*i.e.*, between 40°C to about 100°C) and then subsequently cooled and solidified (*i.e.*, about 20°C to 40°C ), such that the POI is entrapped in a "freezing point depressed solid matrix" (FPDSM) in its solid state. For example, a FPDSM composition of the disclosure comprises at least two FPDSM components, wherein each single (FPDSM) component of the FPDSM composition does not melt below 100°C (*e.g., see* **Table 1**, FPDSM Component Melting Points), and optionally further comprising water, wherein the FPDSM composition is a solid at a temperature between about 20°C to 40°C.

**[0011]** Previous applications of molten solvent systems for solubilizing or stabilizing proteins (*e.g.,* as generally referenced and described above) involves maintenance of the composition in the "liquid state" (*e.g.*, for purposes such as extraction of the POI, solubilization of the POI, biocatalysis using solubilized enzymes, and the like). In contrast to these prior uses of molten solvent systems, the present disclosure is directed to molten solvent systems (*i.e.,* a FPDSM system of the disclosure) wherein a POI is embedded

(*e.g.*, entrapped) within the solvent system as it solidifies, such that the POI is immobilized and thereby not immediately available as a biocatalyst or reactant.

**[0012]** Additionally, Applicant surprisingly discovered herein that it is not necessary to maintain specific molar ratios of the FPDSM components in order to achieve the desired properties of the instant disclosure, namely providing a molten solvent system (FPDSM) formulation wherein the POI is stable prior to solidification of the molten (FPDSM) system, and upon solidification of the FPDSM system, forms a solid matrix composition thereof useful for encapsulating, delivering and stabilizing enzymes at a temperature between about 20°C to 40°C. Furthermore, Applicant discovered that it is not necessary to avoid the addition of water to the FPDSM formulations, and that thermally labile proteins (*e.g.*, enzymes) can retain most of their activity (*i.e.* at least about 70% of their activity), when suspended/entrapped in such FPDSM systems that comprise up to 10% w/w water.

**[0013]** Thus, a surprising advantage of the present disclosure is that functional proteins (*e.g.*, enzymes) suspended within the FPDSM can survive during suspension in the molten FPDSM, thereby maintaining high recovery of functional protein conformation and enzymatic activity, *e.g.*, at least 70% recovery of initial enzyme activity, after being subjected to high temperatures (*i.e.*, temperatures which usually denature most proteins; *e.g.*, temperatures as high as 80°C, 90°C or 100°C) for periods of at least one minute to five minutes, prior to subsequent cooling and solidification from a melt (*e.g.,* in a melt granulation process). Even more surprisingly, the high recovery of active enzyme has been demonstrated for compositions that include water up to 10% w/w. Additionally, the FPDSM (entrapped) enzyme compositions of the disclosure demonstrate excellent storage stability at room temperature (20°C-25°C) for at least one month.

**[0014]** Thus, as set forth below in the Detailed Description, the instant disclosure addresses such long felt and unmet needs for methods, formulations, including granular formulations and compositions thereof which mitigate protein degradation, instability and/or inactivation, including such methods, formulations and compositions which mitigate the degradation and/or loss of activity of solid protein formulations which are stored for a period of time (*i.e.*, hours, days, weeks, months) before their end use.

## SUMMARY OF THE DISCLOSURE

**[0015]** In certain embodiments the present disclosure is directed to stable solid protein compositions, formulations thereof and methods thereof. In certain other embodiments, the disclosure is directed to methods for preparing/formulating one or more "freezing point depressed solid matrix" (FPDSM) systems which stabilize solid protein compositions. In yet other embodiments, the disclosure is directed to methods for inhibiting the degradation of solid protein compositions formulated with one or more FPDSM systems of the disclosure.

**[0016]** Thus, in certain embodiments, the disclosure is directed to a solid enzyme formulation comprising a freezing point depressed solid matrix (FPDSM) system and a solid enzyme composition entrapped therein, wherein the FPDSM system comprises at least two distinct FPDSM components which at least two distinct FPDSM components do not melt at a temperature below 100°C until combined, wherein the combined FPDSM system is a liquid at a temperature between 40°C to about 100°C and a solid at a temperature between about 20°C to 40°C, wherein recovery of the active enzyme from the solidified FPDSM system is at least 70% active enzyme relative to the activity of the solid enzyme composition. In certain other embodiments, the at least two component FPDSM further comprises up to 10% (w/w) water. Thus, in certain embodiments of the formulation, the water content is about 0.05% to 10% w/w. In certain embodiments, the water content is about 1% to about 10% w/w, about 2% to about 10% w/w, about 3% to about 10% w/w, about 4% to about 10% w/w, or about 5% to about 10% w/w.

**[0017]** In other embodiments of the formulation, the FPDSM system comprises a combination of at least two components selected from the group consisting of ammonium chloride, choline chloride, betaine, betaine hydrochloride, citric acid, malonic acid, tartaric acid, fructose, sucrose, glucose, lactose, maltose, urea and sorbitol. In yet other embodiments, the at least two components of the FPDSM system are combined at a molar ratio ranging from about 1:10 to about 10:1, wherein the combined molar ratio of the FPDSM system components are in a liquid state at a temperature between 40°C to about 100°C, and a solid at a temperature between about 20°C to 40°C. In another embodiment of the formulation, the enzyme retains at least 85% enzymatic activity after twenty-four (24) hours of storage at 25°C. In other embodiments, the enzyme retains at least 95% enzymatic activity after twenty-four (24) hours of storage at 25°C. In certain other embodiments, the enzyme retains at least 99% enzymatic activity after twenty-four (24) hours of storage at 25°C. In yet another embodiment, the enzyme retains at least 80% enzymatic activity after seven (7) days of storage at 25°C. In certain other embodiments, the enzyme retains at least 75% enzymatic activity after twenty-eight (28) days of storage at 25°C. In other embodiments, the FPDSM formulated solid enzyme composition retains a higher enzymatic activity than an unformulated solid enzyme composition, wherein both the FPDSM formulated enzyme and the solid enzyme composition are held at the same temperature for the same amount of time before enzymatic activity is assayed.

**[0018]** In other embodiments of the formulation, the enzyme is selected from the group consisting of acetyl esterases, aryl esterases, aminopeptidases, amylases, arabinases, arabinofuranosidases, carbonic anhydrases, carboxypeptidases, catalases, cellulases, chitinases, chymosins, cutinases, deoxyribonucleases, epimerases, esterases, α-galactosidases, β-galactosidases, α-glucanases, glucan lysases, endo-β-glucanases, glucoamylases, glucose oxidases, α-glucosidases, β-glucosidases, glucuronidases, glycosyl hydrolases, hemicellulases, hexose oxidases, hydrolases, invertases, isomerases, laccases, ligases, lipases, lyases, mannosidases, oxidases, oxidoreductases, pectate lyases, pectin acetyl esterases, pectin depolymerases,

pectin methyl esterases, pectinolytic enzymes, perhydrolases, polyol oxidases, peroxidases, phenoloxidases, phytases, polygalacturonases, proteases, peptidases, rhamno-galacturonases, ribonucleases, transferases, transglutaminases, xylanases, hexose oxidases, and combinations thereof.

**[0019]** In another embodiment of the formulation, the FPDSM system comprises choline chloride and citric acid at a molar ratio between about 1:2 (choline chloride/citric acid) to about 2:1 (choline chloride/citric acid). In other embodiments, the FPDSM system comprises choline chloride and sorbitol at a molar ratio between about 2:1 (choline chloride/sorbitol) to about 3:1 (choline chloride/sorbitol). In certain other embodiments, the FPDSM system comprises anhydrous betaine and urea at a molar ratio between about 1:1 (betaine/urea) to about 1:4 (betaine/urea). In another embodiment, the FPDSM system comprises ammonium chloride and urea at a molar ratio between about 1:2 (ammonium chloride/urea) to about 1:3 (ammonium chloride/urea).

**[0020]** In certain other embodiments of the formulation, the FPDSM system comprises at least two different enzymes. In other embodiments, the solid enzyme composition is a spray dried enzyme, a lyophilized enzyme, a precipitated enzyme, a crystalized enzyme, a spray cooled enzyme, spray chilled enzyme or a spray granulated enzyme. In yet other embodiments, the FPDSM formulated solid enzyme comprises a solid form selected from a solid enzyme pellet, a solid enzyme granule, a solid enzyme powder, a solid enzyme film and a solid enzyme extrudate.

**[0021]** Thus, in certain embodiments, the disclosure is related to a solid enzyme composition homogenously distributed and entrapped in a FPDSM system formulation of the disclosure.

**[0022]** In another embodiment, the disclosure is directed to a method for stabilizing a solid enzyme composition from loss of activity, the method comprising (a) selecting and preparing a FPDSM system comprising at least two distinct FPDSM system components and optionally up to 10% water, wherein the FPDSM system components are selected such that when combined and heated, form a liquid at a temperature between 40°C to about 100C, and when subsequently cooled, form a solid at a temperature between about 20°C to 40°C, (b) adding and mixing a solid enzyme composition to the FPDSM system of step (a) at a temperature higher than 40°C and lower than about 100°C, and (c) lowering the temperature of the step (b) mixture to a temperature between about 20°C to 40°C, wherein the FPDSM system solidifies to form a solid FPDSM system that entraps and encapsulates the enzyme. Thus, in certain embodiments of the method, the recovery of the FPDSM formulated enzyme relative to the recovery of solid enzyme composition is at least 70%. In certain other embodiments, the enzyme retains at least 85% enzymatic activity after twenty-four (24) hours of storage at 25°C. In another embodiment, the enzyme retains at least 95% enzymatic activity after twenty-four (24) hours of storage at 25°C. In yet other embodiments, the enzyme retains at least 99% enzymatic activity after twenty-four (24) hours of storage at 25°C. In another embodiment, the enzyme retains at least 80% enzymatic activity after seven (7) days of storage at 25°C. In

certain other embodiments, the enzyme retains at least 75% enzymatic activity after twenty-eight (28) days of storage at 25°C. In another embodiment of the method, the FPDSM formulated solid enzyme composition retains a higher enzymatic activity than an unformulated solid enzyme composition, wherein both the FPDSM formulated enzyme and the solid enzyme composition are stored at the same temperature for the same amount of time before enzymatic activity is assayed.

**[0023]** Thus, in certain embodiments of the method, the FPDSM system comprises a combination of at least two components selected from the group consisting of ammonium chloride, choline chloride, betaine, betaine hydrochloride, citric acid, malonic acid, tartaric acid, fructose, sucrose, glucose, lactose, maltose, urea and sorbitol. In other embodiments, the FPDSM system comprises a combination selected from the group consisting of urea/lactose, urea/glucose, urea/maltose, urea/fructose, urea/ammonium chloride, urea/betaine, urea/betaine hydrochloride, urea/choline chloride, betaine/glucose, betaine/fructose, betaine/lactose, betaine/citric acid, betaine/sucrose, choline chloride/glucose, choline chloride/malonic acid, choline chloride/ tartaric acid, choline chloride/citric acid, choline chloride/ sorbitol, citric acid/glucose and citric acid/maltose. In certain other embodiments, two components of the FPDSM system are combined at a molar ratio ranging from about 1:10 to about 10:1, wherein the combined molar ratio of the FPDSM system components are in a liquid state at a temperature between 40°C to about 100°C, and a solid at a temperature between about 20°C to 40°C. Thus, in certain embodiments of the method, the FPDSM system comprises choline chloride and citric acid at a molar ratio between about 1:2 (choline chloride/citric acid) to about 2:1 (choline chloride/citric acid). In other embodiments, the FPDSM system comprises choline chloride and sorbitol at a molar ratio between about 2:1 (choline chloride/sorbitol) to about 3:1 (choline chloride/sorbitol). In certain other embodiments, the FPDSM system comprises anhydrous betaine and urea at a molar ratio between about 1:1 (betaine/urea) to about 1:4 (betaine/urea). In another embodiment, the FPDSM system comprises ammonium chloride and urea at a molar ratio between about 1:2 (ammonium chloride/urea) to about 1:3 (ammonium chloride/urea). In other embodiments, the FPDSM system comprises betaine and glucose at a molar ratio of about 5:2 (betaine/glucose) and about 0.05% to 10% weight water. In another embodiment, the FPDSM system comprises betaine and urea at a molar ratio of about 1:1 (betaine/glucose) and about 0.05% to 10% weight water. In other embodiments of the method, the FPDSM system comprises urea and glucose at a molar ratio of about 3:1 (urea/glucose) and about 0.05% to 10% weight water. In certain other embodiments, the FPDSM system comprises urea and maltose at a molar ratio of about 6:1 (urea/maltose) and about 0.05% to10% weight water. In yet other embodiments, the FPDSM system comprises urea and lactose at a molar ratio of about 6:1 (urea/lactose) and about 0.05% to 10% weight water. In another embodiment, the FPDSM system comprises urea and lactose at a molar ratio of about 9:1 (urea/lactose) to about 1.5: 1 (urea/lactose)

and about 4.0% to 6.0% weight water. In other embodiments of the method, the water content is about 0.05% to 10% w/w. In certain other embodiments, the water content is about 5% to 10% w/w.

**[0024]** In another embodiment of the method, the enzyme is selected from the group consisting of acetyl esterases, aryl esterases, aminopeptidases, amylases, arabinases, arabinofuranosidases, carbonic anhydrases, carboxypeptidases, catalases, cellulases, chitinases, chymosins, cutinases, deoxyribonucleases, epimerases, esterases, α-galactosidases, β-galactosidases, α-glucanases, glucan lysases, endo-β-glucanases, glucoamylases, glucose oxidases, α-glucosidases, β-glucosidases, glucuronidases, glycosyl hydrolases, hemicellulases, hexose oxidases, hydrolases, invertases, isomerases, laccases, ligases, lipases, lyases, mannosidases, oxidases, oxidoreductases, pectate lyases, pectin acetyl esterases, pectin depolymerases, pectin methyl esterases, pectinolytic enzymes, perhydrolases, polyol oxidases, peroxidases, phenoloxidases, phytases, polygalacturonases, proteases, peptidases, rhamno-galacturonases, ribonucleases, transferases, transglutaminases, xylanases, hexose oxidases, and combinations thereof. In certain embodiments, the FPDSM system comprises at least two different enzymes. Ion certain other embodiments, the solid enzyme composition is a spray dried enzyme, a lyophilized enzyme, a precipitated enzyme, a crystalized enzyme, a spray cooled enzyme, spray chilled enzyme or a spray granulated enzyme. In another embodiment, the solidified FPDSM system of step (c) comprises a solid form selected from a solid pellet, a solid granule, a solid powder, a solid film and a solid extrudate.

**[0025]** Thus, in certain other embodiments the disclosure is related to a solid enzyme composition homogenously distributed and entrapped in a FPDSM system prepared by a method of the disclosure.

**[0026]** In certain other embodiments, the disclosure is related to a method for inhibiting the degradation of a solid enzyme composition stored for at least twenty-four hours at 25°C, the method comprising (a) selecting and preparing a FPDSM system comprising at least two distinct FPDSM system components and optionally up to 10% water, wherein the FPDSM system components are selected such that when combined and heated, form a liquid at a temperature between 40°C to about 100°C, and when subsequently cooled, form a solid at a temperature between about 20°C to 40°C, (b) adding and mixing a solid enzyme composition to the FPDSM system of step (a) at a temperature higher than 40°C and lower than about 100°C, and (c) lowering the temperature of the step (b) mixture to a temperature between about 20°C to 40°C, wherein the FPDSM system solidifies to form a solid FPDSM system that entraps and encapsulates the enzyme. In certain embodiments of the method, the recovery of the FPDSM formulated enzyme relative to the recovery of solid enzyme composition is at least 70%. In another embodiment, the enzyme retains at least 85% enzymatic activity after twenty-four (24) hours of storage at 25°C. In other embodiments, the enzyme retains at least 95% enzymatic activity after twenty-four (24) hours of storage at 25°C. In another embodiment, the enzyme retains at least 99% enzymatic activity after twenty-four (24) hours of storage at 25°C. In certain other embodiments, the enzyme retains at least 80% enzymatic activity after seven (7)

days of storage at 25°C. In other embodiments, the enzyme retains at least 75% enzymatic activity after twenty-eight (28) days of storage at 25°C. In yet another embodiment of the method, the FPDSM formulated solid enzyme composition retains a higher enzymatic activity than an unformulated solid enzyme composition, wherein both the FPDSM formulated enzyme and the solid enzyme composition are stored at the same temperature for the same amount of time before enzymatic activity is assayed.

**[0027]** Thus, in certain other embodiments of the method, the FPDSM system comprises a combination of at least two components selected from the group consisting of ammonium chloride, choline chloride, betaine, betaine hydrochloride, citric acid, malonic acid, tartaric acid, fructose, sucrose, glucose, lactose, maltose, urea and sorbitol. In certain other embodiments, the FPDSM system comprises a combination selected from the group consisting of urea/lactose, urea/glucose, urea/maltose, urea/fructose, urea/ammonium chloride, urea/betaine, urea/betaine hydrochloride, urea/choline chloride, betaine/glucose, betaine/fructose, betaine/lactose, betaine/citric acid, betaine/sucrose, choline chloride/glucose, choline chloride/malonic acid, choline chloride/ tartaric acid, choline chloride/citric acid, choline chloride/ sorbitol, citric acid/glucose and citric acid/maltose. In certain other embodiments of the method, two components of the FPDSM system are combined at a molar ratio ranging from about 1:10 to about 10:1, wherein the combined molar ratio of the FPDSM system components are in a liquid state at a temperature between 40°C to about 100°C, and a solid at a temperature between about 20°C to 40°C.

**[0028]** Thus, in certain embodiments of the method, the FPDSM system comprises choline chloride and citric acid at a molar ratio between about 1:2 (choline chloride/citric acid) to about 2:1 (choline chloride/citric acid). In another embodiment, the FPDSM system comprises choline chloride and sorbitol at a molar ratio between about 2:1 (choline chloride/sorbitol) to about 3:1 (choline chloride/sorbitol). In yet other embodiments, the FPDSM system comprises anhydrous betaine and urea at a molar ratio between about 1:1 (betaine/urea) to about 1:4 (betaine/urea). In certain other embodiments of the method, the FPDSM system comprises ammonium chloride and urea at a molar ratio between about 1:2 (ammonium chloride/urea) to about 1:3 (ammonium chloride/urea). In certain other embodiments, the FPDSM system comprises betaine and glucose at a molar ratio of about 5:2 (betaine/glucose) and about 0.05% to 10% weight water. In other embodiments, the FPDSM system comprises betaine and urea at a molar ratio of about 1:1 (betaine/glucose) and about 0.05% to 10% weight water. In another embodiment, the FPDSM system comprises urea and glucose at a molar ratio of about 3:1 (urea/glucose) and about 0.05% to 10% weight water. In certain other embodiments, the FPDSM system comprises urea and maltose at a molar ratio of about 6:1 (urea/maltose) and about 0.05% to10% weight water. In another embodiment, the FPDSM system comprises urea and lactose at a molar ratio of about 6:1 (urea/lactose) and about 0.05% to 10% weight water. In yet other embodiments, the FPDSM system comprises urea and lactose at a molar ratio of about 9:1 (urea/lactose) to about 1.5: 1 (urea/lactose) and about 4.0% to 6.0% weight water.

**[0029]** Thus, in certain embodiments of the method, the FPDSM system water content is about 0.05% to 10% w/w. In another embodiment, the water content is about 5% to 10% w/w.

**[0030]** In yet other embodiments of the method, the enzyme is selected from the group consisting of acetyl esterases, aryl esterases, aminopeptidases, amylases, arabinases, arabinofuranosidases, carbonic anhydrases, carboxypeptidases, catalases, cellulases, chitinases, chymosins, cutinases, deoxyribonucleases, epimerases, esterases, α-galactosidases, β-galactosidases, α-glucanases, glucan lysases, endo-β-glucanases, glucoamylases, glucose oxidases, α-glucosidases, β-glucosidases, glucuronidases, glycosyl hydrolases, hemicellulases, hexose oxidases, hydrolases, invertases, isomerases, laccases, ligases, lipases, lyases, mannosidases, oxidases, oxidoreductases, pectate lyases, pectin acetyl esterases, pectin depolymerases, pectin methyl esterases, pectinolytic enzymes, perhydrolases, polyol oxidases, peroxidases, phenoloxidases, phytases, polygalacturonases, proteases, peptidases, rhamno-galacturonases, ribonucleases, transferases, transglutaminases, xylanases, hexose oxidases, and combinations thereof. In certain embodiments, the FPDSM system comprises at least two different enzymes. In certain other embodiments of the method, the solid enzyme composition is a spray dried enzyme, a lyophilized enzyme, a precipitated enzyme, a crystalized enzyme, a spray cooled enzyme, spray chilled enzyme or a spray granulated enzyme. In certain other embodiments, the solidified FPDSM system of step (c) comprises a solid form selected from a solid pellet, a solid granule, a solid powder, a solid film and a solid extrudate.

**[0031]** Thus, in certain other embodiments, the disclosure is related to a solid enzyme composition homogenously distributed and entrapped in a FPDSM system prepared by a method of the disclosure.

## BRIEF DESCRIPTION OF DRAWINGS

**[0032]** **Figure 1** shows the viscosity (cP) of a two-component FPDSM system comprising betaine and urea at 95°C, plotted as a function of the betaine mole fraction.

**[0033]** **Figure 2** shows the percent (%) recovery of a subtilisin protease, as the percentage of residual enzyme activity remaining after addition to a molten FPDSM composition, with subsequent solidification at room temperature (21° C in this particular case). The percent residual activity is plotted as a function of water added to a FPDSM system set forth in **Table 3**. Thus, as presented in **FIG. 2**, the following FPDSM solvent systems were tested by the addition of 0%-10% w/w water, with enzyme at the following molar ratios: choline chloride/succinic acid (1:1); urea/ammonium chloride (3:1); urea/ammonium chloride (4:1); urea/ammonium chloride (1:4); urea/glucose (1:1); urea/maltose (1:1); urea/lactose (1:1); betaine/urea (1:1), betaine/urea (1:2), betaine/urea (1:3) and betaine/urea (1:4). Thus, the data presented in **FIG. 2** demonstrate that the addition of up to 10% w/w water to a two-component FPDSM of the disclosure surprisingly does not adversely impact recovered enzymatic activity.

**[0034]** **Figure 3** shows a plot of percent (%) recovery of a subtilisin protease, as a function of the mixing temperature (°C) used to entrap the enzyme in a FPDSM system set forth in **Table 2** of the specification. The compositions of Figure 3 are the same as those of Figure 2; accordingly, the composition with higher percentages of water had lower melting points. Thus, by lowering melting point of the FPDSM, addition of moderate amounts of water may work to reduce the thermally-induced loss of enzyme activity in the course of the mixing step and subsequent solidification, despite the well-known fact that enzyme stability is typically reduced in the presence of water. As presented in **FIG. 3**, the following FPDSM solvent systems were tested with enzyme, at the following molar ratios: choline chloride/succinic acid (1:1); urea/ammonium chloride (3:1); urea/ammonium chloride (4:1); urea/ammonium chloride (1:4); urea/glucose (1:1); urea/maltose (1:1); urea/lactose (1:1); betaine/urea (1:1), betaine/urea (1:2), betaine/urea (1:3) and betaine/urea (1:4). Thus, the data presented in **FIG. 3** demonstrate greater than 70% protease recovery of certain FPDSM compositions including urea/glucose (1:1), urea/lactose (1:1), betaine/urea (1:1) and urea/ammonium chloride (1:4).

## DETAILED DESCRIPTION

**[0035]** The present disclosure is generally related to solid protein compositions and specific formulations thereof which stabilize the solid protein compositions. More specifically, in certain embodiments, the disclosure is directed to formulations which stabilize solid protein compositions and concomitantly mitigate or inhibit certain environmental factors associated with enzymatic and non-enzymatic protein degradation. More particularly, Applicant of the instant disclosure has discovered that certain combinations (*i.e.*, mixtures) of "solvent components" in specified ranges of molar ratios are particularly suitable for mitigating protein degradation, protein instability and/or protein inactivation, most particularly, thermally-induced protein inactivation. Thermally-induced protein inactivation can arise by well-known biophysical and biochemical processes such as protein unfolding, protein aggregation, oxidation, glycation, deamidation, and the like. Storage-stable protein formulations can be produced using a variety of protein formulation, particle formation, and drying processes. For example, as described in further detail below, certain embodiments of the disclosure are directed to storage stable solid protein compositions (*e.g.*, a lyophilized solid protein composition, a spray dried solid protein composition, a spray cooled solid protein composition, a spray chilled solid protein composition, a spray granulated solid protein composition and the like), formulations thereof and methods thereof, wherein the solid protein of interest (POI) is entrapped in a "freezing point depressed solid matrix" (FPDSM) system of the disclosure.

**[0036]** Thus, in certain embodiments, the disclosure is directed to methods for preparing/formulating one or more "freezing point depressed solid matrix" (hereinafter "FPDSM") systems which encapsulate solid protein compositions. In certain other embodiments, the disclosure is related to one or more solid protein

compositions formulated in a FPDSM system described herein. In yet other embodiments, the disclosure is related to methods for inhibiting the degradation of solid protein compositions formulated with one or more FPDSM systems described herein.

**[0037]** Thus, in particular embodiments, the disclosure is related to stable solid protein compositions, formulations thereof, granulation processes thereof and methods thereof, wherein the solid protein of interest is distributed in a (molten liquid) FPDSM system of the disclosure, wherein each individual (FPDSM system) component is a solid that does not melt below 100°C, wherein the combined (molten liquid) FPDSM system solidifies and entraps the protein of interest at a temperature of about 20°C to 40°C. More particularly, certain embodiments are directed to one or more proteins of interest (*e.g.*, an enzyme) formulated in a tunable FPDSM system of the disclosure, wherein the FPDSM system is tuned to be a liquid at a temperature between 40°C to about 100°C, and a solid at a temperature between about 20°C to 40°C. Thus, in other embodiments the disclosure is related to solid protein compositions of interest (*e.g.*, a protease, an amylase, a glucoamylase, a lipase, *etc.*) formulated in a tunable FPDSM system of the disclosure, wherein such FPDSM formulations mitigate the degradation and/or loss of activity of the solid protein composition, particularly solid protein compositions which are stored for a period of time (*i.e.*, hours, days, weeks, months) before their end use.

## *I. Definitions*

**[0038]** In view of the "freezing point depressed solid matrix" systems (or "FPDSM" systems) disclosed herein, solid protein compositions thereof, formulations thereof, granulation processes thereof and methods thereof, the following terms and phrases are defined. Terms not defined herein should be accorded their ordinary meaning as used in the art.

**[0039]** Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which the present compositions, formulations and/or methods apply. Although any methods and materials similar or equivalent to those described herein can also be used in the practice or testing of the present compositions, formulations and methods, representative illustrative methods and materials are now described. All scientific publications, published patent applications and granted patents cited herein are incorporated herein by reference in their entirety.

**[0040]** It is further noted that the claims may be drafted to exclude (*proviso*) any optional element(s). As such, this statement is intended to serve as antecedent basis for use of such exclusive terminology as "solely," "only", "excluding", "not including" and the like, in connection with the recitation of claim elements, or the use of a "negative" or "*proviso*" limitation.

**[0041]** As will be apparent to those of skill in the art upon reading this disclosure, each of the individual embodiments described and illustrated herein has discrete components and features which may be readily

separated from or combined with the features of any of the other several embodiments without departing from the scope or spirit of the present compositions and methods described herein. Any recited method can be carried out in the order of events recited or in any other order which is logically possible.

**[0042]** As used herein, a "freezing point depressed solid matrix" (abbreviated herein as, "FPDSM"), a freezing point depressed solid matrix "system" or a "FPDSM system" may be used interchangeably. Thus, as used herein, a "FPDSM system" comprises at least two (2) distinct FPDSM components, wherein each distinct (FPDSM system) component individually does not melt below 100°C, yet in combination the at least two (2) distinct FPDSM components melt at a temperature between 40°C to about 100°C. In certain embodiments, a "FPDSM system" of the disclosure, comprising at least two (2) distinct FPDSM components, further comprises up to about 10% (w/w) water.

**[0043]** As used herein, a "distinct" FPDSM system component, or simply a "FPDSM component", as used in phrases such as a "distinct FPDSM system component", a "distinct FPDSM solvent component", "individual FPDSM component" and the like includes, but is not limited to, FPDSM components selected from the group consisting of ammonium chloride ($NH_4Cl$), choline chloride, betaine (N,N,N,-trimethylglycine), betaine hydrochloride, citric acid, malonic acid, tartaric acid, sorbitol, sucrose, fructose, glucose, lactose, maltose, urea, and the like.

**[0044]** As used herein, the phrases a "solid protein", a "solid protein composition", a "solid enzyme composition", and the like specifically refer to a protein composition in its solid dried state (*e.g.*, by means of lyophilization, spray drying, crystallization, spray cooling, spray chilling, spray granulated, and the like), in contrast to a "liquid protein composition", which liquid protein compositions are substantially hydrated (dissolved) in water or an aqueous buffer thereof. Thus, as used herein, the terms a "solid" enzyme, a "solid" protein, a "solid" enzyme composition and the like, are equivalent to the terms a "dry" protein, a "dry" enzyme, a "dried" enzyme, a "dried" protein, a "dried" enzyme composition and the like.

**[0045]** As defined herein, the term "protein" will be used hereinafter when referring to a "solid protein" composition of the disclosure. Thus, unless specifically stated otherwise in the specification to be a "liquid protein composition", all such reference to a "protein" equates to a "solid protein composition".

**[0046]** As used herein, terms such as "entrap(s)", entrapped", "embedded", "encapsulate(s)" and the like, as used in phrases such as a "FPDSM system of the disclosure 'entraps' and 'encapsulates' the solid POI" (*e.g.,* when the molten (FPDSM) system is cooled and solidified at ambient temperatures), such terms particularly refer to the ability of the FPDSM system to immobilize (*e.g.*, entrap) and protect (*e.g.*, encapsulate) the POI which is substantially distributed within the surrounding solid matrix FPDSM system, thereby mitigating degradation and/or loss of activity of the solid protein composition entrapped therein, wherein the solid matrix is not a coating surrounding a core

**[0047]** As used herein, phrases such as "molten solvent systems", "molten liquid solvent systems" and "liquid molten solvent systems" may be used interchangeably. As defined above, the individual (*i.e.*, distinct) components of an at least two (2) component FPDSM system do not melt below 100°C (*see*, Table 1). In contrast, a "molten solvent system" particularly refers to a FPDSM system of the disclosure, wherein the at least two (2) FPDSM components have been combined and mixed at a temperature between 40°C to about 100°C, wherein the FPDSM system has been tuned to be in a liquid state at a temperature between 40°C to about 100°C (*i.e.*, melt below about 100°C). Likewise, as used herein, a "solid" or "solidified" FPDSM system of the disclosure particularly refers to the same FPDSM system, wherein the FPDSM system has been cooled to a temperature between about 20°C to 40°C, such that the FPDSM system solidifies, thereby entrapping and encapsulating the solid protein composition therein.

**[0048]** As used herein, the term "room temperature" (RT) means a temperature between about 20°C to about 28°C.

**[0049]** As used herein, the term "ambient temperature" means a temperature between about 20°C to about 40°C.

**[0050]** As used herein, enzyme recovery is calculated as the weight percentage of active enzyme in the solidified FPDSM material relative to the enzyme activity in the initial solid spray dried enzyme powder.

**[0051]** As used herein, a "stable" enzyme composition" (*e.g.*, as used in phrases such as "a stable enzyme composition", "a storage stable enzyme composition" or "a FPDSM stabilized enzyme") particularly refers to a solid enzyme composition formulated in a FPDSM system the disclosure. More particularly, when assaying (measuring) the enzymatic activity of a "stable" (FPDSM formulated) enzyme composition of the disclosure, the enzymatic activity of the "stable" (FPDSM formulated) enzyme composition is relative to (vis-à-vis) the activity of the same (unformulated) enzyme composition (*e.g.*, a solid enzyme, spray-dried control) when the solid enzyme (spray-dried control) is assayed (measured) under the same conditions.

**[0052]** As used herein, the terms "weight percent" or "weight %" are abbreviated hereinafter "wt%" or "% w/w".

**[0053]** As used herein, the term protein or enzyme "melting temperature" (assuming two-state protein folding (F $\leftrightarrow$ U)), is the denaturation midpoint "$T_m$", which is defined as the temperature at which both the folded (F) and unfolded (U) states are equally populated at equilibrium.

**[0054]** As used herein, a "protein of interest" may be abbreviated as "POI", wherein a POI of the disclosure includes, but is not limited to, enzymes, transport proteins, structural proteins, defense or immune related proteins (cytokines; *e.g.*, IL-12), regulatory proteins, receptor proteins, immunoglobulins, cytokines (*e.g.*, IL-12), and the like. Thus, a POI according to the instant disclosure is any solid protein composition contemplated for formulation and stabilization thereof in a FPDSM system of the disclosure.

**[0055]** As defined herein, the terms "polypeptide" and "protein" are used interchangeably and include reference to polymers comprising amino acid residues linked by peptide bonds. The conventional one (1) letter or three (3) letter codes for amino acid residues are used herein. The protein may be linear or branched, it may comprise modified amino acids, and it may be interrupted by non-amino acids.

**[0056]** The terms "polypeptide", "peptide" and "protein" apply to amino acid polymers in which one or more amino acid residues are an artificial chemical analog of a corresponding naturally occurring amino acid, as well as to naturally occurring amino acid polymers. Thus, the terms also encompass amino acid polymers that have been modified naturally or by intervention (*e.g.,* disulfide bond formation, glycosylation, lipidation, acetylation, phosphorylation, or any other manipulation or modification). Also included within the definition are, for example, polypeptides containing one or more analogs of an amino acid (including, for example, unnatural amino acids, *etc.*), as well as other modifications known in the art.

**[0057]** As used herein, a "variant" protein refers to a protein that is derived from a parent (or reference) protein by the substitution, addition, or deletion of one or more amino acids, typically by recombinant DNA techniques. Variant proteins may differ from a parent protein by a small number of amino acid residues and may be defined by their level of primary amino acid sequence homology/identity with a parent (reference) protein.

### *II. Tunable FPDSM Systems*

**[0058]** As described briefly above and detailed in the Experimental Section below, certain embodiments the disclosure are directed to one or more storage stable protein compositions, wherein the protein composition is distributed and encapsulated (entrapped) within an FPDSM system, wherein the FPDSM system comprises at least two FPDSM components (each of which individual FPDSM components does not melt below 100°C), wherein the FPDSM system is fine tuned to be a molten liquid at a temperature between 40°C to about 100°C, and a solid at a temperature between about 20°C to 40°C.

**[0059]** Thus, the processes for producing the FPDSM compositions of the disclosure can be generically described as melt solidification or melt granulation processes. More particularly, in all of these processes, a dry form of the protein (*e.g.,* produced by processes such as lyophilization, spray drying, crystallization, precipitation, spray chilling and the like) is suspended in the molten (liquid) FPDSM solvent system and subsequently solidified at ambient temperatures (*e.g.,* about 20°C to 40°C). Thus, the resulting solidified formulations can be of any size or shape (*i.e.,* monoliths, tablets, granules or powders).

**[0060]** A particularly useful embodiment of melt solidification is melt granulation. For example, in melt granulation processes, the molten FPDSM system (in which the POI is suspended) is atomized or formed into particles or granules, which are flowable and provide protection to the POI. Granules are typically particles with diameters between about 50 microns and 1000 microns, or as large as 5000 microns.

Examples of melt granulation processes included spray cooling, spray chilling, spinning disk atomization, melt extrusion, and the like.

**[0061]** Examples of individual components suitable for combining in a FPDSM system of the disclosure are listed below in **Table 1**. Note that the melting point (in some cases, a decomposition point) of each individual component is greater than 100°C (*i.e.*, none of these individual (distinct) components melts below 100°C). However, as stated above and further discussed below, when the components are combined in mixtures in certain ratios (and/or with the addition of up to 10% w/w water), the resulting (combined) FPDSM systems will melt at a temperature between 40°C to about 100°C, and yet will solidify at a temperature between about 20°C to 40°C.

**TABLE 1**

**MELTING POINT (OR DECOMPOSITION POINT) OF INDIVIDUAL COMPONENTS SUITABLE FOR INCORPORATION INTO AN FPDSM**

| Component | Melting Point (°C) |
|---|---|
| ammonium chloride* | 338 |
| choline chloride* | 302-305 |
| betaine (trimethylglycine)* | 293 - 301 |
| betaine hydrochloride | 241 |
| citric acid | 153-156 |
| malonic acid | 135 |
| tartaric acid | 168-171 |
| fructose* | 103-105 |
| sucrose* | 186 |
| glucose | 146-150 |
| lactose* | 202-203.5 |
| maltose | 102-103 |
| urea | 132-135 |
| sorbitol (anhydrous) | 110 |

* May Decompose

**[0062]** For example, as described herein, a protein of interest (POI) is formulated in a tunable FPDSM system of the disclosure, wherein the POI is combined (added) with a FPDSM system at a temperature between 40°C to about 100°C. For example, once the POI has been mixed with the molten FPDSM system, the combined FPDSM/POI formulation is subsequently cooled to a temperature between about 20°C to 40°C (*i.e.*, a temperature at which the FPDSM system is a solid), wherein the FPDSM encapsulates (entraps)

the protein composition within a solid matrix as the FPDSM system transitions from a liquid state to a solid state during the cooling process.

**[0063]** Thus, in certain embodiments, a protein composition is formulated in a FPDSM system of the disclosure and encapsulated by cooling the FPDSM system as generally described above, wherein the encapsulated POI is substantially stabilized against various forms of environmental protein degradation. More particularly, certain embodiments are related to protein compositions formulated in a FPDSM system of the disclosure, wherein the FPDSM encapsulated composition may be stored at temperatures up to about 40°C or less for a period of time (*e.g.*, an hour, a day, a week, a month, *etc.*) prior the end use of the particular protein (POI) composition.

**[0064]** In addition, an elegant benefit of the tunable FPDSM systems of the instant disclosure, and more particularly the protein compositions formulated therein (and granules thereof), is that the protein composition (or granules thereof) is readily reconstituted for end use by dilution of the solid FPDSM composition in water (or an aqueous buffered solvent). Thus, in certain embodiments, a protein composition is formulated in a FPDSM system of the disclosure, wherein the FPDSM encapsulated composition (or granules thereof) may be held at temperatures up to about 40°C or less for a period of time prior the end use of the particular protein composition, with minimal loss of protein activity thereof.

**[0065]** Thus, as briefly described above, a FPDSM system of the disclosure comprises at least two (2) distinct FPDSM components. In certain other embodiments, a FPDSM system of the disclosure may additionally comprise up to about 10% (w/w) water. In certain embodiments, such distinct FPDSM components are selected from the group consisting of ammonium chloride ($NH_4Cl$), choline chloride, betaine (N,N,N,-trimethylglycine), betaine hydrochloride, citric acid, malonic acid, tartaric acid, sorbitol, sucrose, fructose, glucose, lactose, maltose and urea. In certain other embodiments, an at least two component FPDSM system comprises betaine and monosaccharide, wherein the monosaccharide is selected from the group consisting of glucose, galactose, fructose, mannose, erythrose, ribose, glyceraldehyde, dihydroxyacetone, glycerol, sorbitol, mannitol and dulcitol.

**[0066]** One of skill in the art will appreciate that any POI (*e.g.*, enzyme) contemplated for formulation and stabilization in a FPDSM system of the disclosure will have its own unique thermal denaturation profile and melting temperature ($T_m$). For example, in certain embodiments, optimal FPDSM systems are provided, wherein the protein composition is combined with the FPDSM system at temperatures between 40°C to about 100°C. For example, the $T_m$ of a protein is generally defined as the temperature at which both the folded (F) and unfolded (U) states are equally populated at equilibrium.

**[0067]** For example, one of skill in the art, may readily formulate a novel protein (*e.g.*, a novel enzyme or novel variant thereof) and/or formulate a combination of more than one protein (*e.g.*, a combination of two different enzymes) in a FPDSM system of the disclosure at a temperature of 40°C to about 100°C. Thus,

one skilled in the art, working with a novel protein (of unknown physical properties) to be formulated in such a FPDSM system of the disclosure, may readily determine a denaturation profile and/or a protein melting temperature ($T_m$) profile using routine laboratory techniques and methods available in the art including, but not limited to, differential scanning calorimetry (DSC), circular dichroism (CD), fluorescence anisotropy (polarized light), Fourier transform infrared spectroscopy (FTIR), hydrophobic fluorophores, intrinsic (tryptophan) fluorescence lifetime changes, light scattering, ultracentrifugation sedimentation velocity analysis and the like. In addition, computational and bioinformatics based methods are available and known in the art for predicting protein melting temperature from its primary amino acid sequence (*e.g., see*, Gorania *et al.*, 2010; Ku *et al.*, 2009; Zhang and Maginn, 2013).

**[0068]** As further detailed in the Example section, it has been observed herein that some POIs (*e.g.*, enzymes) remain stable through the melt granulation process at temperatures above the POI's $T_m$. For example, as presented below in the Examples section, in certain embodiments solid (spray dried) subtilisin protease (*i.e.*, Properase® enzyme) was formulated in FPDSM systems of the disclosure at elevated temperatures (*e.g.*, 80-100°C), wherein it is generally known in the art that subtilisin proteases (*e.g.*, including, but not limited to Properase®) are relatively unstable at temperatures of about 55°C and above. Surprisingly, the even presence of water (*e.g.*, water added up to about 10% w/w) within a FPDSM of the disclosure at temperatures near or above the $T_m$ of the POI does not result in appreciable inactivation or destabilization of such entrapped POIs, as shown by the Figures and Examples of the instant disclosure.

**[0069]** Thus, without wishing to be bound by any particular theory, mechanism or mode of action, it is contemplated herein that the water (*e.g.,* up to about 10% w/w) in such FPDSM systems is sequestered, and hence less available than "free" water to interact with the POI in a manner that would lead to inactivation or other manifestations of irreversible structural destabilization. For example, **FIG. 2** of the disclosure demonstrates excellent enzyme recovery in the FDPSM at up to 10% w/w water, wherein it is possible that even more than 10% w/w water can be incorporated into the FDPSM without adverse impact on enzyme recovery. Thus, the melting point of a solid matrix suitable for the formulation of enzymes can be depressed, and thereby "tuned" to more enzyme or protein friendly temperatures, by means of the two levers of the instant disclosure comprising: (1) blending two or more solid components to form a FPDSM blend; and (2) incorporating water, to further depress the melting point. Thus, the compositions of the instant disclosure comprise FPDSM systems suitable for entrapping enzymes and thereby improving their recovery and stability.

**[0070]** Certain embodiments of the disclosure are directed to one or more proteins of interest (*e.g.*, an enzyme) formulated in a tunable FPDSM system of the disclosure, wherein the FPDSM system is tuned to be a liquid at temperatures of 40°C to about 100°C, and a solid at temperatures between about 20°C to 40°C. More particularly, in certain embodiments the disclosure is directed to a protein composition of

interest formulated in a tunable FPDSM system of disclosure, wherein such FPDSM systems significantly mitigate the degradation and/or loss of activity of the protein composition, particularly such formulated protein compositions which are stored for a period of time (*i.e.*, days, weeks, months) before their end use.

**[0071]** Thus, as described below in **Example 1**, various FPDSM system "solvent components" were screened to identify two-component FPDSM systems (**Table 2**) and two-component FPDSM systems which included added water (**Table 3**) meeting specific pre-determined parameters relevant to the stability and formulation requirements of the particular POI (*i.e.*, a subtilisin protease in this particular example). More particularly, as described in Example 1 (and presented in Tables 2 and 3), the following FPDSM system (solvent) components were screened at the indicated (component 1):(component 2) molar ratios specified in the Tables 2 and 3: ammonium chloride, choline chloride, betaine (N,N,N,-trimethylglycine), betaine hydrochloride, citric acid, malonic acid, tartaric acid, sorbitol, fructose, sucrose, glucose, lactose, maltose, urea and water.

**[0072]** For example, the FPDSM system components (Table 2 or Table 3) were screened in the Example 1 with the specific parameters that: (a) the combined FPDSM system components melt (form a liquid) at a temperature between 40°C to about 100°C (*i.e.*, to mitigate thermal denaturation/inactivation of the protein) and (b) that the FPDSM system should quickly solidify at a temperature between about 20°C to 40°C, thereby quickly forming a robust solid matrix, suitable for encapsulation of proteins. As described in **Example 2**, the FPDSM systems meeting the aforementioned screening parameters were subsequently prepared/formulated with an active POI (*i.e.*, a subtilisin protease). More particularly, the POI formulations presented in Example 2 are FPDSM systems comprising added water (*e.g.* 5% w/w), wherein the following solvent component combinations were assessed: (i) anhydrous betaine/urea (1:1 mole ratio) and 5% (weight) water, and (ii) urea/lactose (6:1 mole ratio) and 5% (weight) water.

**[0073]** The recovered protease activity of the Example 2 FPDSM systems were assayed as described in **Example 3**, wherein the percent (%) recovered protease activity of the anhydrous betaine/urea (1:1 mole ratio) + 5% water formulation was 71.8% and the percent (%) recovered protease activity of the urea/lactose (6:1 mole ratio) + 5% water was 87.3%. (*e.g., s*ee, **Table 5**)

**[0074]** In addition, the ability to fine tune such FPDSM systems are demonstrated in **Example 4**. For example, certain FPDSM systems which did not melt (*i.e.*, form a liquid) at a temperature feasible for formulating the subtilisin protease (*i.e.*, at a temperature of 40°C to about 100°C were re-formulated (*i.e.*, fine-tuned) with the addition of water (*e.g.*, up to about 10% w/w). Thus, as presented in Example 4 (**Table 6**), the ability to fine tune such FPDSM systems by the addition of a specified weight percent of water, thereby lowering the FPDSM system mixing temperature, significantly enhances the recovered protease activity of these formulations (*e.g., see*, Table 6, urea/ammonium chloride (1:4) + 10% water and betaine/urea (1:1) + 5% water). Furthermore, as presented in Table 6, even when the added water does not

appear to significantly lower the mixing temperature of the FPDSM system required to melt the mixture of components (*e.g.*, *see*, Table 6, "Mixing Temperatures"), the added water results in an increase in the recovered protease activity from such formulations (*e.g.*, *see* Table 6, "Properase® Relative Residual Activity"). For example, a comparison of the urea/glucose (1:1) + 5% water (FPDSM system) relative to the urea/glucose (1:1) + 10% water (FPDSM system), indicates that the water addition does not significantly reduce the mixing temperature going from 5% water (*i.e.*, 80°C) to 10% water (*i.e.*, 80°C), whereas the recovered protease activity from the same formulations indicate that the water in such FPDSM systems improves the recovery of active protease (*e.g.*, the Properase® relative residual activity of the urea/glucose (1:1) + 5% water (FPDSM system) was 79.7%, whereas the Properase® relative residual activity of the urea/glucose (1:1) + 10% water (FPDSM system) increased to 93%).

**[0075]** **Example 5** (*see*, **FIG. 3**) provides an exemplary analysis of the protease activity (*i.e.*, Properase®) as a function of different mixing temperatures, which analysis is readily extended to other proteins of interest contemplated herein. For example, as shown in FIG. 3., there was an evident decrease in protease activity as the mixing temperature generally exceeded 80°C.

**[0076]** **Example 6** and **Example 7** of the disclosure further describe methods for granulating such protein encapsulated FPDSM systems, which protein/FPDSM system granules are particularly suitable for short-term or long-term storage thereof.

**[0077]** **Example 8** of the disclosure demonstrates the storage stability of such protein/FPDSM system formulations. For example, a subtilisin protease was formulated in a FPDSM system of the disclosure, wherein the storage stability of the protease/FPDSM system formulation was evaluated at 25°C for a period of 28 days. More specifically, as presented in **Table 7**, the protease activity at days 7, 14, 21 and 28, remained well above 98% recovered activity. Furthermore, when compared to the activity of the control protease (*i.e.*, spray dried protease) at days 7, 14, 21 and 28, the recovered protease activity of all FPDSM systems demonstrate that greater protease activity was maintained relative to the spray dried protease.

**[0078]** **Example 9** of the disclosure further describes a second POI (*i.e.*, an amylase) formulated and stabilized in a FPDSM system of the disclosure. More particularly, Example 9 describes the maximum solubility of an α-amylase protein of interest in a choline chloride/urea (1:2 ratio) FPDSM system (*e.g.*, *see*, **FIG. 4**).

**[0079]** Thus, in certain embodiments, the disclosure is directed to such FPDSM systems. In other embodiments, the disclosure is related to one or more solid protein compositions formulated and stabilized in such FPDSM systems of the disclosure. In certain other embodiments, the disclosure is related to storage stable solid enzyme compositions comprising a FPDSM system formulation disclosed herein, wherein such (FPDSM stabilized) solid enzyme compositions retain at least about 50% to 100% enzymatic activity after at least 24-hours of storage at 25°C.

## III. *FPDSM Stabilized Proteins of Interest and Recovered Activity Thereof*

**[0080]** A protein of interest (POI) can be any endogenous or heterologous protein or a variant thereof. The protein can contain one or more disulfide bridges or is a protein whose functional form is a monomer or a multimer, *i.e.*, the protein has a quaternary structure and is composed of a plurality of identical (homologous) or non-identical (heterologous) subunits. As described *supra*, certain embodiments of the disclosure are directed to one or more proteins of interest formulated in a protein stabilizing FPSDM system of the disclosure.

**[0081]** Thus, in certain embodiments, a protein of interest (POI) is an enzyme, a hormone, a growth factor, a cytokine, an antibody, a receptor, and the like. In certain embodiments, a POI is an enzyme selected from acetyl esterases, aryl esterases, aminopeptidases, amylases, arabinases, arabinofuranosidases, carboxypeptidases, catalases, cellulases, chitinases, chymosin, cutinases, deoxyribonucleases, epimerases, esterases, α-galactosidases, β-galactosidases, α-glucanases, glucan lysases, endo-β-glucanases, glucoamylases, glucose oxidases, α-glucosidases, β-glucosidases, glucuronidases, hemicellulases, hexose oxidases, hydrolases, invertases, isomerases, laccases, lipases, lyases, mannosidases, oxidases, oxidoreductases, pectate lyases, pectin acetyl esterases, pectin depolymerases, pectin methyl esterases, pectinolytic enzymes, perhydrolases, polyol oxidases, peroxidases, phenoloxidases, phytases, polygalacturonases, proteases, rhamno-galacturonases, ribonucleases, thaumatin, transferases, transport proteins, transglutaminases, xylanases, hexose oxidases, and the like.

**[0082]** Thus, in certain embodiments, a POI or a variant POI thereof is an enzyme selected from Enzyme Commission (EC) Number EC 1, EC 2, EC 3, EC 4, EC 5 or EC 6.

**[0083]** For example, in certain embodiments a POI is an oxidoreductase enzyme, including, but not limited to, an EC 1 (oxidoreductase) enzyme selected from EC 1.10.3.2 (*e.g.*, a laccase), EC 1.10.3.3 (*e.g.*, L-ascorbate oxidase), EC 1.1.1.1 (*e.g.*, alcohol dehydrogenase), EC 1.11.1.10 (*e.g.*, chloride peroxidase), EC 1.11.1.17 (*e.g.*, peroxidase), EC 1.1.1.27 (*e.g.*, L-lactate dehydrogenase), EC 1.1.1.47 (*e.g.*, glucose 1-dehydrogenase), EC 1.1.3.X (*e.g.*, glucose oxidase), EC 1.1.3.10 (*e.g.*, pyranose oxidase), EC 1.13.11.X (*e.g.*, dioxygenase), EC 1.13.11.12 (*e.g.*, lineolate 13S-lipozygenase), EC 1.1.3.13 (*e.g.*, alcohol oxidase), EC 1.14.14.1 (*e.g.*, monooxygenase), EC 1.14.18.1 (*e.g.*, monophenol monooxigenase) EC 1.15.1.1 (*e.g.*, superoxide dismutase), EC 1.1.5.9 (formerly EC 1.1.99.10, *e.g.*, glucose dehydrogenase), EC 1.1.99.18 (*e.g.*, cellobiose dehydrogenase), EC 1.1.99.29 (*e.g.*, pyranose dehydrogenase), EC 1.2.1.X (*e.g.*, fatty acid reductase), EC 1.2.1.10 (*e.g.*, acetaldehyde dehydrogenase), EC 1.5.3.X (*e.g.*, fructosyl amine reductase), EC 1.8.1.X (*e.g.*, disulfide reductase) and EC 1.8.3.2 (*e.g.*, thiol oxidase).

**[0084]** In certain embodiments a POI is a transferase enzyme, including, but not limited to, an EC 2 (transferase) enzyme selected from EC 2.3.2.13 (*e.g.*, transglutaminase), EC 2.4.1.X (*e.g.*,

hexosyltransferase), EC 2.4.1.40 (*e.g.*, alternasucrase), EC 2.4.1.18 (*e.g.*, 1,4 alpha-glucan branching enzyme), EC 2.4.1.19 (*e.g.*, cyclomaltodextrin glucanotransferase), EC 2.4.1.2 (*e.g.*, dextrin dextranase), EC 2.4.1.20 (*e.g.*, cellobiose phosphorylase), EC 2.4.1.25 (*e.g.*, 4-alpha-glucanotransferase), EC 2.4.1.333 (*e.g.*, 1,2-beta-oligoglucan phosphor transferase), EC 2.4.1.4 (*e.g.*, amylosucrase), EC 2.4.1.5 (*e.g.*, dextransucrase), EC 2.4.1.69 (*e.g.*, galactoside 2-alpha-L-fucosyl transferase), EC 2.4.1.9 (*e.g.*, inulosucrase), EC 2.7.1.17 (*e.g.*, xylulokinase), EC 2.7.7.89 (formerly EC 3.1.4.15, *e.g.*, [glutamine synthetase]-adenylyl-L-tyrosine phosphorylase), EC 2.7.9.4 (*e.g.*, alpha glucan kinase) and EC 2.7.9.5 (*e.g.*, phosphoglucan kinase).

**[0085]** In other embodiments a POI is a hydrolase enzyme, including, but not limited to, an EC 3 (hydrolase) enzyme selected from EC 3.1.X.X (*e.g.*, an esterase), EC 3.1.1.1 (*e.g.*, pectinase), EC 3.1.1.14 (*e.g.*, chlorophyllase), EC 3.1.1.20 (*e.g.*, tannase), EC 3.1.1.23 (*e.g.*, glycerol-ester acylhydrolase), EC 3.1.1.26 (*e.g.*, galactolipase), EC 3.1.1.32 (*e.g.*, phospholipase A1), EC 3.1.1.4 (*e.g.*, phospholipase A2), EC 3.1.1.6 (*e.g.*, acetylesterase), EC 3.1.1.72 (*e.g.*, acetylxylan esterase), EC 3.1.1.73 (*e.g.*, feruloyl esterase), EC 3.1.1.74 (*e.g.*, cutinase), EC 3.1.1.86 (*e.g.*, rhamnogalacturonan acetylesterase), EC 3.1.1.87 (*e.g.*, fumosin B1 esterase), EC 3.1.26.5 (*e.g.*, ribonuclease P), EC 3.1.3.X (*e.g.*, phosphoric monoester hydrolase), EC 3.1.30.1 (*e.g.*, Aspergillus nuclease S1), EC 3.1.30.2 (*e.g.*, Serratia marcescens nuclease), EC 3.1.3.1 (*e.g.*, alkaline phosphatase), EC 3.1.3.2 (*e.g.*, acid phosphatase), EC 3.1.3.8 (*e.g.*, 3-phytase), EC 3.1.4.1 (*e.g.*, phosphodiesterase I), EC 3.1.4.11 (*e.g.*, phosphoinositide phospholipase C), EC 3.1.4.3 (*e.g.*, phospholipase C), EC 3.1.4.4 (*e.g.*, phospholipase D), EC 3.1.6.1 (*e.g* arylsufatase), EC 3.1.8.2 (*e.g.*, diisopropyl-fluorophosphatase), EC 3.2.1.10 (*e.g.*, oligo-1,6-glucosidase), EC 3.2.1.101 (*e.g.*, mannan endo-1,6-alpha-mannosidase), EC 3.2.1.11 (*e.g.*, alpha-1,6-glucan-6-glucanohydrolase), EC 3.2.1.131 (*e.g.*, xylan alpha-1,2-glucuronosidase), EC 3.2.1.132 (*e.g.*, chitosan N-acetylglucosaminohydrolase), EC 3.2.1.139 (*e.g.*, alpha-glucuronidase), EC 3.2.1.14 (*e.g.*, chitinase), EC 3.2.1.151 (e.g., xyloglucan-specific endo-beta-1,4-glucanase), EC 3.2.1.155 (*e.g.*, xyloglucan-specific exo-beta-1,4-glucanase), EC 3.2.1.164 (*e.g.*, galactan endo-1,6-beta-galactosidase), EC 3.2.1.17 (*e.g.*, lysozyme), EC 3.2.1.171 (*e.g.*, rhamnogalacturonan hydrolase), EC 3.2.1.174 (*e.g.*, rhamnogalacturonan rhamnohydrolase), EC 3.2.1.2 (*e.g.*, beta-amylase), EC 3.2.1.20 (*e.g.*, alpha-glucosidase), EC 3.2.1.22 (*e.g.*, alpha-galactosidase), EC 3.2.1.25 (*e.g.*, beta-mannosidase), EC 3.2.1.26 (*e.g.*, beta-fructofuranosidase), EC 3.2.1.37 (*e.g.*, xylan 1,4-beta-xylosidase), EC 3.2.1.39 (*e.g.*, glucan endo-1,3-beta-D-glucosidase), EC 3.2.1.40 (*e.g.*, alpha-L-rhamnosidase), EC 3.2.1.51 (*e.g.*, alpha-L-fucosidase), EC 3.2.1.52 (*e.g.*, beta-N-Acetylhexosaminidase), EC 3.2.1.55 (*e.g.*, alpha-N-arabinofuranosidase), EC 3.2.1.58 (*e.g.*, glucan 1,3-beta-glucosidase), EC 3.2.1.59 (*e.g.*, glucan endo-1,3-alpha-glucosidase), EC 3.2.1.67 (*e.g.*, galacturan 1,4-alpha-galacturonidase), EC 3.2.1.68 (*e.g.*, isoamylase), EC 3.2.1.7 (*e.g.*, 1-beta-D-fructan fructanohydrolase), EC 3.2.1.74 (*e.g.*, glucan 1,4-β-glucosidase), EC 3.2.1.75 (*e.g.*, glucan endo-1,6-beta-glucosidase), EC 3.2.1.77

(*e.g.*, mannan 1,2-(1,3)-alpha-mannosidase), EC 3.2.1.80 (*e.g.*, fructan beta-fructosidase), EC 3.2.1.82 (*e.g.*, exo-poly-alpha-galacturonosidase), EC 3.2.1.83 (*e.g.*, kappa-carrageenase), EC 3.2.1.89 (*e.g.*, arabinogalactan endo-1,4-beta-galactosidase), EC 3.2.1.91 (*e.g.*, cellulose 1,4-beta-cellobiosidase), EC 3.2.1.96 (*e.g.*, mannosyl-glycoprotein endo-beta-N-acetylglucosaminidase), EC 3.2.1.99 (*e.g.*, arabinan endo-1,5-alpha-L-arabinanase), EC 3.4.X.X (*e.g.*, peptidase), EC 3.4.11.X (*e.g.*, aminopeptidase), EC 3.4.11.1 (*e.g.*, leucyl aminopeptidase), EC 3.4.11.18 (*e.g.*, methionyl aminopeptidase), EC 3.4.13.9 (*e.g.*, Xaa-Pro dipeptidase), EC 3.4.14.5 (*e.g.*, dipeptidyl-peptidase IV), EC 3.4.16.X (*e.g.*, serine-type carboxypeptidase), EC 3.4.16.5 (*e.g.*, carboxypeptidase C), EC 3.4.19.3 (*e.g.*, pyroglutamyl-peptidase I), EC 3.4.21.X (*e.g.*, serine endopeptidase), EC 3.4.21.1 (*e.g.*, chymotrypsin), EC 3.4.21.19 (*e.g.*, glutamyl endopeptidase), EC 3.4.21.26 (*e.g.*, prolyl oligopeptidase), EC 3.4.21.4 (*e.g.*, trypsin), EC 3.4.21.5 (*e.g.*, thrombin), EC 3.4.21.63 (*e.g.*, oryzin), EC 3.4.21.65 (*e.g.*, thermomycolin), EC 3.4.21.80 (*e.g.*, streptogrisin A), EC 3.4.22.X (*e.g.*, cysteine endopeptidase), EC 3.4.22.14 (*e.g.*, actinidain), EC 3.4.22.2 (*e.g.*, papain), EC 3.4.22.3 (*e.g.*, ficain), EC 3.4.22.32 (*e.g.*, stem bromelain), EC 3.4.22.33 (*e.g.*, fruit bromelain), EC 3.4.22.6 (e.g., chymopapain), EC 3.4.23.1 (e.g., pepsin A), EC 3.4.23.2 (e.g., pepsin B), EC 3.4.23.22 (e.g., endothiapepsin), EC 3.4.23.23 (*e.g.*, mucorpepsin), EC 3.4.23.3 (*e.g.*, gastricsin), EC 3.4.24.X (*e.g.*, metalloendopeptidase), EC 3.4.24.39 (*e.g.*, deuterolysin), EC 3.4.24.40 (v., serralysin), EC 3.5.1.1 (*e.g.*, asparaginase), EC 3.5.1.11 (*e.g.*, penicillin amidase), EC 3.5.1.14 (*e.g.*, N-acyl-aliphatic-L-amino acid amidohydrolase), EC 3.5.1.2 (*e.g.*, L-glutamine amidohydrolase), EC 3.5.1.28 (*e.g.*, N-acetylmuramoyl-L-alanine amidase), EC 3.5.1.4 (*e.g.*, amidase), EC 3.5.1.44 (*e.g.*, protein-L-glutamine amidohydrolase), EC 3.5.1.5 (*e.g.*, urease), EC 3.5.1.52 (*e.g.*, peptide-N(4)-(N-acetyl-beta-glucosaminyl)asparagine amidase), EC 3.5.1.81 (*e.g.*, N-Acyl-D-amino-acid deacylase), EC 3.5.4.6 (*e.g.*, AMP deaminase) and EC 3.5.5.1 (*e.g.*, nitrilase).

**[0086]** In other embodiments a POI is a lyase enzyme, including, but not limited to, an EC 4 (lyase) enzyme selected from EC 4.1.2.10 (*e.g.*, mandelonitrile lyase), EC 4.1.3.3 (*e.g.*, N-acetylneuraminate lyase), EC 4.2.1.1 (*e.g.*, carbonate dehydratase), EC 4.2.2.- (*e.g.*, rhamnogalacturonan lyase), EC 4.2.2.10 (*e.g.*, pectin lyase), EC 4.2.2.22 (*e.g.*, pectate trisaccharide-lyase), EC 4.2.2.23 (*e.g.*, rhamnogalacturonan endolyase) and EC 4.2.2.3 (*e.g.*, mannuronate-specific alginate lyase).

**[0087]** In certain other embodiments a POI is an isomerase enzyme, including, but not limited to, an EC 5 (isomerase) enzyme selected from EC 5.1.3.3 (*e.g.*, aldose 1-epimerase), EC 5.1.3.30 (*e.g.*, D-psicose 3-epimerase), EC 5.4.99.11 (*e.g.*, isomaltulose synthase) and EC 5.4.99.15 (*e.g.*, (1→4)-α-D-glucan 1-α-D-glucosylmutase).

**[0088]** In yet other embodiments, a POI is a ligase enzyme, including, but not limited to, an EC 6 (ligase) enzyme selected from EC 6.2.1.12 (*e.g.*, 4-coumarate:coenzyme A ligase) and EC 6.3.2.28 (*e.g.*, L-amino-acid alpha-ligase).

**[0089]** Thus, in certain embodiments, a POI of the disclosure is an industrial protease or amylase enzyme, which protease or amylase is stabilized and formulated in an FPDSM system of the disclosure. For example, in certain embodiments, a POI is neutral protease (or "metalloproteases") or an alkaline (or "serine") protease. For example, *Bacillus* subtilisin proteins (enzymes) are exemplary serine proteases for use and stabilization in a FPDSM composition of the disclosure. More particularly, a wide variety of *Bacillus* subtilisins have been identified and sequenced, for example, subtilisin 168, subtilisin BPN', subtilisin Carlsberg, subtilisin DY, subtilisin 147 and subtilisin 309 (*e.g.*, WO 1989/06279 and Stahl *et al.*, 1984). Likewise, numerous references provide examples of variant proteases suitable for formulation in a FPDSM system of the disclosure, such as PCT Publication Nos. WO1999/20770; WO1999/20726; WO1999/20769; WO1989/06279; U.S. RE34,606; U.S. Patent Nos. 4,914,031; 4,980,288; 5,208,158; 5,310,675; 5,336,611; 5,399,283; 5,441,882; 5,482,849; 5,631,217; 5,665,587; 5,700,676; 5,741 ,694; 5,858,757; 5,880,080; 6,197,567 and 6,218,165.

**[0090]** Thus, in other embodiments, a POI is an amylase formulated and stabilized in an FPDSM system of the disclosure. A wide variety of amylase enzymes and variants thereof are known to one skilled in the art. For example, International PCT Publication NO. WO2006/037484 and WO 2006/037483 describe variant α-amylases having improved solvent stability, Publication No. WO1994/18314 discloses oxidatively stable α-amylase variants, Publication No. WO1999/19467, WO2000/29560 and WO2000/60059 disclose Termamyl-like α-amylase variants, Publication No. WO2008/112459 discloses α-amylase variants derived from *Bacillus sp.* number 707, Publication No. WO1999/43794 discloses maltogenic α-amylase variants, Publication No. WO1990/11352 discloses hyper-thermostable α-amylase variants, Publication No. WO2006/089107 discloses α-amylase variants having granular starch hydrolyzing activity.

**[0091]** In other embodiments, a POI formulated and stabilized in a FPDSM system of the disclosure is a peptide, a peptide hormone, a growth factor, a clotting factor, a chemokine, a cytokine, a lymphokine, an antibody, a receptor, an adhesion molecule, a microbial antigen (*e.g.*, HBV surface antigen, HPV E7, etc.), variants thereof, fragments thereof and the like. Other types of proteins (or variants thereof) of interest may be those that are capable of providing nutritional value to a food or to a crop. Non-limiting examples include plant proteins that can inhibit the formation of anti-nutritive factors and plant proteins that have a more desirable amino acid composition (*e.g.*, a higher lysine content than a non-transgenic plant).

**[0092]** As described *supra*, certain embodiments are directed to FPDSM stabilized protein compositions. More particularly, certain embodiments are related to a solid enzyme composition stabilized in a FPDSM system of the disclosure. For example, in certain embodiments, the activity of an enzyme of interest (*e.g.*, a protease) is measured before being formulated in a FPDSM system and/or immediately after being formulated in a FPDSM system. In certain other embodiments, the activity of an FPDSM formulated

enzyme composition (*e.g.*, a protease, an amylase) is measured after two (2) hours, after twelve (12) hours, after one (1) day, after seven (7) days, after twenty-one (21) days, *etc.* of storage at room temperature (RT; about 20-25°C). Thus, certain embodiments, are related to recovered enzymatic activity of a FPDSM formulated enzyme composition which has been stored for a period of time prior to its end-use.

**[0093]** There are various assays known to those of ordinary skilled in the art for detecting and measuring activity of proteins. In particular, for proteases, there are assays based on the release of acid-soluble peptides from casein or hemoglobin measured as absorbance at 280 nm or colorimetrically, using the Folin method (*e.g.*, Bergmeyer et al., 1984). Other assays involve the solubilization of chromogenic substrates (*see e.g.*, Ward, 1983). Other exemplary assays include succinyl-Ala-Ala-Pro-Phe-para-nitroanilide assay (SAAPFpNA) and the 2,4,6-trinitrobenzene sulfonate sodium salt assay (TNBS assay). Numerous additional references known to those in the art provide suitable methods (*see e.g.*, Wells *et al.*, 1983; Christianson *et al.*, 1994 and Hsia *et al.*, 1999). International PCT Publication No. WO2014/164777 discloses Ceralpha α-amylase activity assays useful for amylase activities described herein. Additional means for determining the levels of a protein of interest include, but are not limited to, the use of immunoassays with either polyclonal or monoclonal antibodies specific for the protein. Examples include enzyme-linked immunosorbent assay (ELISA), radioimmunoassay (RIA), fluorescence immunoassay (FIA), and fluorescent activated cell sorting (FACS).

## EXAMPLES

**[0094]** Certain aspects of the present disclosure may be further understood in light of the following examples, which should not be construed as limiting. Modifications to materials and methods will be apparent to those skilled in the art.

## EXAMPLE 1

## FREEZING POINT DEPRESSED SOLID MATRIX COMPOSITIONS THAT MEET PHYSICAL REQUIREMENTS AS AN ENCAPSULATING MATRIX FOR SOLID PROTEIN COMPOSITIONS

### *A. Screening*

**[0095]** In the present example, Applicant screened various solvent components (*see*, **Table 1**) to identify solvent component mixtures (*see,* **Tables 2** and **3**) meeting specific pre-determined parameters which are relevant to the stability and formulation requirements of one or more protein compositions of the disclosure. For example, in certain embodiments, the solvent component mixtures were screened for solvent component mixtures having melting points lower than 100°C, and an ability to solidify at a temperature

above 20°C. Other considerations for solvent component mixtures may include suitable viscosity and non-hygroscopic behavior.

**[0096]** *Initial Screen*: Compositions with the potential to form FPDSM solvent systems are summarized in Table 2 and Table 3. Fifty (50) gram master mixes of each solvent component was prepared at five (5) different molar ratios. Twenty-two (22) different solvent systems were created resulting in one hundred ten (110) total initial samples.

**[0097]** *Melting Temperature*: To determine melting temperatures, two (2) gram samples were placed into test tubes in a heat block in duplicate. The samples were heated at 100°C, 80°C, 60°C, and 40°C for an initial screen. The amount of time taken for the sample to form a homogenous liquid was recorded and physical observations were noted. An *infra*-red (IR) gun was used to confirm heating temperatures.

**[0098]** *Solidification*: Because many compositions solidify at a temperature below their melting point, separate observations were made of the solidification behavior. To test the ability of the solvent component mixtures to solidify, samples that could melt (*i.e.*, form a molten liquid) were poured onto a sheet of aluminum foil at room temperature (20-25°C). The presence or absence of solidification was determined by visual observation of the sample cooled to ambient temperature (*i.e.*, between about 20°C to about 40°C). The pH of the component mixtures was recorded using litmus paper. As Tables 2 and 3 indicate, only certain combinations and ratios of component mixtures melt and solidify in the specified temperature ranges of the disclosure, making them suitable as FPDSMs for encapsulation of enzymes or other POIs.

**[0099]** *Melting Point Determination by Differential Scanning Calorimetry*: Differential scanning calorimetry (DSC) was performed on a TA Instruments DSC Q2000 thermal analyzer in nitrogen atmosphere. The DSC was used to determine the melting temperatures of the samples *via* heat flow. Ten (10) mg of each of the solvent samples were inserted into a low mass pan. The pan was clamped with a lid, and inserted into the DSC apparatus. The sample was heated from 20°C to 100°C at a heating rate of 10°C $min^{-1}$, was held isothermal at 100°C for a few minutes, then cooled to 20°C at 5°C $min^{-1}$, and heated back to 100°C at a heating rate of 10°C $min^{-1}$. An empty pan sealed with a lid was used as reference. Melting point of the FDPSM composition was determined by the first major peak in the heating cycle of the DSC thermogram.

**[0100]** *Viscosity*: A Brookfield viscometer equipped with Spindle 18 was used to determine the viscosity of the betaine/urea compositions at different molar ratios. About Ten (10) grams of each composition was placed in the sample container and heated to about 10°C above the apparent melting temperature (*i.e.*, the apparent $T_m$; identified from the initial screen with a continuous running water bath attached to a thermocouple). The viscosity, measured in cP (centiPoise), was recorded after one (1) minute. **Table 4** and **FIG. 1** show the viscosity of betaine/urea compositions at different molar ratios of mixture components at 95°C.

**TABLE 2**

**TWO-COMPONENT SOLVENT MIXTURES SCREENED AT FIVE DIFFERENT MOLAR RATIOS, FOR SUITABILITY AS FPDSM MIXTURES**

| Component 1 | Component 2 | Molar Ratio | Melts at 40-100°C | Solidifies at 20-40°C | pH |
|---|---|---|---|---|---|
| choline chloride | malonic acid | 1:3 | Yes | No | 1 |
| choline chloride | malonic acid | 1:2 | Yes | No | 1 |
| choline chloride | malonic acid | **1:1** | Yes | No | 1 |
| choline chloride | malonic acid | 2:1 | Yes | No | 1 |
| choline chloride | malonic acid | 3:1 | Yes | No | 1 |
| choline chloride | urea | 1:3 | Yes | No | 8 |
| choline chloride | urea | **1:2** | Yes | No | 8 |
| choline chloride | urea | 1:1 | Yes | No | 8 |
| choline chloride | urea | 2:1 | Yes | No | 8 |
| choline chloride | urea | 3:1 | Yes | No | 8 |
| choline chloride | phenylacetic acid | 1:3 | Yes | No | 4 |
| choline chloride | phenylacetic acid | 1:2 | Yes | No | 4 |
| choline chloride | phenylacetic acid | **1:1** | Yes | No | 4 |
| choline chloride | phenylacetic acid | 2:1 | Yes | No | 4 |
| choline chloride | phenylacetic acid | 3:1 | Yes | No | 4 |
| choline chloride | tartaric acid | 1:2 | No | No | 4 |
| choline chloride | tartaric acid | 1:1 | No | No | 4 |
| choline chloride | tartaric acid | **2:1** | No | No | 4 |
| choline chloride | tartaric acid | 3:1 | No | No | 4 |
| choline chloride | tartaric acid | 4:1 | No | No | 4 |
| choline chloride | citric acid | 1:3 | No | No | 3 |
| choline chloride | citric acid | 1:2 | Yes | Yes | 3 |
| choline chloride | citric acid | **1:1** | Yes | Yes | 3 |
| choline chloride | citric acid | 2:1 | Yes | Yes | 3 |
| choline chloride | citric acid | 3:1 | No | No | 3 |
| choline chloride | glucose | 1:1 | Yes | No | 6 |
| choline chloride | glucose | 2:1 | Yes | No | 6 |
| choline chloride | glucose | **4:1** | Yes | No | 6 |
| choline chloride | glucose | 6:1 | Yes | No | 6 |

TABLE 2 (*Continued*)

TWO-COMPONENT SOLVENT MIXTURES SCREENED AT FIVE DIFFERENT MOLAR RATIOS

| Component 1 | Component 2 | Molar Ratio | Melts at 40-100°C | Solidifies at 20-40°C | pH |
|---|---|---|---|---|---|
| choline chloride | glucose | 8:1 | Yes | No | 6 |
| choline chloride | Sorbitol | 1:3 | Yes | No | 4 |
| choline chloride | Sorbitol | 1:2 | Yes | No | 4 |
| choline chloride | Sorbitol | **1:1** | Yes | No | 4 |
| choline chloride | Sorbitol | 2:1 | Yes | Yes | 4 |
| choline chloride | Sorbitol | 3:1 | Yes | Yes | 4 |
| citric acid | glucose | 1:4 | No | No | |
| citric acid | glucose | 1:3 | No | No | |
| *citric acid | glucose | **1:2** | No | No | |
| citric acid | glucose | 1:1 | No | No | |
| citric acid | glucose | 2:1 | No | No | |
| citric acid | maltose | 1:3 | No | No | |
| citric acid | maltose | 1:2 | No | No | |
| citric acid | maltose | 1:1 | No | No | |
| citric acid | maltose | 2:1 | No | No | |
| citric acid | maltose | 3:1 | No | No | |
| citric acid | betaine monohydrate | 1:3 | Yes | No | |
| citric acid | betaine monohydrate | 1:2 | Yes | No | |
| citric acid | betaine monohydrate | 1:1 | Yes | No | |
| citric acid | betaine monohydrate | 2:1 | Yes | No | |
| citric acid | betaine monohydrate | 3:1 | Yes | No | |
| ammonium chloride | urea | 1:4 | No | No | |
| ammonium chloride | urea | 1:3 | Yes | Yes | 9 |
| *ammonium chloride | urea | **1:2** | Yes | Yes | 9 |
| ammonium chloride | urea | 1:1 | No | No | |
| ammonium chloride | urea | 2:1 | No | No | |
| betaine anhydrous | urea | 1:4 | Yes | Yes | 9 |
| betaine anhydrous | urea | 1:3 | Yes | Yes | 9 |
| *betaine anhydrous | urea | **1:2** | Yes | Yes | 9 |
| betaine anhydrous | urea | 1:1 | Yes | Yes | 9 |
| betaine anhydrous | urea | 2:1 | No | No | |
| betaine anhydrous | sucrose | 1:1 | No | No | |
| betaine anhydrous | sucrose | 2:1 | No | No | |
| *betaine anhydrous | sucrose | **4:1** | No | No | |
| betaine anhydrous | sucrose | 6:1 | No | No | |
| betaine anhydrous | sucrose | 8:1 | No | No | |
| betaine anhydrous | glucose | 1:2 | No | No | |
| betaine anhydrous | glucose | 1:1 | No | No | |
| *betaine anhydrous | glucose | **5:2** | No | No | |
| betaine anhydrous | glucose | 7:2 | No | No | |

**TABLE 2 (*Continued*)**

**TWO-COMPONENT SOLVENT MIXTURES SCREENED AT FIVE DIFFERENT MOLAR RATIOS**

| Component 1 | Component 2 | Molar Ratio | Melts at 40-100°C | Solidifies at 20-40°C | pH |
|---|---|---|---|---|---|
| betaine anhydrous | glucose | 5:1 | No | No | |
| urea | fructose | 1:3 | Yes | No | |
| urea | fructose | 1:2 | Yes | No | |
| urea | fructose | 1:1 | Yes | No | |
| urea | fructose | 2:1 | Yes | No | |
| urea | fructose | 3:1 | Yes | No | |
| urea | glucose | 1:3 | Yes | No | |
| urea | glucose | 1:2 | Yes | No | |
| urea | glucose | 1:1 | Yes | No | |
| urea | glucose | 2:1 | No | No | |
| urea | glucose | 3:1 | No | No | |
| urea | maltose | 1:2 | Yes | No | |
| urea | maltose | 1:1 | Yes | No | |
| urea | maltose | 2:1 | Yes | No | |
| urea | maltose | 4:1 | Yes | No | |
| urea | maltose | 6:1 | Yes | No | |
| urea | lactose | 1:2 | No | No | |
| urea | lactose | 1:1 | No | No | |
| urea | lactose | 2:1 | No | No | |
| urea | lactose | 4:1 | No | No | |
| urea | lactose | 6:1 | No | No | |
| betaine monohydrate | fructose | 1:2 | No | No | |
| betaine monohydrate | fructose | 1:1 | No | No | |
| betaine monohydrate | fructose | 5:2 | No | No | |
| betaine monohydrate | fructose | 7:2 | No | No | |
| betaine monohydrate | fructose | 5:1 | No | No | |
| betaine monohydrate | citric acid | 1:3 | No | No | |
| betaine monohydrate | citric acid | 1:2 | No | No | |
| betaine monohydrate | citric acid | 1:1 | No | No | |
| betaine monohydrate | citric acid | 5:2 | No | No | |
| betaine monohydrate | citric acid | 7:2 | No | No | |
| betaine monohydrate | lactose | 1:1 | No | No | |
| betaine monohydrate | lactose | 2:1 | No | No | |
| betaine monohydrate | lactose | 4:1 | No | No | |
| betaine monohydrate | lactose | 6:1 | No | No | |
| betaine monohydrate | lactose | 8:1 | No | No | |

**TABLE 3**

**TWO-COMPONENT SOLVENT MIXTURES, WITH ADDED WATER, SCREENED AT DIFFERENT MOLAR RATIOS**

| Component 1 | Component 2 | $H_2O$ (Wt % $H_2O$ added) | Molar Ratio Component 1 and 2 | Melts at 40-100°C | Solidifies at 20-40°C | pH |
|---|---|---|---|---|---|---|
| choline chloride | glucose | 0 | 4:1 | Yes | No | 7 |
| choline chloride | glucose | 2.5 | 4:1 | Yes | No | 7 |
| choline chloride | glucose | 5 | 4:1 | Yes | No | 7 |
| choline chloride | glucose | 10 | 4:1 | Yes | No | 7 |
| citric acid | glucose | 0 | 1:2 | No | No | 3 |
| citric acid | glucose | 2.5 | 1:2 | No | No | 3 |
| citric acid | glucose | 5 | 1:2 | No | No | 3 |
| citric acid | glucose | 10 | 1:2 | No | No | 3 |
| betaine | glucose | 0 | 4:1 | No | No | 8 |
| betaine | glucose | 2.5 | 4:1 | No | No | 8 |
| betaine | glucose | 5 | 4:1 | No | No | 8 |
| betaine | glucose | 10 | 4:1 | Yes | No | 8 |
| betaine | glucose | 0 | 5:2 | No | No | 8 |
| betaine | glucose | 2.5 | 5:2 | No | No | 8 |
| betaine | glucose | 5 | 5:2 | Yes | Yes | 8 |
| betaine | glucose | 10 | 5:2 | Yes | Yes | 8 |
| urea | fructose | 0 | 1:1 | No | No | 8 |
| urea | fructose | 2.5 | 1:1 | Yes | No | 8 |
| urea | fructose | 5 | 1:1 | Yes | No | 8 |
| urea | fructose | 10 | 1:1 | Yes | No | 8 |
| urea | glucose | 0 | 3:1 | No | No | 8 |
| urea | glucose | 2.5 | 3:1 | No | No | 8 |
| urea | glucose | 5 | 3:1 | Yes | Yes | 8 |
| urea | glucose | 10 | 3:1 | Yes | Yes | 8 |
| urea | maltose | 0 | 6:1 | No | No | 8 |
| urea | maltose | 2.5 | 6:1 | No | No | 8 |
| urea | maltose | 5 | 6:1 | Yes | Yes | 8 |
| urea | maltose | 10 | 6:1 | Yes | Yes | 8 |
| urea | lactose | 0 | 6:1 | No | No | 8 |
| urea | lactose | 2.5 | 6:1 | No | No | 8 |
| urea | lactose | 5 | 6:1 | Yes | Yes | 8 |
| urea | lactose | 10 | 6:1 | Yes | Yes | 8 |
| citric acid | maltose | 0 | 1:1 | No | No | 3 |
| citric acid | maltose | 2.5 | 1:1 | No | No | 3 |
| citric acid | maltose | 5 | 1:1 | No | No | 3 |
| citric acid | maltose | 10 | 1:1 | No | No | 3 |

**TABLE 4**

**VISCOSITY OF MOLTEN BETAINE/UREA COMPOSITIONS**

| FPDSM | Molar Ratio | Betaine Mole Fraction | Viscosity (cP) |
|---|---|---|---|
| **Anhydrous Betaine/Urea** | 1:3 | 0.25 | 51.6 |
| | 1:2 | 0.33 | 100.9 |
| | 1:1 | 0.50 | 113.8 |
| | 2:1 | 0.67 | 212.0 |

**EXAMPLE 2**

**PREPARATION OF FPDSM COMPOSITIONS INCORPORATING ENZYME USING MELT GRANULATION PROCESS**

**[0101]** As stated briefly above in Example 1, the solvent component mixtures contemplated in the present disclosure (*i.e.*, for use in formulating and/or stabilizing one or more protein compositions of the disclosure) need to (A) melt at a temperature between 40°C to about 100°C; and (B) quickly solidify at a temperature between about 20°C to 40°C, thereby quickly forming a robust protein (encapsulated) solid mixture. Thus, in the present example, samples of solvent component mixtures were prepared as follows: water at a specific weight percent (wt %) was added to a container, followed by the addition of thirty (30) grams of the solvent mixture components in the specified molar ratio. The solvent components and water were then mixed at 10°C above the predicted melting temperature on a hot plate. Once a fully homogenous molten liquid state was formed, the temperature was lowered by 10°C. Approximately two (2) grams of spray dried solid enzyme (*i.e.*, Properase®, Dansico US Inc.; a *B. alkalophilus* subtilisin protease) was added to the solvent component/water mixture and hand-mixed for sixty (60) seconds on the hot plate. The subtilisin protease preparation was subsequently poured onto a sheet of aluminum foil and left at room temperature (*i.e.*, 21°C in this particular case) to solidify.

**[0102]** More specifically, the solvent components tested in the present example were: (1) anhydrous betaine (*i.e.*, N,N,N-trimethylglycine), (2) urea, (3) lactose and (4) water. More particularly, the following solvent component mixtures were formulated for encapsulating and stabilizing the solid enzyme (Properase®): (A) anhydrous betaine/urea (1:1 mole ratio) and 5% (weight) water at 85°C, and (B) urea/lactose (6:1 mole ratio) and 10% (weight) water at 80°C (*e.g.*, *see* Table 5).

## EXAMPLE 3

## ENZYME RECOVERY OF FPDSM FORMULATED ENZYMES PREPARED WITH MELT GRANULATION

**[0103]** To assess enzymatic activity of FPDSM formulated (protease) enzyme (*i.e.,* Properase®; formulated as described above in Example 2), the solidified enzyme cast from encapsulation was transferred to a tared 250 mL Nalgene bottle and weighed. One hundred (100) grams of Tris buffer (pH 8.6) was added to the Nalgene bottle, and shaken overnight *via* a shaker to dissolve the protease. The resulting dissolved liquid (subtilisin protease) preparation was tested for proteolytic activity against the substrate N-succinyl-L-Ala-L-Ala-L-Pro-L-Phe-*p*-nitroanilide (hereinafter, "Suc-AAPF-pNA"; *Sigma, St. Louis, MO, Product No. S-7388*), wherein Suc-AAPF-pNA was used as to make standard curve. The Suc-AAPF-pNA control and UFC purified protease (Properase®) were used as standards, wherein Suc-AAPF-pNA was used as substrate (as generally described in Example 4 of U.S. Patent Publication No. US2015/0147768).

**[0104]** For example, the Suc-AAPF-pNA assay was generally performed as follows: In order to determine protease activity, the hydrolysis of N-succinyl-L-alanyl-L-alanyl-L-prolyl-L-phenyl-p-nitroanilide (suc-AAPF-pNA) was measured. The reagent solutions used were: 100 mM Tris/HCl, pH 8.6, containing 0.005% TWEEN®-80 (Tris dilution buffer); 100 mM Tris buffer, pH 8.6, containing 10 mM CaCl.sub.2 and 0.005% TWEEN®-80 (Tris/Ca buffer); and 160 mM suc-AAPF-pNA in DMSO (suc-AAPF-pNA stock solution) (Sigma: S-7388). To prepare a suc-AAPF-pNA working solution, 1 ml suc-AAPF-pNA stock solution was added to 50 ml Tris/Ca buffer and mixed repeatedly. The assay was performed by adding 5 µl of diluted culture supernatant to each well containing 150 µl Tris dilution buffer, immediately followed by the addition of 100 µl of 2 mg/ml suc-AAPF-pNA working solution. The solutions were mixed for 5 seconds, and the absorbance change in kinetic mode (11 readings in 2 minutes) was read at 405 nm in an MTP reader, at 25°C. The protease activity was expressed as AU absorbance at 405 nm.

**[0105]** The solvent component mixtures set forth below in **Table 5** yielded robust, homogenous films when cooled in ambient temperature. Spray dried Properase ® enzyme powder was dispersed in the molten FPDSM system, then poured and cast onto an aluminum foil sheet and allowed to cool and solidify by the procedure described in Example 1. The solid FPDSM material with entrapped enzyme was then dissolved and assayed for enzyme activity as described about. Enzyme recovery was calculated as the weight percentage of active enzyme in the solid FPDSM material relative to the enzyme activity in the initial solid spray dried enzyme powder.

**TABLE 5**
**PROPERASE® ENZYME RECOVERY**

| Two-Component FPDSM System with Added Water | % Enzyme Recovery |
|---|---|
| [1]Anhydrous Betaine/Urea (1:1) + 5% weight water | 71.8 % |
| [2]Urea/Lactose (6:1) + 10 % weight water | 87.3 % |

[1]Anhydrous Betaine/Urea (1:1) + 5% weight water; heated to 85°C, enzyme added and hand-mixed for 60 seconds.
[2]Urea/Lactose (6:1) + 10 % weight water; heated to 80°C, enzyme added and hand-mixed for 60 seconds.

## EXAMPLE 4

## ENZYME RECOVERY VERSUS WATER COMPONENT ADDTION

**[0106]** In the instant example, water was added to two (2) component FPDSM systems having a melting point above 100°C, which is generally denaturing to most protein. Thus, water was added and mixed with the first and second solvent components presented in Table 6, between a range of 5-10% (weight) water. The water addition had the effect of lower the melting temperature of the FPDSM, and accordingly the mixing temperature required for melting and dispersing the added solid enzyme. The recovered (enzyme) activity from melt granulation process was calculated by dividing the residual activity of enzyme by the initial activity of the solid enzyme prior to addition to the FPDSM. Notably, as set forth in FIG. 2 and Table 6 below, the addition of 5-10% water to the solvent component mixtures does not result in a decrease of the observed enzymatic activity, but evidently results in improved recovery of activity, relative to compositions without added water.

**TABLE 6**

**ENZYME RECOVERY OF FPDSM FORMULATED ENZYMES PREPARED WITH MELT GRANULATION**

| Component 1 and Component 2 | Molar Ratio | (%) Water Added | Mixing Temp (°C) | Properase® Recovered Activity (%) |
|---|---|---|---|---|
| choline chloride/succinic acid | 1:1 | 0% | 80 | 3.6 |
| urea/ammonium chloride | 3:1 | 0% | 110 | 0.7 |
| urea/ammonium chloride | 4:1 | 0% | 110 | 3.3 |
| urea/ammonium chloride | 1:4 | 10% | 85 | 71.8 |
| urea/glucose | 1:1 | 5% | 80 | 79.7 |
| urea/glucose | 1:1 | 10% | 80 | 93.0 |
| urea/lactose | 6:1 | 10% | 80 | 87.3 |
| urea/maltose | 6:1 | 10% | 80 | 49.3 |
| betaine/urea | 1:1 | 0% | 95 | 48.8 |
| betaine/urea | 1:1 | 5% | 85 | 71.8 |
| betaine/urea | 1:2 | 0% | 105 | 9.9 |
| betaine/urea | 1:3 | 0% | 105 | 10.7 |
| betaine/urea | 1:4 | 0% | 105 | 1.5 |

**EXAMPLE 5**

**ENZYME RECOVERY VERSUS TEMPERATURE**

**[0107]** In the instant example, Properase® protease enzyme was added to FPDSM solvent component mixtures at different temperatures (*e.g., see*, FIG. 3). For example, specific temperatures were chosen wherein a specific solvent component mixture would remain a molten liquid system. Thus, as set forth in FIG. 3, the Properase® enzymatic activity (as measured by the "Suc-AAPF-pNA assay" described above) was recorded and plotted as a function of temperature. As presented in **FIG. 3**, there is an evident decrease in protease activity at mixing temperatures generally exceeding 80-85°C.

## EXAMPLE 6

## ENZYME GRANULATION USING THE SPINNING DISK TECHNIQUE

**[0108]** In the instant example, a protein of interest is formulated in an approximately 420 grams of a solvent component mixture comprising urea/betaine (1:1) + water (4.5%) formulation, according to the following protocol.

**[0109]** *Materials:* Betaine ($M_W$: 117.15), Urea ($M_W$: 60.06), Properase® enzyme (approximately 20g), a Water/Oil Bath Set at 95°C and a Water/Oil Bath Set at 85°C.

**[0110]** *Procedure:* Prepare two water baths, at 95°C and 85°C

(1) Add Materials (within about 5 minutes):
   (a) Add 20g $dH_20$ to empty tared container,
   (b) Add 135.33 g urea to container, and
   (c) Add 264.67 g betaine to container,

(2) Melt materials to form a homogenous molten FPDMS composition (within about 1 hour):
   (a) Heat in 95°C hot bath with occasional hand-mixing until fully homogenous (20-30 minutes), and
   (b) Once homogenous, transfer container to 85°C water bath (20 minutes) to equilibrate at 85°C,

(3) Disperse enzyme into molten FPDMS composition (within about 5 minutes)
   (a) Add 20g enzyme (approximately 4.5wt%),
   (b) Stir thoroughly for 60 seconds (precisely) to make sure enzyme is fully dispersed (residence time) before dispensing onto a spinning disk for granulation,

(4) Dispense the enzyme preparation onto a 4" spinning disk running at 6000 rpm immediately after 60 seconds,

(5) Atomize the enzyme preparation into ambient atmosphere at 20-25°C, and

(6) Collect particles at a minimum distance of 15 feet below the disk surface level.

## EXAMPLE 7

## ENZYME GRANULATION USING THE SPINNING DISK TECHNIQUE

**[0111]** In the instant example, a protein of interest (POI) was formulated in an approximately 100g solvent component mixture comprising urea/lactose (6:1) and water (4.4%), according to a similar protocol as described above in Example 6.

**[0112]** *Materials:* Lactose ($M_W$: 342.30), Urea ($M_W$: 60.06), Properase® enzyme (approximately 7g), a Water/Oil Bath Set at 95°C and a Water/Oil Bath Set 85°C.

**[0113]** *Procedure:* Set up / Get ready a 95°C and an 85°C hot water bath.

(1) Add Materials (within about 5 minutes):
   (a) Added 4.43g $dH_20$ to empty tared container,
   (b) Added 45.42g urea to container, and
   (c) Added 43.15g lactose to container,

(2) Melted to Homogenous (within about 1 hour):
   (a) Heated in 95°C hot bath with occasional hand-mixing until fully homogenous (20-30 minutes), and
   (b) Once homogenous, transfer container to 85°C water bath (20 minutes) to equilibrate at 85°C,

(3) Dispersed Enzyme into molten FPDMS composition (within about 5 minutes):
   (a) Added 7g enzyme (approximately 7wt%),

(4) Stirred thoroughly for 60 seconds (precisely) to make sure enzyme is fully dispersed (residence time) before dispensing onto a spinning disk for granulation. Dispensed onto a 4" spinning disk running at 6000 rpm immediately after 60 seconds,

(5) Atomized the enzyme preparation into ambient atmosphere at 20-25°C, and

(6) Collected particles at a minimum distance of 15 feet below the disk surface level.

## EXAMPLE 8

## STORAGE STABILITY OF ENZYME FORMULATED IN UREA/LACTOSE SOLVENT COMPONENT MIXTURES

**[0114]** In general, most protein compositions and formulations thereof are stored for a period of time prior to their end use. It is therefore imperative that such proteins (*e.g.*, enzymes) are stable against various forms of protein degradation (*e.g.*, heat, oxidation, UV light degradation, contaminants, and the like) for periods of time of at least 24 hours, preferably at least about 1-28 days, and more preferably for at least 7 weeks. Thus, in the instant example, a protease enzyme (*e.g.*, Properase®), encapsulated by a two-component FPDSM system (*i.*e., comprising urea and lactose) plus added water), was assessed for storage stability at a storage temperature of about 25°C for 28 days.

**[0115]** More particularly, the solvent component mixtures were prepared using lactose and urea with a bulk congealing method as described in the following exemplary procedure for a 30 g batch, consisting of 90 mol% urea and 10 mol% lactose (*i.e.*, urea/lactose; 9:1) and approximately 4.5 wt% water and approximately 5.4 wt% (Properase®) enzyme.

(1) About 1.35 g $dH_20$ was added to empty tared container,

(2) About 16.54 g urea was added to the container from Step 1,

(3) About 10.48 g lactose was added to the container from Step 2,

(4) The materials from Step 3 were hand-stirred with a stainless-steel spatula,

(5) The container was placed in a water bath set 10°C above the melting point of the FPDSM system,

(6) The mixture was hand-stirred often for approximately 5 minutes until the sample was a homogenous liquid,

(7) About 1.62 g (5.4 wt%) Properase® was added to the container, and stirred gently for 60 seconds,

(8) The formulation was then cast on a sheet of aluminum foil to solidify,

(9) The solid cast was broken into small pieces that were ground using a Retsch Ultra Centrifugal Mill. The ground materials were subsequently screened using a Retsch Vibratory Sieve Shaker and size-classified.

(10) The particles of -25 mesh fraction (< 710 um) were analyzed for enzyme activity during storage at normal room temperature (20-25° C) for 4 weeks. The particles were stored in screw capped (HDPE) Nalgene™ bottles. The protease activity analysis data presented in Table 7, was performed using the Suc-AAPF-pNA substrate and assay described in Example 3.

**[0116]** As presented in Table 7 below, the residual protease activity was greater than 88% during the first month (*i.e.*, 28-days) of storage for all urea/lactose solvent composition ratios tested (*i.e.*, 9:1, 8.5:1.5, 3:1 and 6:4). More particularly, with the exception of the observed 89% residual protease activity (recovered) form the urea/lactose solvent component mixture (at mole ratio 8.5:1.5) at day 14, the residual protease

activity (recovered) from the urea/lactose solvent component mixtures (*i.e.*, 9:1, 8.5:1.5, 3:1 and 6:4 urea/lactose solvent component mixtures) at day 7, 14, 21 and 28, remained well above 98% recovered activity. Furthermore, protease formulated in urea/lactose solvent component mixtures (*i.e.*, 9:1, 8.5:1.5, 3:1 and 6:4) demonstrated stable protease activity during storage at room temperature, comparable to that of the spray dried Properase®.

**TABLE 7**

**TWENTY-EIGHT DAY ROOM TEMPERATURE STABILITY OF PROPERASE® FORMULAUTED IN UREA/LACTOSE SOLVENT COMPONENT MIXTURES WITH ADDED WATER**

| **Formulation urea/lactose (mol:mol) + 4.5% water** | **Storage Period (*in days*)** | | | |
|---|---|---|---|---|
| | **7** | **14** | **21** | **28** |
| | **Relative Residual Activity** | | | |
| 9:1 | 100% | 103% | 102% | 98% |
| 8.5:1.5 | 100% | 89% | 102% | 99% |
| 3:1 | 100% | 105% | 104% | 106% |
| 6:4 | 100% | 103% | 106% | 103% |
| | | | | |
| Spray Dried Properase® | 100% | 97% | 99% | 95% |

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## CLAIMS

1. A solid enzyme formulation comprising a freezing point depressed solid matrix (FPDSM) system and a solid enzyme composition entrapped therein, wherein the FPDSM system comprises at least two distinct FPDSM components which at least two distinct FPDSM components do not melt at a temperature below 100°C until combined, wherein the combined FPDSM system is a liquid at a temperature between 40°C to about 100°C and a solid at a temperature between about 20°C to 40°C, wherein recovery of the active enzyme from the solidified FPDSM system is at least 70% active enzyme relative to the activity of the solid enzyme composition.

2. The formulation of claim 1, wherein the at least two component FPDSM further comprises up to 10% (w/w) water.

3. The formulation of claim 1, wherein the FPDSM system comprises a combination of at least two components selected from the group consisting of ammonium chloride, choline chloride, betaine, betaine hydrochloride, citric acid, malonic acid, tartaric acid, fructose, sucrose, glucose, lactose, maltose, urea and sorbitol.

4. The formulation of claim 2, wherein the FPDSM system comprises a combination of at least two components selected from the group consisting of ammonium chloride, choline chloride, betaine, betaine hydrochloride, citric acid, malonic acid, tartaric acid, fructose, sucrose, glucose, lactose, maltose, urea and sorbitol.

5. The formulation of claim 4 or claim 5, wherein two components of the FPDSM system are combined at a molar ratio ranging from about 1:10 to about 10:1, wherein the combined molar ratio of the FPDSM system components are in a liquid state at a temperature between 40°C to about 100°C, and a solid at a temperature between about 20°C to 40°C.

6. The formulation of claim 2, wherein the water content is about 0.05% to 10% w/w.

7. The formulation of claim 2, wherein the water content is about 5% to 10% w/w.

8. The formulation of claim 1, wherein the enzyme retains at least 85% enzymatic activity after twenty-four (24) hours of storage at 25°C.

9. The formulation of claim 1, wherein the enzyme retains at least 95% enzymatic activity after twenty-four (24) hours of storage at 25°C.

10. The formulation of claim 1, wherein the enzyme retains at least 99% enzymatic activity after twenty-four (24) hours of storage at 25°C.

11. The formulation of claim 1, wherein the enzyme retains at least 80% enzymatic activity after seven (7) days of storage at 25°C.

12. The formulation of claim 1, wherein the enzyme retains at least 75% enzymatic activity after twenty-eight (28) days of storage at 25°C.

13. The formulation of claim 1, wherein the FPDSM formulated solid enzyme composition retains a higher enzymatic activity than an unformulated solid enzyme composition, wherein both the FPDSM formulated enzyme and the solid enzyme composition are stored at the same temperature for the same amount of time before enzymatic activity is assayed.

14. The formulation of claim 1, wherein the enzyme is selected from the group consisting of acetyl esterases, aryl esterases, aminopeptidases, amylases, arabinases, arabinofuranosidases, carbonic anhydrases, carboxypeptidases, catalases, cellulases, chitinases, chymosins, cutinases, deoxyribonucleases, epimerases, esterases, α-galactosidases, β-galactosidases, α-glucanases, glucan lysases, endo-β-glucanases, glucoamylases, glucose oxidases, α-glucosidases, β-glucosidases, glucuronidases, glycosyl hydrolases, hemicellulases, hexose oxidases, hydrolases, invertases, isomerases, laccases, ligases, lipases, lyases, mannosidases, oxidases, oxidoreductases, pectate lyases, pectin acetyl esterases, pectin depolymerases, pectin methyl esterases, pectinolytic enzymes, perhydrolases, polyol oxidases, peroxidases, phenoloxidases, phytases, polygalacturonases, proteases, peptidases, rhamno-galacturonases, ribonucleases, transferases, transglutaminases, xylanases, hexose oxidases, and combinations thereof.

15. The formulation of claim 1 or claim 2, wherein the FPDSM system comprises choline chloride and citric acid at a molar ratio between about 1:2 (choline chloride/citric acid) to about 2:1 (choline chloride/citric acid).

16. The formulation of claim 1 or claim 2, wherein the FPDSM system comprises choline chloride and sorbitol at a molar ratio between about 2:1 (choline chloride/sorbitol) to about 3:1 (choline chloride/sorbitol).

17. The formulation of claim 1 or claim 2, wherein the FPDSM system comprises anhydrous betaine and urea at a molar ratio between about 1:1 (betaine/urea) to about 1:4 (betaine/urea).

18. The formulation of claim 1 or claim 2, wherein the FPDSM system comprises ammonium chloride and urea at a molar ratio between about 1:2 (ammonium chloride/urea) to about 1:3 (ammonium chloride/urea).

19. The formulation of claim 1, wherein the FPDSM system comprises at least two different enzymes.

20. The formulation of claim 1, wherein the solid enzyme composition is a spray dried enzyme, a lyophilized enzyme, a precipitated enzyme, a crystalized enzyme, a spray cooled enzyme, spray chilled enzyme or a spray granulated enzyme.

21. A solid enzyme composition homogenously distributed and entrapped in a FPDSM system formulation of claim 2.

22. The solid enzyme composition of claim 20 or claim 21, comprising a solid form selected from a solid enzyme pellet, a solid enzyme granule, a solid enzyme powder, a solid enzyme film and a solid enzyme extrudate.

23. A solid enzyme composition homogenously distributed and entrapped in a FPDSM system formulation of claim 1.

24. A method for stabilizing a solid enzyme composition from loss of activity, the method comprising:
    (a) selecting and preparing a FPDSM system comprising at least two distinct FPDSM system components and optionally up to 10% water, wherein the FPDSM system components are selected such that when combined and heated, form a liquid at a temperature between 40°C to about 100C, and when subsequently cooled, form a solid at a temperature between about 20°C to 40°C,

(b) adding and mixing a solid enzyme composition to the FPDSM system of step (a) at a temperature higher than 40°C and lower than about 100°C, and

(c) lowering the temperature of the step (b) mixture to a temperature between about 20°C to 40°C, wherein the FPDSM system solidifies to form a solid FPDSM system that entraps and encapsulates the enzyme.

25. The method of claim 24, wherein the recovery of the FPDSM formulated enzyme relative to the recovery of solid enzyme composition is at least 70%.

26. The method of claim 24, wherein the enzyme retains at least 85% enzymatic activity after twenty-four (24) hours of storage at 25°C.

27. The method of claim 24, wherein the enzyme retains at least 95% enzymatic activity after twenty-four (24) hours of storage at 25°C.

28. The method of claim 24, wherein the enzyme retains at least 99% enzymatic activity after twenty-four (24) hours of storage at 25°C.

29. The method of claim 24, wherein the enzyme retains at least 80% enzymatic activity after seven (7) days of storage at 25°C.

30. The method of claim 24, wherein the enzyme retains at least 75% enzymatic activity after twenty-eight (28) days of storage at 25°C.

31. The method of claim 24, wherein the FPDSM formulated solid enzyme composition retains a higher enzymatic activity than an unformulated solid enzyme composition, wherein both the FPDSM formulated enzyme and the solid enzyme composition are stored at the same temperature for the same amount of time before enzymatic activity is assayed.

32. The method of claim 24, wherein the FPDSM system comprises a combination of at least two components selected from the group consisting of ammonium chloride, choline chloride, betaine, betaine hydrochloride, citric acid, malonic acid, tartaric acid, fructose, sucrose, glucose, lactose, maltose, urea and sorbitol.

33. The method of claim 24, wherein the FPDSM system comprises a combination selected from the group consisting of urea/lactose, urea/glucose, urea/maltose, urea/fructose, urea/ammonium chloride, urea/betaine, urea/betaine hydrochloride, urea/choline chloride, betaine/glucose, betaine/fructose, betaine/lactose, betaine/citric acid, betaine/sucrose, choline chloride/glucose, choline chloride/malonic acid, choline chloride/ tartaric acid, choline chloride/citric acid, choline chloride/ sorbitol, citric acid/glucose and citric acid/maltose.

34. The method of claim 24, wherein two components of the FPDSM system are combined at a molar ratio ranging from about 1:10 to about 10:1, wherein the combined molar ratio of the FPDSM system components are in a liquid state at a temperature between 40°C to about 100°C, and a solid at a temperature between about 20°C to 40°C.

35. The method of claim 24, wherein the FPDSM system comprises choline chloride and citric acid at a molar ratio between about 1:2 (choline chloride/citric acid) to about 2:1 (choline chloride/citric acid).

36. The method of claim 24, wherein the FPDSM system comprises choline chloride and sorbitol at a molar ratio between about 2:1 (choline chloride/sorbitol) to about 3:1 (choline chloride/sorbitol).

37. The method of claim 24, wherein the FPDSM system comprises anhydrous betaine and urea at a molar ratio between about 1:1 (betaine/urea) to about 1:4 (betaine/urea).

38. The method of claim 24, wherein the FPDSM system comprises ammonium chloride and urea at a molar ratio between about 1:2 (ammonium chloride/urea) to about 1:3 (ammonium chloride/urea).

39 The method of claim 24, wherein the FPDSM system comprises betaine and glucose at a molar ratio of about 5:2 (betaine/glucose) and about 0.05% to 10% weight water.

40. The method of claim 24, wherein the FPDSM system comprises betaine and urea at a molar ratio of about 1:1 (betaine/glucose) and about 0.05% to 10% weight water.

41. The method of claim 4 wherein the FPDSM system comprises urea and glucose at a molar ratio of about 3:1 (urea/glucose) and about 0.05% to 10% weight water.

42. The method of claim 24, wherein the FPDSM system comprises urea and maltose at a molar ratio of about 6:1 (urea/maltose) and about 0.05% to10% weight water.

43. The method of claim 24, wherein the FPDSM system comprises urea and lactose at a molar ratio of about 6:1 (urea/lactose) and about 0.05% to 10% weight water.

44. The method of claim 24, wherein the FPDSM system comprises urea and lactose at a molar ratio of about 9:1 (urea/lactose) to about 1.5: 1 (urea/lactose) and about 4.0% to 6.0% weight water.

45. The method of claim 24, wherein the water content is about 0.05% to 10% w/w.

46. The method of claim 24, wherein the water content is about 5% to 10% w/w.

47. The method of claim 24, wherein the enzyme is selected from the group consisting of acetyl esterases, aryl esterases, aminopeptidases, amylases, arabinases, arabinofuranosidases, carbonic anhydrases, carboxypeptidases, catalases, cellulases, chitinases, chymosins, cutinases, deoxyribonucleases, epimerases, esterases, α-galactosidases, β-galactosidases, α-glucanases, glucan lysases, endo-β-glucanases, glucoamylases, glucose oxidases, α-glucosidases, β-glucosidases, glucuronidases, glycosyl hydrolases, hemicellulases, hexose oxidases, hydrolases, invertases, isomerases, laccases, ligases, lipases, lyases, mannosidases, oxidases, oxidoreductases, pectate lyases, pectin acetyl esterases, pectin depolymerases, pectin methyl esterases, pectinolytic enzymes, perhydrolases, polyol oxidases, peroxidases, phenoloxidases, phytases, polygalacturonases, proteases, peptidases, rhamno-galacturonases, ribonucleases, transferases, transglutaminases, xylanases, hexose oxidases, and combinations thereof.

48. The method of claim 24, wherein the FPDSM system comprises at least two different enzymes.

49. The method of claim 24, wherein the solid enzyme composition is a spray dried enzyme, a lyophilized enzyme, a precipitated enzyme, a crystalized enzyme, a spray cooled enzyme, spray chilled enzyme or a spray granulated enzyme.

50. The method of claim 24, wherein the solidified FPDSM system of step (c) comprises a solid form selected from a solid pellet, a solid granule, a solid powder, a solid film and a solid extrudate.

51. A solid enzyme composition homogenously distributed and entrapped in a FPDSM system prepared by the method of claim 24.

52. A method for inhibiting the degradation of a solid enzyme composition stored for at least twenty-four hours at 25°C, the method comprising:

(a) selecting and preparing a FPDSM system comprising at least two distinct FPDSM system components and optionally up to 10% water, wherein the FPDSM system components are selected such that when combined and heated, form a liquid at a temperature between 40°C to about 100C, and when subsequently cooled, form a solid at a temperature between about 20°C to 40°C,

(b) adding and mixing a solid enzyme composition to the FPDSM system of step (a) at a temperature higher than 40°C and lower than about 100°C, and

(c) lowering the temperature of the step (b) mixture to a temperature between about 20°C to 40°C, wherein the FPDSM system solidifies to form a solid FPDSM system that entraps and encapsulates the enzyme.

53. The method of claim 52, wherein the recovery of the FPDSM formulated enzyme relative to the recovery of solid enzyme composition is at least 70%.

54. The method of claim 52, wherein the enzyme retains at least 85% enzymatic activity after twenty-four (24) hours of storage at 25°C.

55. The method of claim 52, wherein the enzyme retains at least 95% enzymatic activity after twenty-four (24) hours of storage at 25°C.

56. The method of claim 52, wherein the enzyme retains at least 99% enzymatic activity after twenty-four (24) hours of storage at 25°C.

57. The method of claim 52, wherein the enzyme retains at least 80% enzymatic activity after seven (7) days of storage at 25°C.

58. The method of claim 52, wherein the enzyme retains at least 75% enzymatic activity after twenty-eight (28) days of storage at 25°C.

59. The method of claim 52, wherein the FPDSM formulated solid enzyme composition retains a higher enzymatic activity than an unformulated solid enzyme composition, wherein both the FPDSM formulated enzyme and the solid enzyme composition are stored at the same temperature for the same amount of time before enzymatic activity is assayed.

60. The method of claim 52, wherein the FPDSM system comprises a combination of at least two components selected from the group consisting of ammonium chloride, choline chloride, betaine, betaine hydrochloride, citric acid, malonic acid, tartaric acid, fructose, sucrose, glucose, lactose, maltose, urea and sorbitol.

61. The method of claim 52, wherein the FPDSM system comprises a combination selected from the group consisting of urea/lactose, urea/glucose, urea/maltose, urea/fructose, urea/ammonium chloride, urea/betaine, urea/betaine hydrochloride, urea/choline chloride, betaine/glucose, betaine/fructose, betaine/lactose, betaine/citric acid, betaine/sucrose, choline chloride/glucose, choline chloride/malonic acid, choline chloride/ tartaric acid, choline chloride/citric acid, choline chloride/ sorbitol, citric acid/glucose and citric acid/maltose.

62. The method of claim 52, wherein two components of the FPDSM system are combined at a molar ratio ranging from about 1:10 to about 10:1, wherein the combined molar ratio of the FPDSM system components are in a liquid state at a temperature between 40°C to about 100°C, and a solid at a temperature between about 20°C to 40°C.

63. The method of claim 52, wherein the FPDSM system comprises choline chloride and citric acid at a molar ratio between about 1:2 (choline chloride/citric acid) to about 2:1 (choline chloride/citric acid).

64. The method of claim 52, wherein the FPDSM system comprises choline chloride and sorbitol at a molar ratio between about 2:1 (choline chloride/sorbitol) to about 3:1 (choline chloride/sorbitol).

65. The method of claim 52, wherein the FPDSM system comprises anhydrous betaine and urea at a molar ratio between about 1:1 (betaine/urea) to about 1:4 (betaine/urea).

66. The method of claim 52, wherein the FPDSM system comprises ammonium chloride and urea at a molar ratio between about 1:2 (ammonium chloride/urea) to about 1:3 (ammonium chloride/urea).

67 The method of claim 52, wherein the FPDSM system comprises betaine and glucose at a molar ratio of about 5:2 (betaine/glucose) and about 0.05% to 10% weight water.

68. The method of claim 52, wherein the FPDSM system comprises betaine and urea at a molar ratio of about 1:1 (betaine/glucose) and about 0.05% to 10% weight water.

69. The method of claim 52, wherein the FPDSM system comprises urea and glucose at a molar ratio of about 3:1 (urea/glucose) and about 0.05% to 10% weight water.

70. The method of claim 52, wherein the FPDSM system comprises urea and maltose at a molar ratio of about 6:1 (urea/maltose) and about 0.05% to10% weight water.

71. The method of claim 52, wherein the FPDSM system comprises urea and lactose at a molar ratio of about 6:1 (urea/lactose) and about 0.05% to 10% weight water.

72. The method of claim 52, wherein the FPDSM system comprises urea and lactose at a molar ratio of about 9:1 (urea/lactose) to about 1.5: 1 (urea/lactose) and about 4.0% to 6.0% weight water.

73. The method of claim 52, wherein the water content is about 0.05% to 10% w/w.

77. The method of claim 52, wherein the water content is about 5% to 10% w/w.

78. The method of claim 52, wherein the enzyme is selected from the group consisting of acetyl esterases, aryl esterases, aminopeptidases, amylases, arabinases, arabinofuranosidases, carbonic anhydrases, carboxypeptidases, catalases, cellulases, chitinases, chymosins, cutinases, deoxyribonucleases, epimerases, esterases, α-galactosidases, β-galactosidases, α-glucanases, glucan lysases, endo-β-glucanases, glucoamylases, glucose oxidases, α-glucosidases, β-glucosidases, glucuronidases, glycosyl hydrolases, hemicellulases, hexose oxidases, hydrolases, invertases, isomerases, laccases, ligases, lipases, lyases, mannosidases, oxidases, oxidoreductases, pectate lyases, pectin acetyl esterases, pectin depolymerases, pectin methyl esterases, pectinolytic enzymes, perhydrolases, polyol oxidases, peroxidases, phenoloxidases, phytases, polygalacturonases, proteases, peptidases, rhamno-galacturonases, ribonucleases, transferases, transglutaminases, xylanases, hexose oxidases, and combinations thereof.

79. The method of claim 52, wherein the FPDSM system comprises at least two different enzymes.

80. The method of claim 52, wherein the solid enzyme composition is a spray dried enzyme, a lyophilized enzyme, a precipitated enzyme, a crystalized enzyme, a spray cooled enzyme, spray chilled enzyme or a spray granulated enzyme.

81. The method of claim 52, wherein the solidified FPDSM system of step (c) comprises a solid form selected from a solid pellet, a solid granule, a solid powder, a solid film and a solid extrudate.

82. A solid enzyme composition homogenously distributed and entrapped in a FPDSM system prepared by the method of claim 52.

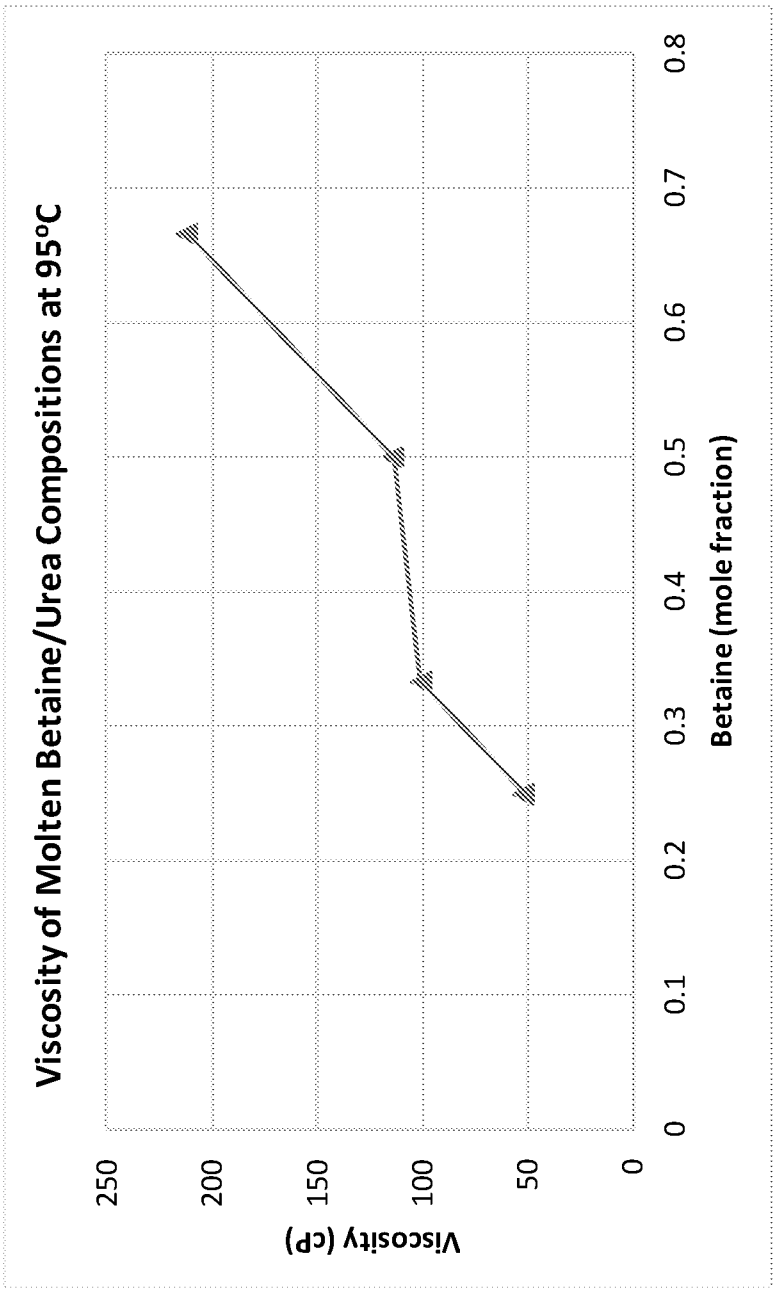
Viscosity of Molten Betaine/Urea Compositions at 95°C
Viscosity (cP)
250
200
150
100
50
0
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
Betaine (mole fraction)

FIG. 1

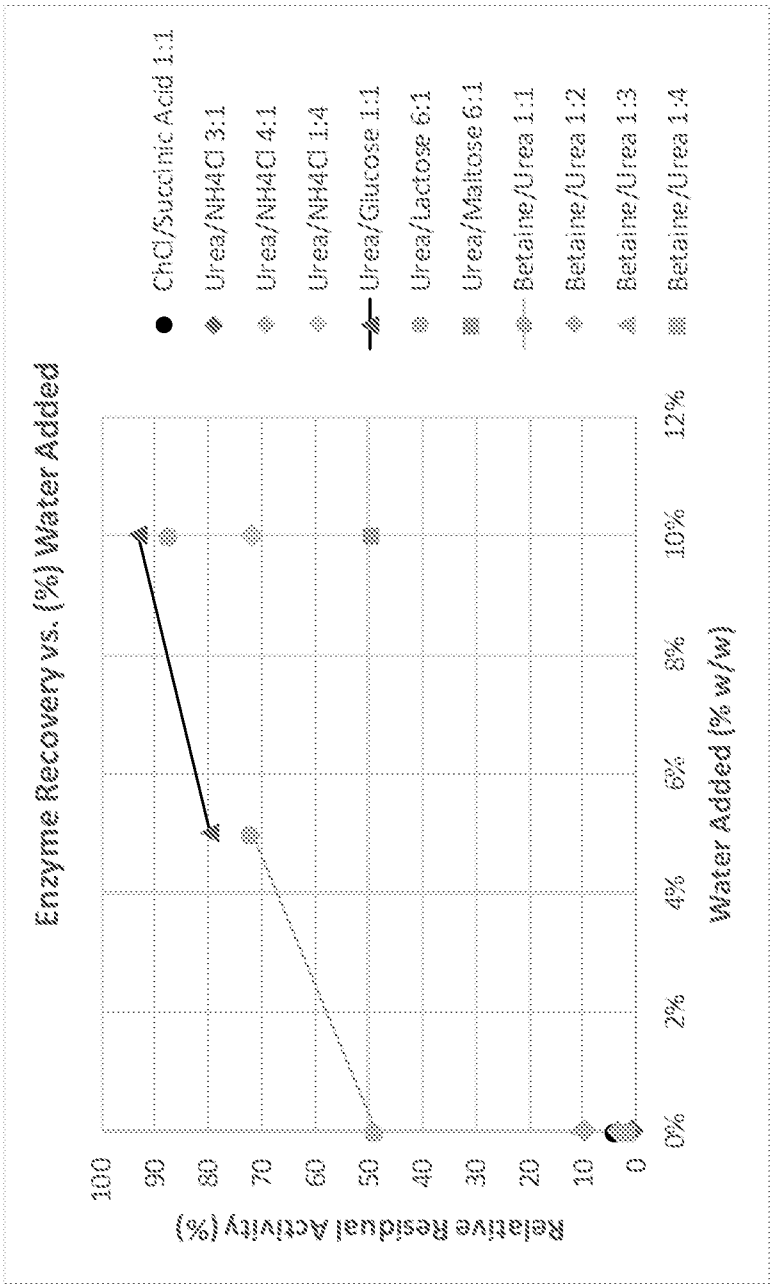
Enzyme Recovery vs. (%) Water Added
Relative Residual Activity (%)
Water Added (% w/w)
0%
2%
4%
6%
8%
10%
12%
0
10
20
30
40
50
60
70
80
90
100
ChCl/Succinic Acid 1:1
Urea/NH4Cl 3:1
Urea/NH4Cl 4:1
Urea/NH4Cl 1:4
Urea/Glucose 1:1
Urea/Lactose 6:1
Urea/Maltose 6:1
Betaine/Urea 1:1
Betaine/Urea 1:2
Betaine/Urea 1:3
Betaine/Urea 1:4

FIG. 2

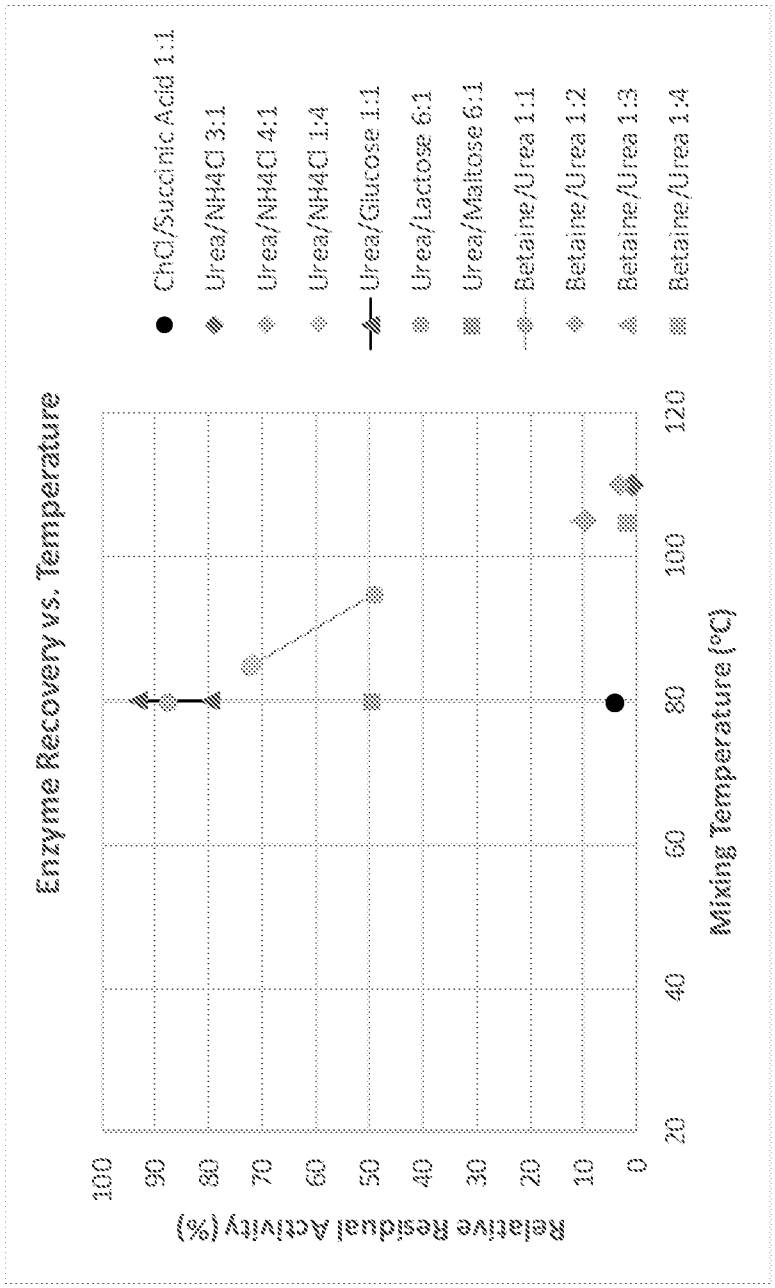
Enzyme Recovery vs. Temperature
Relative Residual Activity (%)
Mixing Temperature (°C)
ChCl/Succinic Acid 1:1
Urea/NH4Cl 3:1
Urea/NH4Cl 4:1
Urea/NH4Cl 1:4
Urea/Glucose 1:1
Urea/Lactose 6:1
Urea/Maltose 6:1
Betaine/Urea 1:1
Betaine/Urea 1:2
Betaine/Urea 1:3
Betaine/Urea 1:4

FIG. 3

# INTERNATIONAL SEARCH REPORT

International application No
PCT/US2018/058665

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K9/16 A61K38/43
ADD.

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)
A61K

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)
EPO-Internal, WPI Data, BIOSIS, CHEM ABS Data, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | WO 2016/149636 A1 (DANISCO US INC)<br>22 September 2016 (2016-09-22)<br>page 7, paragraphs 14, 16-17<br>pages 10-11, paragraph 26-33<br>page 13, paragraph 42<br>page 16, paragraph 52<br>page 19, paragraph 64<br>page 39; table 3<br>pages 50-51; example 7<br>claims 1, 6-7, 18, 20, 24-31<br>-----<br>-/-- | 1-82 |

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

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

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"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

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"&" 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 2019 | 12/02/2019 |

| Name and mailing address of the ISA/ | Authorized officer |
|---|---|
| European Patent Office, P.B. 5818 Patentlaan 2<br>NL - 2280 HV Rijswijk<br>Tel. (+31-70) 340-2040,<br>Fax: (+31-70) 340-3016 | Lemarchand, Aude |

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Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

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Information on patent family members

International application No
PCT/US2018/058665

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| | | TW 201446149 A | 16-12-2014 |
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| | | CA 2904765 A1 | 04-09-2014 |
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| | | JP 2018113982 A | 26-07-2018 |
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